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Critical Role of c-Jun N-terminal Kinase Signaling in Binge Alcohol-Driven Atrial Arrhythmic Remodeling

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CRITICAL ROLE OF C-JUN N-TERMINAL KINASE SIGNALING IN BINGE ALCOHOL-DRIVEN ATRIAL ARRHYTHMIC REMODELING

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LIST OF ABBREVIATIONS

AF	atrial fibrillation
AT	atrial tachycardia
ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
BAC	blood alcohol concentration
BMI	body mass index
BW	body weight
CAD	coronary artery disease
CaMKII	Ca^{2+}/calmodulin-dependent kinase II
CHF	congestive heart failure
CICR	Ca^{2+}-induced Ca^{2+} release
Cx43	connexin43
DAD	delayed after-depolarization
DM	diabetes mellitus
EAD	early after-depolarization
EF	ejection fraction
ECM	extracellular matrix
ERK	extracellular signal-regulated kinase

FS	fractional shortening
HF	heart failure
HTN	hypertension
IP/IP-ed	immunoprecipitation/immunoprecipitated
JNK	c-Jun N-terminal kinase
JNK1dn	dominant-negative overexpression of JNK1
JNK2dn	dominant-negative overexpression of JNK2
JNK1/2dn	dominant-negative overexpression of JNK1 and JNK2
HHS	holiday heart syndrome
HW	heart weight
LW	lung weight
NCX	Na^+/Ca^{2+} exchanger
RA	right atrium
ROS	reactive oxygen species
RyR	ryanodine receptor
SERCA	sarco/endo reticulum Ca^{2+} ATPase
SR	sarcoplasmic reticulum
WT	wildtype
wt-CaMKII	wild-type CaMKII
vv-CaMKII	Met280/281Val mutated CaMKII

ABSTRACT

Holiday Heart Syndrome (HHS) is cardiac arrhythmia induced by binge alcohol consumption, a drinking pattern affects 38 million adults in our society. Atrial fibrillation (AF) is the most frequently diagnosed arrhythmia in HHS and causes significant morbidity and mortality. Despite the intensive prevention effort nationwide, the binge drinking population keeps rising. However, no effective treatment strategies are available for binge drinking induced AF due to the unknown underlying mechanisms. In this thesis, I aim to elucidate the mechanisms of binge alcohol-promoted atrial arrhythmogenicity by using a mouse model of repeated binge alcohol exposure established in the Ai Lab.

The Ai Lab has recently discovered that the activation of stress-response kinase c-Jun N-terminal kinase (JNK) plays an essential role in atrial arrhythmogenicity. Interestingly, previous research also documented that alcohol promotes JNK activation in non-atrial tissue. However, it is unknown whether JNK plays a role in binge alcohol-induced atrial arrhythmogenicity. In this thesis, I found increased JNK activation in repeated binge alcohol-exposed mouse/rabbit atria. In addition, abolishing JNK pathway with dominant negative JNK overexpression in the

heart (JNK1/2dn mice) successfully suppressed binge alcohol-promoted atrial arrhythmia.

Aberrant Ca^{2+} activities, especially Ca^{2+} waves, play a pivotal role in arrhythmogenicity. Ca^{2+}/calmodulin-dependent kinase II (CaMKII) is a well-established arrhythmogenic molecule that regulates multiple cardiac Ca^{2+} handling proteins. Previous research has shown that CaMKII-dependent phosphorylation of ryanodine receptor (RyR), the main Ca^{2+} release channel on the sarcoplasmic reticulum (SR), results in enhanced SR Ca^{2+} leak and the rise of Ca^{2+} waves. SR Ca^{2+} overload has also been shown to sensitize RyR and promote diastolic SR Ca^{2+} leak. However, it is unknown whether Ca^{2+} mishandling plays a vital role in binge drinking-induced atrial arrhythmia. In this thesis, I found an increased frequency of Ca^{2+} waves in atrial tissue from binge alcoho- exposed mice, while abolishing the JNK pathway in JNK1/2dn mice precluded the Ca^{2+} wave occurrence. CaMKII activation and CaMKII-dependent RyR phosphorylation were both enhanced after alcohol exposure. On the other hand, a single dose of CaMKII inhibitor (KN93) treatment after binge alcohol exposure reversed the atrial Ca^{2+} waves and atrial arrhythmogenicity in WT mice. Alcohol-treated atrial myocytes (HL-1 cells) revealed enhanced diastolic SR Ca^{2+} leak, while suppressing JNK or CaMKII both alleviated the SR Ca^{2+} leak, further indicating alcohol induced JNK/CaMKII contributes to arrhythmogenic Ca^{2+} mishandling. Further studies showed alcohol exposure promotes CaMKII activation in a JNK-dependent manner, but not via direct

oxidation of redox-sensitive amino acid sites (Met280/281) on CaMKII. Besides CaMKII-dependent RyR phosphorylation, enhanced SR Ca^{2+} load can also promote SR Ca^{2+} leak via sensitizing the SR luminal Ca^{2+} sensor of RyR. I found increased SR Ca^{2+} load in alcohol-treated atrial myocytes via a JNK-dependent yet CaMKII-independent mechanism. Ablating SR Ca^{2+} luminal sensor with RyR E4872Q$^{+/-}$ mutation alleviated the binge alcohol or JNK activation-induced increase in Ca^{2+} waves and atrial arrhythmogenicity.

In conclusion, the key findings of this thesis work are: 1) repeated binge alcohol leads to enhanced activation of stress-response kinase; 2) JNK2 phosphorylates CaMKII, a pro-arrhythmic molecule, and promotes CaMKII-dependent diastolic SR Ca leak via increased RyR channel activity; 3) JNK2 elevates SR Ca load via JNK-enhanced SERCA uptake. Findings in this work suggest that JNK inhibition could be a therapeutic strategy for binge alcohol prompted atrial arrhythmias.

CHAPTER ONE

LITERATURE REVIEW

1.1 Binge Drinking Promotes Atrial Fibrillation

1.1.1 Holiday Heart Syndrome and Binge Drinking-caused Atrial Fibrillation

Binge drinking causes a significantly enhanced propensity of cardiac arrhythmias, a condition which is known as the Holiday Heart Syndrome (HHS) (Beets et al., 2009; Ettinger et al., 1978; Mitka, 2009). HHS was first recognized in the early 70's when Ettinger et al. found an association between alcohol intoxication and cardiac arrhythmias (Ettinger, et al., 1978). Such cases typically happen more frequently on or immediately after weekends or holidays such as Christmas or New Year's Eve during which heavy alcohol consumption is frequent (Ettinger, 1984; Ettinger, et al., 1978).

Among the different cardiac rhythm disturbances observed in patients suffering from HHS, atrial fibrillation (AF) is the most frequently diagnosed arrhythmia (Ettinger, et al., 1978; Mandyam et al., 2012; Tonelo, Providencia, & Goncalves, 2013). In early clinical case reports, Thornton documented spontaneous AF after one episode of binge drinking (85-200g alcohol) in non-alcoholic patients with no evidence of underlying diseases or cardiac abnormalities (Thornton, 1984).

In a test by Engel et al. on the potential contribution of whiskey consumption to AF, two out of three non-alcoholic patients developed AF or atrial flutter after one binge dose (Engel & Luck, 1983). Clinical evidences suggest that one-third of all new-onset AF cases are related to alcohol intoxication (Hansson, Madsen-Hardig, & Olsson, 2004; Lowenstein, Gabow, Cramer, Oliva, & Ratner, 1983; Maryniak et al., 2006; Peter, Gracey, & Beach, 1968; E. C. Rich, Siebold, & Campion, 1985). Moreover, binge drinking promotes AF onset in both regular and non-regular drinkers (Engel & Luck, 1983; Krishnamoorthy, Lip, & Lane, 2009; Thornton, 1984). Compared with non-binge drinkers, binge drinkers have a significantly increased risk (29%) of AF (Y. Liang et al., 2012).

Although it is commonly believed that moderate drinking may offer cardioprotective effects (O'Keefe, Bhatti, Bajwa, DiNicolantonio, & Lavie, 2014), accumulating evidence has established that excessive binge drinking increases all-cause mortality (Makela, Paljarvi, & Poikolainen, 2005; Murray et al., 2002) by provoking cardiac arrhythmia and even cardiac death (Chenet, McKee, Leon, Shkolnikov, & Vassin, 1998). For instance, in a study conducted in Moscow, it was found that death rates from cardiac causes were significantly higher on Saturdays, Sundays and Mondays than during the rest of the week due to the fact that binge drinking on weekends is a common phenomena (Chenet, et al., 1998). Alcohol-induced cardiac diseases are common among patients with chronic heavy alcohol consumption (for instance, 35 drinks per week for men) (Guzzo-Merello et al., 2015;

Larsson, Drca, & Wolk, 2014; Mukamal, Tolstrup, Friberg, Jensen, & Gronbaek, 2005; Sano et al., 2014). However, evidence indicates that binge drinking actually causes similar incidence of sudden cardiac death as chronic heavy drinking (Wannamethee & Shaper, 1992). More alarmingly, binge alcohol not only increases the cardiac risks for patients with preexisting cardiac conditions (such as coronary artery disease (CAD), and ischemic cardiomyopathy) (Trejbal & Mitro, 2008), it also affects young and apparently healthy individuals without clinical evidence of heart diseases (Tonelo, et al., 2013). Regardless of underlying cardiovascular diseases (including hypertension (HTN), ischemic heart disease and cardiomyopathy), significantly higher percentage of patients diagnosed with AF report incidences of heavy alcohol consumption within 2 days compared to non-AF patients (Koskinen, Kupari, Leinonen, & Luomanmaki, 1987). Indeed, according to the statistics, patients suffering from binge drinking-induced AF are usually without clinical evidence of cardiac diseases or other conditions that could increase the risk of arrhythmias (Ettinger, 1984; Ettinger, et al., 1978).

1.1.2 Binge Drinking in Young Adults and the Health Risk of Repeated Binge Drinking

Binge drinking is an excessive alcohol consumption pattern (>=60g pure alcohol or about 5 drinks for men or 4 drinks for women within 2 hours, blood alcohol concentration (BAC) above 80mg/dL)(Corbin et al., 2014; "National Institute on Alcohol Abuse and Alcoholism Moderate & binge drinking," 2012). Although

extensive prevention effort has been made nationwide, the prevalence of binge drinking remains on the rise (Mathurin & Deltenre, 2009; Pincock, 2003). Currently, the binge drinking pattern has been identified in 18% of the drinking population, which accounts for 38 million of the adult population in the U.S. (Corbin, et al.., 2014; Djousse et al.., 2004; "National Institute on Alcohol Abuse and Alcoholism Moderate & binge drinking," 2012; "Vital signs: binge drinking prevalence, frequency, and intensity among adults - United States, 2010," 2012). Young adults are a population particularly affected by binge drinking (Beets, et al., 2009; Mitka, 2009). In our society, 1 in 3 young drinkers transition into binge drinking within 12 months after their first drink (H. G. Cheng & Anthony, 2016). Binge drinking is also a frequent phenomenon among college students, and is more commonly observed in men than in women (Patrick, Terry-McElrath, Kloska, & Schulenberg, 2016). Considering the previous finding that binge drinking can cause AF even in patients without clinical indications of increased cardiac risk (Tonelo, et al., 2013), the reported trend of binge drinking in young adults is indeed an alarming health risk.

Although binge drinkers are usually not alcohol-dependent (Esser et al., 2014), such individuals tend to repeat binge drinking within a short period of time. On average, binge drinkers (1 in 6 adults in U.S.) tend to binge drink about 4 times per month, consuming approximately 8 drinks per episode ("Vital signs: binge drinking prevalence, frequency, and intensity among adults-U.S., 2010," 2012). A follow up study shows that about 30% of binge drinkers report more than10 binge

episodes while 15% report 5-9 binge episodes within a 30-day period (Esser, et al., 2014). Although binge alcohol-induced AF tend to be paroxysmal with electrocardiograms and lab results reverse to normal after the onset of AF without intervention, AF recurrences are frequently observed in patients who continue binge drinking (Ettinger, 1984; Ettinger, et al., 1978; Krishnamoorthy, et al., 2009). In this light, it is of vital importance to identify the mechanisms of alcohol-induced AF, which can endow us with strategies to alleviate binge alcohol-induced paroxysmal AF and prevent the development of persistent AF.

1.1.3 AF Causes Significant Morbidity and Mortality

It has been commonly perceived that the consequences of AF were less grave compared to ventricular arrhythmia, where sudden cardiac death usually arises. Yet multiple sources of evidence have indicated that AF, the most commonly diagnosed cardiac arrhythmia, brings a 40-90% increased all-cause mortality (Benjamin et al., 2009; Benjamin et al., 1998; Krahn, Manfreda, Tate, Mathewson, & Cuddy, 1995) to the patient and significant financial burden to our society (Calkins et al., 2012).

The most prominent complication of AF is stroke. It is well-known that AF decreases left atria contraction efficiency thus impairs blood flow in the left atria which further enhances thrombogenesis (Watson, Shantsila, & Lip, 2009). In the Framingham Cohort Study, the risk of stroke is 5.6 times higher in AF patients compared to the counterparts with normal sinus rhythm (Kannel, Abbott, Savage, & McNamara, 1983). Also, compared with non-AF associated strokes, AF-associated

strokes lead to poorer prognosis and higher mortality (Lip, 2013). Besides stroke, AF aggravates existing cardiovascular diseases. For instance, it is estimated that AF occurs in 15-30% of heart failure (HF) patients, bringing in potentially deleterious hemodynamic consequences. Such effects include but are not limited to the loss of atrial systole, the loss of atrial-ventricular synchrony and irregularity in the pulse interval, which all accelerate the progression of ventricular dysfunction (Carson et al., 1993; D. M. Clark, Plumb, Epstein, & Kay, 1997). As a result, AF marks a tripled risk for death from HF (Carson, et al., 1993; Dries et al., 1998; Krahn, et al., 1995), and even the new onset of AF in HF patients projects a 2-fold increase in mortality and 4.5-fold increase in all-cause hospitalization (Aleong, Sauer, Davis, & Bristow, 2014).

Unfortunately, current AF management strategies are ineffective. Anti-coagulation therapy has been the cornerstone of preventing AF-induced stroke; however, in certain population of treated AF patient, stroke still prevails (Touze & Ciocanu, 2014). Endocardial catheter ablation therapy, especially the ablation of the pulmonary vein region, has been the most effective approach of rhythm control (Furberg et al., 1994). However, in the general population of AF patients, no significant benefit was found in ablation treatment compared to therapy without ablation in long term studies, while the patient population need re-ablation ranges up to 54% (Skelly et al., 2015). Surgical ligation or excision of left atria where 90% of the thrombi occurs in AF patients is standard of care in AF patient who undergo open-chest surgeries. However, the evidence for long-term efficacy and safety is

insufficient to recommend this approach to all AF patients (Aryana, Saad, & d'Avila, 2012).

Statistics have shown that AF accounts for more than 350,000 U.S. hospital admissions annually (Wattigney, Mensah, & Croft, 2003) and costs the U.S. health care system approximately $26 billion each year (Calkins, et al., 2012). Binge alcohol consumption has been shown to be one of the most significant risk factors for AF besides old age, male gender and HF (Magnani et al., 2011). As has been discussed, binge alcohol consumption not only affects large percentage of population but also shows a trend of rise, thus it is of pressing need to understand the nature of binge alcohol prompted AF.

1.1.4 The Underlying Mechanisms of Binge Alcohol-caused AF are Currently Unknown

To date, the underlying mechanisms of how binge drinking causes atrial arrhythmias remain unclear. One of the previous studies suggests that acute alcohol consumption can affect intra-atrial conduction in healthy men, yet the patient sample size is small in this study and the AF inducibility is not assessed (Sengul et al., 2011). Animal models have been used to study the binge alcohol induced arrhythmia. For instance, atrial tachyarrhythmia, including AF and atrial flutter, are induced in pigs after acute alcohol infusion with fast electrical pacing (burst pacing) (Anadon et al., 1996). On the contrary, Gao et al. found that acute alcohol infusion (0.4-1.6g/kg, bodyweight (BW)) delays the onset time of acetylcholine-$CaCl_2$-

induced AF in mice. While the contradicting results could be due the different nature of AF induction protocol (Y. Gao et al., 2012), it is notable that the BAC in both cases are well above baseline. This suggests that such models focus more on the direct effect of acute alcohol toxicity on atrial arrhythmia rather than the arrhythmogenic remodeling of the atrial tissue. However, previous clinical findings have shown that alcohol-induced arrhythmias usually occur when BAC recovers to normal (J. C. Clark, 1988; Denison, Jern, Jagenburg, Wendestam, & Wallerstedt, 1994), suggesting that the timeframe of the cardiac risk post binge alcohol is likely during the alcohol recovery/withdraw period which are not mimicked by the current animal studies (Anadon, et al., 1996; Y. Gao, et al., 2012). Recently, the Ai Lab developed a mouse model of repeated binge alcohol exposure and recovery (2g/kg BW, I.P., every other day, a total of four doses) that mimics the human drinking pattern during the holiday season. This mouse model will be used in my thesis study to explore the underlying mechanism of atrial arrhythmogenic substrate remodeling in repeated binge alcohol consumption.

1.2 Functional Role of Stress-response Kinase c-Jun N-terminal Kinase in Alcohol–caused Tissue Injury

1.2.1 Alcohol Exposure Promotes the Activation of c-Jun N-terminal Kinase, a Stress-responsive Kinase that Contributes to Alcohol-evoked Organ Damage

Extensive studies suggest that alcohol exposure leads to the activation of c-Jun N-terminal Kinase (JNK), a critical member of the stress-response kinase MAPKs

(mitogen-activated protein kinases), in various organs including the liver, the pancreas, and the heart (Aroor, James, Jackson, & Shukla, 2010; C. H. Lang, Derdak, & Wands, 2014; Lee, Aroor, & Shukla, 2002; S. Y. Li, Gilbert, Li, & Ren, 2009; Masamune, Kikuta, Satoh, Satoh, & Shimosegawa, 2002; McCarroll et al., 2003; Meriin et al., 1999; Nishitani & Matsumoto, 2006). For instance, alcohol exposure causes JNK activation in hepatocytes in mice (Lee, et al., 2002). Acute alcohol perfusion (50mM) in the rat liver also causes tissue damage, especially lipid peroxidation, via JNK activation (Nishitani & Matsumoto, 2006). Additionally, alcohol exposure leads to JNK activation which mediates apoptosis in gastric mucosal epithelial cells and gastric ulcer, while JNK inhibition effectively attenuates the alcohol-prompted alterations (X. J. Luo et al., 2013). Mice fed with alcohol demonstrate enhanced JNK activation in cardiac tissue, reduced cell shortening of cardiac myocytes and cardiac hypertrophy (S. Y. Li, et al., 2009). Moreover, studies also showed that acute alcohol treatment increases phosphorylated JNK but not other MAP kinases (including, extracellular signal-regulated kinases (ERK), and MAPK-p38) in isolated mouse ventricular myocytes (Duan et al., 2002; X. Zhang et al., 2005; X. Zhang et al., 2003). Also, acute alcohol treatment (8%, 1hour (hr)) results in enhanced JNK phosphorylation in ventricular cell line H9C2 while the other MAPKs are not affected (Meriin, et al., 1999). In summary, alcohol exposure causes JNK activation in various organs including the heart.

1.2.2 JNK, a Stress-responsive Protein Kinase

JNK, a Ser/Thr kinase, is an important member of stress-responsive MAPK family. JNK has three isoforms (JNK1, JNK2 and JNK3), with the major cardiac isoforms being JNK1 and JNK2 (Q. Liang et al., 2003). The structure of JNK shares similarities with other MAPKs by exhibiting typical eukaryotic protein kinase fold. In the protein kinase fold of JNK, the C-terminal domain is rich in α-helices and the N-terminal domain is rich in β-sheets. The two domains are connected by two peptide segments, which form a groove that is used for JNK substrates binding (Brown, Noble, Endicott, & Johnson, 1999; Kimberly, Zheng, Town, Flavell, & Selkoe, 2005). When JNK is activated by dual phosphorylation on a specific Thr-X-Tyr motif by upstream kinases MKK4 and MKK7 (Davis, 2000), the relative position of the two domains changes so the amino acid residues in the catalytic center are aligned to bind and phosphorylate JNK substrate (Bogoyevitch & Kobe, 2006). As a Ser/Thr kinase, activated JNK phosphorylates its substrates on Ser/Thr residues in a consensus sequence of Pro-X-Thr/Ser-Pro (Bogoyevitch & Kobe, 2006). Identified JNK substrates include but are not limited to c-Jun, JunB, ATF2, c-Myc and p53 (Bogoyevitch & Kobe, 2006). The development and application of JNK inhibitors is a currently very active field of research, which is discussed in Clinical Implications, Chapter Nine, page 170-171.

1.2.3 The Mechanisms of Alcohol-induced JNK Activation

Accumulating evidence suggests that alcohol metabolism leads to enhanced JNK activation. Alcohol metabolism primarily takes place in the liver, while the heart, brain and kidney also metabolize alcohol (Dinu, Nechifor, & Movileanu, 2005; Liew et al., 2013; Rodrigo & Rivera, 2002). Alcohol dehydrogenase (ADH) in the cytoplasm and CYP2E1 in microsome metabolize alcohol and produce acetaldehyde, while aldehyde dehydrogenase (ALDH) in the mitochondria continues the oxidation to produce acetate and in the meantime, giving rise to reactive oxygen species (ROS, e.g. mitochondrial superoxide(Murphy, 2009))(Gonthier, Jeunet, & Barret, 1991; Kukielka, Dicker, & Cederbaum, 1994). Acetaldehyde, a key alcohol metabolite, has been shown to promote JNK activation and to stimulate the production of mitochondrial ROS (R. Guo & Ren, 2010). In a mouse model of chronic alcohol intake, increased expression and activation of JNK and enhanced phosphorylation of JNK substrate c-Jun are observed in cardiac tissue, while overexpressing ALDH that attenuates JNK activation by clearing acetaldehyde (S. Y. Li, et al., 2009). In addition to acetaldehyde, the elevation of ROS also enhances JNK activation. In a mouse model of chronic alcohol administration, ROS production and JNK phosphorylation are enhanced, while inhibiting CYP2E1 abolishes the rise in JNK activation (R. H. Zhang et al., 2013). Jin et al. have shown that enhanced ROS production by CYP2E1 in alcohol exposure promotes the activation of JNK, which further enhances the transcription of CYP2E1 in SP-1-dependent pathways, forming an augmentation signaling loop

between ROS and JNK (Cederbaum, Lu, & Wu, 2009; Jin, Ande, Kumar, & Kumar, 2013; Knockaert, Fromenty, & Robin, 2011). Although alcohol-induced ROS has been shown to cause cell injury via direct oxidation of proteins, the detrimental effects of ROS are largely through ROS-regulated activation of JNK signaling cascades (Schattenberg & Czaja, 2014). This is evidenced by the striking rescue effects of JNK inhibition on alcohol-caused cell death in various cell types, such as hepatic cells and neurons (Cabrales-Romero Mdel et al., 2006; Kamata et al., 2005; Morio et al., 2013; Shah, Yoon, & Kim, 2015). Taken together, alcohol metabolism elevates the activation of JNK in acetaldehyde/ROS-mediated pathways, and alcohol exposure causes tissue and organ damage largely via JNK activation. Moreover, the Ai Lab recently discovered that JNK activation promoted atrial arrhythmia in the aged heart.

1.2.4 JNK Activation Promotes Cardiac Arrhythmia

Emerging evidence suggest that JNK activation promotes arrhythmia. Clinical findings suggest that human AF propensity increases with aging (Benjamin et al., 1994; Go et al., 2001; M. W. Rich, 2009). Recently, the Ai Lab discovered that JNK activation, and specifically, the activation of JNK2, is enhanced in human atria during aging (Figures 1A, 1B). In a rat aging model, increased JNK phosphorylation is also associated with increased burst-pacing induced AF, while inhibiting JNK phosphorylation alleviated AF propensity (D. Xu et al., 2012). Also, enhanced JNK activation contributes to increased AF inducibility in aged rabbits and in young rabbits challenged with the JNK activator anisomycin (Yan et al., 2013). These results strongly suggest that JNK

activation plays a significant and contributive role in the development of atrial arrhythmia. JNK activation has also been found in the reperfusion phase of the ischemic reperfusion injury, which is known to be associated with a high propensity of arrhythmia (Bogoyevitch et al., 1996; Siow, Choy, Leung, & O, 2000). JNK phosphorylation is also significantly increased in a mouse model of Rheb1 mutation (a small GTPase) in which malignant arrhythmia, HF and premature death were observed (Y. Cao et al., 2013). In summary, JNK could be a critical molecule that promotes arrhythmias.

Although it has been demonstrated that JNK activation promotes atrial arrhythmia in anisomycin-treated (pharmacological JNK activator) rabbits, it remains unknown how JNK activation promotes atrial arrhythmogenicity. Thus, one aim of my thesis is to test the hypothesis that JNK activation also plays critical roles in repeated binge alcohol-induced atrial arrhythmia.

Figure 1

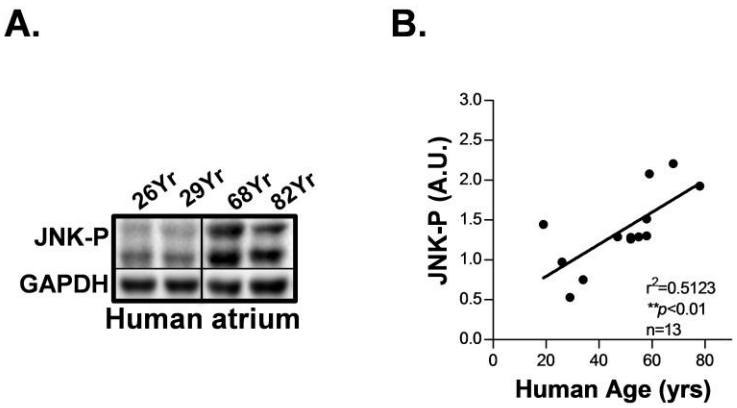

Figure 1 Activated JNK is associated with enhanced atrial arrhythmogenicity. **A)** Representative immunoblotting images and **B***)* Quantitative data showing increased phosphorylated JNK (JNK-P) in aged human atria. (Yan et al. manuscript in submission; *I would like to thank Ms.Weiwei Zhao for assistance on immunoblotting*).

1.3 Abnormal Ca^{2+} Handling and Ca^{2+} Triggered Arrhythmic Activities in Alcohol- caused Atrial Arrhythmia

1.3.1 Alcohol Promotes Cellular Abnormal Ca^{2+} Activities

A large spectrum of *in vivo* and *in vitro* models of alcohol exposure have demonstrated the alcohol-induced alteration in cellular Ca^{2+} handling in the diverse drinking patterns that have been observed in humans. Alcohol exposure has been shown to cause aberrant Ca^{2+} activities (such as Ca^{2+} waves and Ca^{2+} sparks) and altered intracellular Ca^{2+} dynamics in a large variety of animal/cell models.

In animal models of alcohol exposure, typical short term *in vivo* alcohol treatment includes single dose to 1 week of alcohol gavage (Z. Ren, Yang, et al., 2016) or I.P. injection(Ge, Guo, & Ren, 2011; R. Guo & Ren, 2010; Qiu et al., 2016). On the other hand, long term *in vivo* alcohol treatment typically ranges from 2 weeks to several months. For instance, murine models of long term consumption are usually constructed via feeding animals with alcohol-containing diet (Aistrup, Kelly, Piano, & Wasserstrom, 2006; R. Guo, Xu, Babcock, Zhang, & Ren, 2015), or I.P. injections for 2 week to 6 months (Almehmadi et al., 2014; Khan & Pandy, 2016).

Isolated cells from alcohol-exposed animals are a powerful resource to study alcohol-induced cellular Ca^{2+} mishandling. For instance, the Ren Lab have shown that ventricular myocytes from a repeated binge alcohol-exposed mice (3 g/kg/day, BW, 3 days) exhibit decreased intracellular Ca^{2+} rise, prolonged Ca^{2+} decay and reduced SERCA Ca^{2+} uptake in respond to electrical stimuli compared to myocytes

from sham mice (Ge, et al., 2011). The Wasserstrom Lab have shown that Ca^{2+} dynamics in ventricular myocytes from long term alcohol treated rats (1-3 month) alter in a time-dependent manner, specifically, ventricular myocytes from 1-month alcohol-treated rats demonstrate increased sarcoplasmic reticulum (SR, the major Ca^{2+} storage site inside the cell) Ca^{2+} content while 3-month alcohol treatment decreased SR Ca^{2+} content; yet in both groups, Ca^{2+} spark frequency is increased (Aistrup, et al., 2006). Moreover, in a rat model of fetal alcohol syndrome in which the mice are exposed to prenatal alcohol (6 g/kg/day BW, 12 days), both resting and peak level of intracellular Ca^{2+} in neonatal ventricular myocytes are increased compared to control (J. Ren et al., 2002). Such diverse results suggest that the alterations of Ca^{2+} handling from isolated ventricular myocytes are highly dependent upon alcohol exposure model.

In vitro alcohol exposure also elicits a wide spectrum of intracellular Ca^{2+} response depending on the differences in alcohol exposure model and cell type. For instance, acute alcohol exposure (1-100mM, 15-30min) in pancreatic acinar cells increases Ca^{2+} wave propagation speed which leads to enhanced protease activation (Orabi et al., 2011). The Ren Lab also found that further acute alcohol treatment (52 mM and 140 mM, 10 min) on prenatal alcohol-exposed neonatal mouse ventricular myocytes depresses the SR Ca^{2+} content (J. Ren, et al., 2002). In a cellular model of fetal alcohol syndrome, treating cultured piglet neonatal coronary arterial smooth muscle cells with alcohol (10-25 mM) for an extended period (7 days) results in

elevation of intracellular Ca^{2+} level (Altura, Zhang, Cheng, & Altura, 1996). Similarly, *in vitro* alcohol treatment can cause intracellular Ca^{2+} mishandling, and the alterations are highly dependent upon the alcohol treatment model.

To date, alcohol-caused cellular Ca^{2+} mishandling haven been documented in different cell types, moreover, different alcohol treatment strategy can elicit distinct intracellular Ca^{2+} response. The next question is that what is functional consequence of these aberrant Ca^{2+} activities?

1.3.2 Abnormal Ca^{2+} Waves are Critical for Arrhythmia Initiation

Abnormal Ca^{2+} activities are known to be a critical contributor in the initiation of arrhythmias (Figure 2) (Pogwizd & Bers, 2004; Rubart & Zipes, 2005). In a normal systolic phase of a cardiac cycle, the plasma membrane of cardiac myocytes depolarizes and leads to the opening of L-type Ca^{2+} channels. Ryanodine receptors (RyR), the main Ca^{2+} release channel on the sarcoplasmic reticulum (SR), open in response to Ca^{2+} entry via L-type Ca^{2+} channels and transiently increase intracellular Ca^{2+} (Bers, 2000). The rise of intracellular Ca^{2+} drives the activation of contractile machinery (Bers, 2000). RyR channels are usually closed during diastole, however, diastolic RyR opening is enhances in certain pathological conditions. The increased RyR opening promotes the spontaneous diastolic SR Ca^{2+} release that can evoke abnormal Ca^{2+} activities, such as Ca^{2+} waves (Ai, Curran, Shannon, Bers, & Pogwizd, 2005; Bers, 2002, 2014; H. Cheng, Lederer, Lederer, & Cannell, 1996; N. Li et al., 2014; Respress et al., 2012; Venetucci, Trafford, O'Neill, & Eisner, 2008). The

formation of Ca^{2+} waves involves the local Ca^{2+}-induced- Ca^{2+}-release (CICR) which is facilitated by the existence of hundreds of RyRs in a single Ca^{2+} release unit (Franzini-Armstrong, Protasi, & Ramesh, 1999; Soeller, Crossman, Gilbert, & Cannell, 2007). Enhanced diastolic SR Ca^{2+} leak through RyR channels, which increase intracellular Ca^{2+} concentration, resulting in the activation of the electrogenic Na^{+}/Ca^{2+} exchanger (NCX)(Lehnart et al., 2008; Sugai et al., 2009). Upon activation, NCX allows 3 Na^{+} into the cytosol in exchange of 1 Ca^{2+} out of the cell which further depolarizes the plasma membrane and causes arrhythmogenic delayed after depolarizations (DADs) (Bers, 2000, 2014; Maruyama et al., 2010; Rubart & Zipes, 2005). Once DADs reach the activation threshold of the Na^{+} channel, ectopic beats arise, which could further depolarize the surrounding cardiac tissue and culminate into episodes of arrhythmia (Maruyama, et al., 2010). Work in the 1990s has shown that large majority of arrhythmias (>90%) were initiated by nonreentrant mechanisms involving DAD and early afterdepolarization (EAD)(Pogwizd & Corr, 1992), while both mechanisms depend on aberrant Ca^{2+} released from SR (Burashnikov & Antzelevitch, 2003; P. S. Chen et al., 2012; Choi & Salama, 2000; Zhao et al., 2012).

Figure. 2

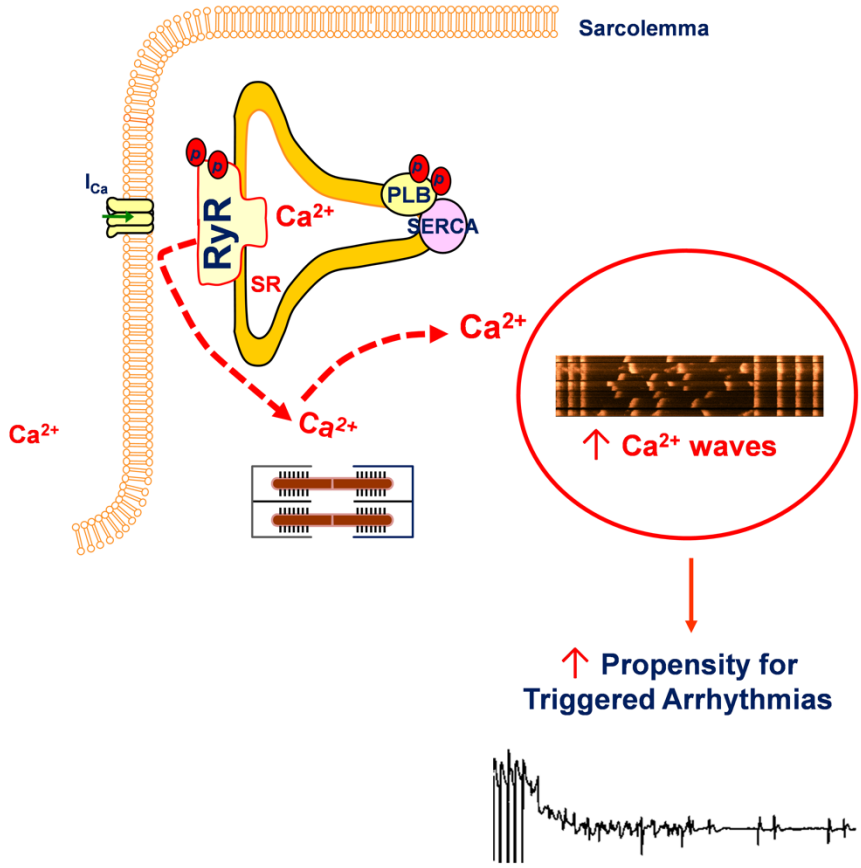

Figure 2. Schematic of SR Ca^{2+} leak induced arrhythmogenic Ca^{2+} waves and arrhythmia. In certain pathological conditions, including AF and heart failure, diastolic SR Ca^{2+} leak from RyR increases local Ca^{2+} concentration which promotes the reverse opening of NCX that leads to the arise of DAD, ectopic beats and triggered arrhythmias.

Increased diastolic SR Ca^{2+} leak leads to abnormal Ca^{2+} waves, which is arrhythmogenic. Ca^{2+} waves have been observed in various cardiac conditions involving arrhythmogenesis (W. Chen et al., 2014; Ferrier, 1977; Gonano et al., 2011; Kass, Lederer, Tsien, & Weingart, 1978; Kass, Tsien, & Weingart, 1978; Lederer & Tsien, 1976; Nuss, Kaab, Kass, Tomaselli, & Marban, 1999; Pogwizd & Bers, 2004). For instance, Ca^{2+} waves are pivotal for the initiation of ventricular tachyarrhythmia in HF (Pogwizd & Bers, 2004). Ca^{2+} waves also underlie the cause of arrhythmia due to digoxin toxicity where DADs are first observed (Gonano, et al., 2011; Venetucci, et al., 2008). Ca^{2+} waves also underlie catecholaminergic polymorphic ventricular tachycardia due to mutations in RyR and other Ca^{2+} handling proteins (Priori & Chen, 2011).

Further studies in animal models provide abundant evidence supporting the arrhythmogenic roles of Ca^{2+} waves. For instance, in a mouse model of dilated cardiomyopathy carrying a loss-of-function mutation of SR Ca^{2+} binding protein (histidine-rich Ca^{2+}-binding protein with S96A mutation), Ca^{2+} wave frequency in isolated ventricular myocytes is 10-fold higher compared to wildtype (WT) littermates, meanwhile arrhythmogenicity is also enhanced (Singh et al., 2013). Challenging the hearts from a transgenic (Tg) mouse line carrying RyR mutation (R2474S) with high Ca^{2+}concentration perfusion or isopreterenol provides evidence that increased frequency of Ca^{2+} waves is associated with ventricular ectopy and spontaneous ventricular tachycardia (Lehnart, et al., 2008). Similar observations are made in a Tg mouse line with mutations in β-adrenergic/PKA pathways (Haghighi et al., 2015;

Schulte et al., 2016). Ca^{2+} waves and SR Ca^{2+} mishandling also underlie atrial arrhythmia. The increased frequency of spontaneous Ca^{2+} release events are associated with increased episodes of inducible AF in a model of junctophilin-2 mutation than in WT mice (Beavers et al., 2013). Using confocal Ca^{2+} imaging combined with pseudo-ECG recording, Xie et al. even directly showed that burst pacing-induced atrial Ca^{2+} waves proceeded atrial arrhythmia (Xie et al., 2013). At the same time, the ectopic Ca^{2+} activity are sensitive to RyR channel blocker, such as ryanodine, which further suggest the involvement of RyR dysfunction in the rise of Ca^{2+} waves (Maruyama, et al., 2010). Zhang et al. also have also shown that ablating Ca^{2+} waves in ventricular myocyte with pharmacological reagents (non-β-blocking R-carvedilol) alleviates the stress-induced ventricular arrhythmia (J. Zhang et al., 2015). In summary of the previously discussed data from different animal models, such findings establish a logic line that SR Ca^{2+} release due to RyR remodeling promotes the rising of Ca^{2+} waves and further triggers arrhythmia. Thus, it is of vital importance to understand whether Ca^{2+} waves also play pivotal roles in repeated binge-drinking caused atrial arrhythmias.

1.3.3 Repeated Binge Alcohol Exposure Mouse Model and Alcohol-treated Cell Model used in This Thesis

Although as previously introduced in Chapter One (section 1.3.1), different animal and cell models of alcohol exposure have been used it study alcohol-induced Ca^{2+} mishandling, to date, there is not an animal model that mimics the human holiday drinking pattern with alternating binge alcohol episodes and recovery and is

used in studying the atrial Ca^{2+} mishandling. In this thesis, I used a mouse model developed in the Ai Lab that exhihibits alternated binge alcohol episode (2 g/kg BW, I.P., for a total of 4 doses, every other day) and recovery (Figure 5A) to study atrial intracellular Ca^{2+} activity after repeated binge alcohol exposures. The detailed characterization of this model will be described in Chapter Three, page 45-49.

During binge drinking, peak BAC is approximately 50mM in young and healthy adults (230 mg/dL) (McMillen, Hillis, & Brown, 2009), which drops to about 17.4mM (80 mg/dL, legal intoxication level) 2 hours after a binge alcohol episode of 4-5 drinks ("Vital signs: binge drinking prevalence, frequency, and intensity among adults-U.S., 2010," 2012). A common strategy to design *in vitro* alcohol exposure model is to mimic the *in vivo* BAC with the alcohol concentration in the cell medium. However, due to the fact that *in vivo* alcohol metabolism follows the first-order elimination kinetics, alcohol is usually cleared from the system within hours (Greiffenstein, Mathis, Stouwe, & Molina, 2007; Matyas et al., 2016). This could presents a challenge for *in vitro* cell models to closely mimic the pattern of alcohol level decline in animal body. In my thesis study, medium containing 50 mM alcohol was used to treat HL-1 cells (an atrial cell line (Claycomb et al., 1998; S. M. White, Constantin, & Claycomb, 2004)), and the medium alcohol concentration dropped to approximately 15 mg/dL after 24 hours (3.3 mM, Figure 19B), which is still significantly higher than sham control level (15.79 ± 0.42 mg/dL vs. 0.29 ± 0.23 mg/dL in sham, n= 5, 4, N = 2, $p<0.001$; Figure 19B). These *in vitro* models of alcohol

exposure are discussed in details Chapter Six, page 99-101. With this mouse and cell model, I aim at exploring the alcohol-prompted cardiac remodeling including Ca^{2+} mishandling and the underlying mechanisms.

1.3.4 CaMKII-dependent RyR Phosphorylation Promotes SR Ca^{2+} Leak and Ca^{2+} Waves

Accumulating evidence has established that increased RyR opening contributes to enhanced SR Ca^{2+} leak and the downstream arrhythmogenic events (D. A. Eisner, Kashimura, O'Neill, Venetucci, & Trafford, 2009; D. A. Eisner, Kashimura, Venetucci, & Trafford, 2009). RyR is a phospho-protein that can be targeted by various kinases (Houser, 2014; O'Brien, Venturi, & Sitsapesan, 2015). CaMKII, a pro-arrhythmic Ser/Thr kinase, is able to phosphorylate RyR at the site of Ser2815 that leads to enhanced diastolic SR Ca^{2+} leak (Camors & Valdivia, 2014; Greenstein, Foteinou, Hashambhoy-Ramsay, & Winslow, 2014).

The activation of CaMKII has been found in multiple pathological conditions of the heart involving both atria and ventricle, and in almost every case, CaMKII activation promotes the phosphorylation of RyR and downstream Ca^{2+} mishandling. For instance, enhanced CaMKII activation has been found in human chronic AF (Heijman, Voigt, Nattel, & Dobrev, 2014; Heijman, Voigt, Wehrens, & Dobrev, 2014). CaMKII activation and CaMKII-dependent RyR phosphorylation are enhanced in aged mice which demonstrated increased propensity for intraesophageal atrial electrical stimulation induced atrial arrhythmia (X. Guo, Yuan, Liu, & Fang, 2014).

Also, electrical stimulation enhances CaMKII activity that results in increased SR Ca^{2+} leak in isolated atrial myocytes (Qin et al., 2011). In both human ischaemic and dilated cardiac myopathy, enhanced CaMKII activation and CaMKII-dependent RyR phosphorylation contribute to increased frequency of diastolic Ca^{2+} sparks, Ca^{2+} waves and spontaneous transients, while CaMKII inhibition (with AIP) reduces SR Ca^{2+} leak by 80% (Fischer et al., 2014; Maier et al., 2003; Sossalla et al., 2010). Moreover, overexpression of CaMKII increases SR Ca^{2+} leak, leading to increased DAD and EAD at isopreterenol challenge. This further demonstrates the arrhythmogenic role of CaMKII activation (Sag et al., 2009).

On the other hand, inhibiting CaMKII or blocking CaMKII-dependent RyR phosphorylation has been shown to alleviate the spontaneous Ca^{2+} events and arrhythmia. For instance, spontaneous Ca^{2+} waves in isopreterenol-challenged ventricular myocytes are largely ablated by treatment of KN93, a CaMKII inhibitor (Curran et al., 2010). Said et al. found that inhibiting CaMKII in ischemia/reperfusion injury decreased the propensity of EADs and premature ectopic beats (Said et al., 2011). In a model of digoxin toxicity, suppressing CaMKII with KN93 or AIP also suppresses Ca^{2+} waves hence arrhythmia (Gonano, et al., 2011). In a mouse model of dilated cardiomyopathy induced by mutation of SR Ca^{2+} binding protein (SR histidine-rich protein with S96A mutation that causes human ventricular arrhythmia), CaMKII-dependent phosphorylation of RyR is increased in ventricular myocytes while KN93 treatment alleviated the Ca^{2+} wave in isolated

myocytes (Singh, et al., 2013). Also, knocking in mutant RyR-S2815A (ablating the CaMKII phosphorylation site) protects mouse heart from HF after cardiac pressure overload (induced via transverse aortic constriction), also, ventricular myocytes from the mutant mice demonstrated less SR Ca^{2+} leak compared to WT littermates after the same transverse aortic constriction manipulation (Respress, et al., 2012). Overall, evidence from various arrhythmogenic cardiac disease models suggests a similar pathway: enhanced CaMKII activation promotes SR Ca^{2+} leak and SR Ca^{2+} waves through hyperphosphorylation of RyR.

1.3.5 The Mechanisms of CaMKII Activation in Alcohol Exposure

Canonical CaMKII activation depends on Ca^{2+} and calmodulin. As intracellular Ca^{2+} concentration increases, Ca^{2+} binds to calmodulin forming Ca^{2+}/calmodulin complex which can bind to inactivated CaMKII. The binding of Ca^{2+}/calmodulin to CaMKII causes a configuration change of CaMKII that promotes its autophophorylation at Thr286 residue thus CaMKII become activated (Schulman & Greengard, 1978). Besides canonical activation, CaMKII can also be activated by direct oxidation of redox-sensitive amino acid sites Met280/281(Erickson, 2014; Erickson, He, Grumbach, & Anderson, 2011; Luczak & Anderson, 2014). Evidence of redox-sensitive CaMKII activation in the cardiovascular system is abundant. For instance, cardiac myocytes briefly treated with H_2O_2 demonstrate increased CaMKII in both autophosphorylation and direct oxidation, while KN93 fully reverses H_2O_2-induced CaMKII activation (Song et al., 2011). In H_2O_2-treated ventricular myocytes,

oxidation-induced CaMKII activation also promotes SR Ca^{2+} leak similar to the canonically-activated CaMKII (H. Li et al., 2013). In a mouse model of early stage diabetes, increased CaMKII oxidation promotes CaMKII-dependent RyR phosphorylation and spontaneous Ca^{2+} release events in the heart that can be suppressed by CaMKII inhibition (Sommese et al., 2016). Overall, ROS-induced CaMKII activation exerts similar functional consequences in promoting RyR phosphorylation and Ca^{2+} mishandling as that of activated CaMKII via the canonical pathways.

Alcohol has been shown to elevate ROS in multiple sources of organ and tissue where alcohol metabolism takes place, and the heart has been proved to be one of such organs (Dinu, et al., 2005; Liew, et al., 2013; Rodrigo & Rivera, 2002), which could in turn promote CaMKII activation. CaMKII activation has also been found in alcohol exposure. The majority of these studies were performed in the neural system in which the major subtype of CaMKII is CaMKIIα and CaMKIIβ (Hell, 2014). For instance, chronic alcohol intake upregulates CaMKII expression in reward-related brain region (Faccidomo, Reid, Agoglia, Ademola, & Hodge, 2016). CaMKII phosphorylation and CaMKII-dependent AMPA receptor phoshprylation are both enhanced in mouse amygdala 24 days of after alcohol drinking (Salling et al., 2016). On the contrary, immediately after binge-drinking, CaMKII phosphorylation is decreased in the amygdala from adolescent but not adult mice (Agoglia, Holstein, Reid, & Hodge, 2015). CaMKII activation is also enhanced in alcohol-induced

conditioned place preference in mice (Easton et al., 2013). Interestingly, recent data have shown that CaMKII is a downstream effector of ROS, while inhibiting CaMKII decreases ROS production suggesting a positive-feedback loop between ROS production and CaMKII activation (Nishio et al., 2012; Odagiri et al., 2009; Zhu et al., 2014).

To date, the status of CaMKII activation in binge alcohol exposed heart remains unknown. Also, knowledge of whether direct oxidation plays a major role in alcohol-related CaMKII activation in the heart is lacking. In this thesis, CaMKII activation status and activation mechanisms will be explored in both binge alcohol exposed mouse hearts and alcohol-treated atrial cells. Also, I will examine whether alcohol promotes CaMKII activation in a ROS-dependent manner in HL-1 atrial myocytes and HEK293 cells with the overexpression of genetically modified CaMKII proteins.

1.3.6 CaMKII-dependent RyR Function in Alcohol Exposure

Although alcohol has been shown to activate CaMKII in neuronal systems in different models (Faccidomo, et al., 2016; Salling, et al., 2016), and CaMKII-dependent RyR phosphorylation can increase RyR opening probability (Camors & Valdivia, 2014; Sommese, et al., 2016), previous studies yielded limited and inconclusive results on the impact of alcohol on RyR opening probability. Ye et al. found that acute alcohol treatment (10-100 mM) decreased RyR channel activity in SR vesicles extracted from HEK293 cells overexpressing rabbit cardiac RyR2 (Ye,

Jian, Jaggar, Bukiya, & Dopico, 2014). Yet RyR from frog skeletal muscles are shown to be insensitive to 2.2-217mM alcohol treatment (Oba, Ishikawa, Murayama, Ogawa, & Yamaguchi, 2000; Oba, Koshita, & Yamaguchi, 1997), on the other hand, the opening probability of alcohol-treated RyR channels increased significantly by adding additional H_2O_2 (10-100mM) although H_2O_2 alone did not increase RyR opening probability (Oba, et al., 2000). The different results may be attributed to different treatment condition or even different sources and extraction methods of the RyR channels. Recently, collaborative work from the Fill Lab shows acute alcohol treatment (39mM, 30min) increased RyR single channel opening probability, while inhibiting JNK abolished the alcohol-induced alterations (Figures 3B). This result suggests that acute alcohol treatment increases RyR single channel activity via JNK activation. Immunoblotting studies further showed the presence of SERCA, RyR, JNK2, CaMKII in human atrial SR vesicles used in RyR single channel recordings while the JNK1 level is very low (Figure 3C). On the other hand, JNK 1 and JNK2 are both present in whole human atrial tissue homogenates, suggesting that the different distribution of JNK1 and JNK2 in different cell compartments.

Figure 3

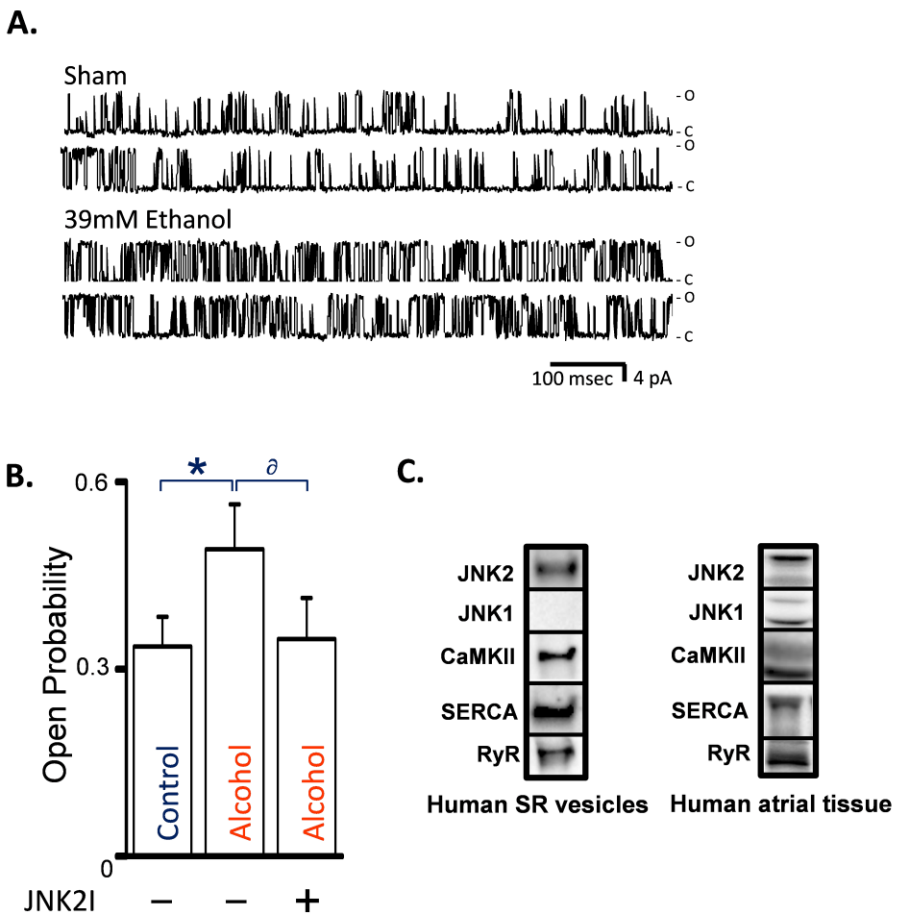

Figure 3. Alcohol treatment increases RyR single channel opening probability **A)** Example recordings showed increased RyR single channel opening in alcohol (39mM) treated human SR vesicles **B)** Summarized data showing increased RyR single channel opening probability which was reversed by JNK2 inhibitor JNK2I (JNK inhibitor-IX). *Courtesy of Fill Lab for collaborative work* **(A-B)** *with Dr.Ai on RyR single channel recording.* **C)** Immunoblotting showing that isolated human SR vesicles used for single channel recording contain JNK2, CaMKII, SERCA, and RyR, but not JNK1; on the other hand, immunoblotting of human atrial tissue showed the presence of JNK1 protein in addition to the other proteins. *I would like to thank Ms.Weiwei Zhao for assistance on immunoblotting.* All data were presented as Mean ± SEM. One-Way ANOVA test with Tukey post hoc test was used to compare the mean of selected groups.

1.4 SR Ca^{2+} Overload as Another Potential Mechanism in Alcohol-JNK Promoted Abnormal Ca^{2+} Waves

1.4.1 SR Ca^{2+} Overload can Promote Arrhythmogenic Spontaneous Ca^{2+} Release

In addition to increased CaMKII-dependent phosphorylation of RyR that increases the SR Ca^{2+} leak, SR Ca^{2+} overload has also been recognized as mechanism of arrhythmogenesis via triggered Ca^{2+} activities (W. Chen, et al., 2014; T. Guo, Gillespie, & Fill, 2012; Marx et al., 2000; Shannon, Pogwizd, & Bers, 2003; Venetucci, Trafford, & Eisner, 2007). Accumulating evidence suggests that SR Ca^{2+} overload can increase the RyR channel activity by triggering the RyR luminal Ca^{2+} sensor hence promoting spontaneous Ca^{2+} release events (J. Zhang et al., 2014a).

SR Ca^{2+} overload has been found in various cardiac conditions (Bompotis et al., 2015; Lindegger, Hagen, Marks, Lederer, & Kass, 2009; Neef et al., 2010; Pluteanu et al., 2015; Voigt et al., 2014; Yeh et al., 2008), and is associated with an increased frequency of triggered activities and enhanced arrhythmias (Nassal, Wan, Laurita, & Cutler, 2015; Yeh, et al., 2008). For instance, Ca^{2+} overload due to altered cAMP metabolism promotes spontaneous Ca^{2+} release that culminates into DADs and ventricular arrhythmia via triggered activities (Lerman, Belardinelli, West, Berne, & DiMarco, 1986). Another prominent example is the mechanism of digoxin toxicity. In this case, increased SR Ca^{2+} load induced by increased intracellular Na^+ level due to inhibited Na^+/K^+ ATPase promotes arrhythmia via increased

spontaneous SR Ca^{2+} release (D. A. Eisner & Lederer, 1980; D. A. Eisner, Lederer, & Vaughan-Jones, 1981; Venetucci, et al., 2008). Ca^{2+} waves and sparks are also observed in SR Ca^{2+} overload situation during ischemia and reperfusion injury in Langendorff-perfused intact hearts (Mattiazzi, Argenziano, Aguilar-Sanchez, Mazzocchi, & Escobar, 2015). Spontaneous SR Ca^{2+} release has also been documented in cell models of SR Ca^{2+} overload. For instance, in ventricular myocytes, increasing SR Ca^{2+} load (by treatment of forskolin) enhances the frequency of spontaneous Ca^{2+} waves (Curran, et al., 2010). Also, increasing SR Ca^{2+} load by elevated extracellular Ca^{2+} level (10mM) has been shown to promote Ca^{2+} sparks and Ca^{2+} waves in ventricular myocytes (H. Cheng, et al., 1996).

Interestingly, increased SR Ca^{2+} load has also been observed when myocytes are challenged with alcohol or increased ROS. For instance, treating ventricular myocytes with H_2O_2 (200μM) led to SR Ca^{2+} overload mediated by CaMKII activation, which further enhanced SR Ca^{2+} leak (H. Li, et al., 2013). In animal models of alcohol exposure, SR Ca^{2+} content is increased in rat mesenteric lymphatic vessels after the treatment of intragastric administration of alcohol (Souza-Smith, Kerut, Breslin, & Molina, 2015). Emerging evidence also indicates that the alcohol-induced alteration in SR Ca^{2+} load in cardiac myocytes can be biphasic and the results largely depend on the alcohol treatment strategy. For instance, ventricular myocytes from 1-month alcohol fed rats showed increased SR Ca^{2+} load while cells from 3-month alcohol fed rats showed decreased SR Ca^{2+} load; on the other hand, transient high concentration

of alcohol (1.5%) significantly decreased SR Ca^{2+} load in isolated sham ventricular myocytes, while low concentrations of alcohol (0.15-0.3%) did not exert a significant impact (Aistrup, et al., 2006).

It is well-acknowledged that atrial SR Ca^{2+} content is higher than that of ventricular myocytes (Walden, Dibb, & Trafford, 2009). Thus, this theoretically would make atrial myocytes more prone to spontaneous diastolic SR Ca^{2+} release (Bers, 2014; Chelu et al., 2009; Neef, et al., 2010; Venetucci, et al., 2008). On the other hand, ablating the RyR luminal Ca^{2+} sensing function by mutating an essential amino acid site (E4872Q) on the RyR inner loop region has been shown to both decrease spontaneous Ca^{2+} waves and alleviate susceptibility to stress-induced arrhythmia (J. Zhang, et al., 2014a), further suggesting that SR luminal Ca^{2+} sensing plays an important role in arrhythmogenicity. However, whether binge alcohol alters SR Ca^{2+} content in atrial myocytes remains unknown to date.

1.4.2 Sarcoplasmic Reticulum Ca^{2+} ATPase (SERCA) Activity Is Pivotal to SR Ca^{2+} Load

SERCA is the primary Ca^{2+} pump that restores Ca^{2+} to the SR and maintains intracellular Ca^{2+} homeostasis, which results in the relaxation of myocytes during diastole (D. Eisner, Bode, Venetucci, & Trafford, 2013; Inesi, Ebashi, & Watanabe, 1964; Inesi, Sumbilla, & Kirtley, 1990; Stammers et al., 2015; Zarain-Herzberg, MacLennan, & Periasamy, 1990). The role of SERCA in building SR Ca^{2+} load to the threshold of generating Ca^{2+} waves has been well-recognized. Lukyanenko et al.

demonstrated that increasing extracellular Ca^{2+} concentration promotes the occurrence of Ca^{2+} waves while inhibiting SERCA with thapsigargin ablates the Ca^{2+} waves (Lukyanenko, Subramanian, Gyorke, Wiesner, & Gyorke, 1999). It is also suggested in mice experiencing fast atrial pacing, CaMKII activation could increase SR Ca^{2+} load by enhancing SERCA activity (Chelu, et al., 2009). Increased SERCA abundance and activity have also been implicated in increased SR Ca^{2+} load (Nassal, et al., 2015; Voigt, et al., 2014). Furthermore, in cardiac myocytes from a mouse model of heterozygous SERCA knockout, where SERCA-dependent Ca^{2+} reuptake is slower than control, Ca^{2+} waves occur in lower frequency when cells are challenged with increased external Ca^{2+} (10mM) (Stokke et al., 2011). To date, the SERCA function in alcohol-exposed atrial myocytes has not been explored. In this thesis, whether alcohol treatment alters SERCA function will be explored as a potential mechanism of alcohol-induced alteration in SR Ca^{2+} load.

1.5 Other Potential Arrhythmogenic Substrates that Contribute to Alcohol-prompted AF

It is well known that abnormal Ca^{2+} activities prompts arrhythmias via triggered mechanism, on the other hand, electrical remodeling of cardiac membrane ion channels (Ca^{2+} channels and K^{+} channels) have been found to be associated with the development of AF (Christ et al., 2004; Nattel, Maguy, Le Bouter, & Yeh, 2007), studies suggest that the atrial ion channel remodeling occur long before the onset of AF, and the correlation with the occurrence of sustained AF is in patients and animal

modes are not clear (Kanagaratnam, Kojodjojo, & Peters, 2008; van der Velden et al., 2000). The contribution of ion channels to AF occurance and AF-induced ion channel remodeling are still controversial. For instance, increased vagal activity has been shown to promote AF by stabilizing atrial reentry (Kneller et al., 2002) , however, the expression of inward-rectifier K^+ channel, which mediates cardiac vagal effect, is decreased in AF patients (Dobrev et al., 2005). Moreover, PKC inhibition reduces inward rectifier K^+ channel, yet in AF patients, PKC is upregulated (Voigt et al., 2007). These evidences bring controversy on whether inward-rectifier K^+ channel is a major contributor to AF via mediating vagal activity.

The development of reentrant substrate promotes the sustaining of arrhythmia. The Ai Lab and others have recently discovered that JNK activation promotes the remodeling of connexin43 (Cx43), the major gap junction protein in atria and ventricles (Jones & Lancaster, 2015; Yan, et al., 2013). The gap junction remodeling further causes decreased conduction velocity and altered conduction patterns in both atrial tissue and in atrial cell monolayers. Such alterations in action potential conduction form reentrant substrates and enhance the propensity for arrhythmia (Jones & Lancaster, 2015; Yan, et al., 2013). However, my preliminary data using optical mapping to measure atrial conduction velocity showed that there is no significantly decreased atrial conduction velocity in repeated binge alcohol exposed mice (Figure 4A). Also, the Ai Lab showed unaltered Cx43 abundance in mouse atria

in alcohol-exposed mice compared to sham controls (Figure 4B). These data suggest that gap junction may not be a major player in alcohol-evoked atrial arrhythmias.

Figure 4

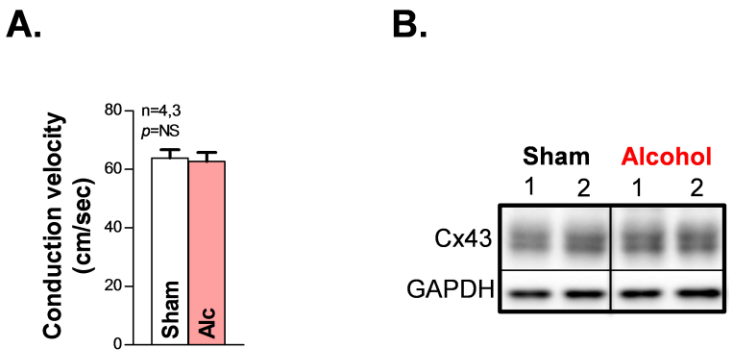

Figure 4. Cardiac conduction velocity and Cx43 expression in binge drinking-exposed mouse heart **A)** Unaltered atrial action potential conduction velocity **B)** Unaltered Cx43 expression level compared to sham controls. *I would like to thank Ms.Weiwei Zhao for assistance on immunoblotting.* All data were presented as Mean ± SEM. Unpaired Student *t*-test was used to compare the mean of selected groups.

Besides gap junctions, fibrosis can form non-conductive zones that block the action potential propagation and promote reentry. Indeed, structural remodeling has been observed in various arrhythmogenic cardiac diseases in human (Ohkubo et al., 2010; Stevenson, Weiss, Wiener, & Nademanee, 1989) and in animal models (Nisbet et al., 2016). It has been shown that JNK activation plays active roles in fibrosis formation. For instance, Cardin et al. have also shown that in a burst pacing-induced congestive heart failure (CHF) canine model, increased level of angiotensin and MAPK (JNK, MAPK-p38 and ERK) activation promote fibrosis formation in the atria (Cardin et al., 2003).

Previous research suggests that cardiac interstitial fibrosis formation relies on the activation of inflammatory cytokines and growth factors, including but not limited to angiotensin II, TGF-β and platelet-derived growth factor, which contributes to the activation of cardiac fibroblast via complex cell signaling cascades (Czubryt, 2012; Nattel, Burstein, & Dobrev, 2008). It is known that angiotensin II promotes the expression of TGF-β, which promotes the production of angiotensin II that forms a signaling amplification loop (Rosenkranz, 2004). Angiotensin II-induced JNK activation has been shown to promote fibroblast activation (Hunyady & Catt, 2006; H. D. Xiao et al., 2004). Activated fibroblasts are capable of generating large amount of extracellular matrix (ECM) and contribute to ECM remodeling (Crawford, Haudek, Cieslik, Trial, & Entman, 2012). The fibrosis formation typically happens in the time scale of weeks after the tissue insult (Czubryt, 2012), as Cardin

et al. have observed that burst pacing induced atrial fibrosis peaks at 5 weeks after burst pacing (Cardin, et al., 2003). Also, cardiac fibrosis is usually observed in long term alcohol exposed-human (Sokolova, 2016) and animals (W. Liu, Li, Tian, Xu, & Zhang, 2011; Steiner, Pruznak, Navaratnarajah, & Lang, 2015; Vasdev, Chakravarti, Subrahmanyam, Jain, & Wahi, 1975). In my thesis study, the mice were exposed to only one week of repeated binge alcohol at the time of terminal study. Thus it is very unlikely that the fibrosis formation plays a major role in the arrhythmogenic remodel. However, the fibrosis formation status will be tested in future studies.

Taken together, considering the essential role of aberrant Ca^{2+} activities (especially Ca^{2+} waves) in arrhythmogenicity, I focus on exploring the role of alcohol-driven JNK activation in Ca^{2+} triggered arrhythmia activities using our repeated binge alcohol model in my thesis work.

CHAPTER TWO

HYPOTHESES AND SPECIFIC AIMS

Binge alcohol drinking dramatically increases the risk for atrial fibrillation (AF), the most common cardiac arrhythmias. Clinical findings have shown that one-third of all new-onset AF cases are related to alcohol intoxication. Binge drinking-evoked AF leads to significant morbidity and mortality among otherwise healthy individuals, and imposes a huge economic burden on our society. To date, effective treatment for binge drinking-induced AF remains ineffective. This is largely due to an incomplete understanding of the underlying mechanisms of alcohol-induced AF genesis.

The Ai Lab has recently discovered that increased activation of the stress-activated kinase c-Jun N-terminal kinase (JNK) promotes atrial arrhythmia in aged rabbits and mice. Alcohol exposure has been found to lead to the activation of JNK in various animal models. However, the functional impact of alcohol-induced JNK activation on atrial arrhythmogenesis is completely unknown to date.

Aberrant sarcoplasmic reticulum (SR) Ca^{2+} activities (including Ca^{2+} waves) are responsible for enhanced arrhythmogenesis in diseased hearts. Ca^{2+} waves stem

from increased SR Ca^{2+} leak from the Ca^{2+}-induced-Ca^{2+}-release (CICR) channel ryanodine receptor (RyR) on the SR. Recent findings in the Fill Lab demonstrated that alcohol exposure leads to increased RyR channel opening probability in isolated human atrial SR vesicles, while JNK specific inhibition completely abolishes this alcohol action on RyR channel activity. All these findings made me hypothesize that JNK activation plays pivotal role in alcohol-induced aberrant SR Ca^{2+} activities. Therefore, in Aim1 I will **explore the role of alcohol-prompted JNK activation in the inducibility of atrial arrhythmias and aberrant atrial Ca^{2+} activities (Ca^{2+} waves and altered intracellular Ca^{2+} decay) in a newly developed mouse model of repeated binge alcohol exposure**.

The next goal is to determine the underlying mechanisms of alcohol-enhanced JNK prompting arrhythmogenic Ca^{2+} activities. It is well established that Ca^{2+} waves stem from diastolic SR Ca^{2+} leak. CaMKII is a well-known pro-arrhythmic molecule that critically contributes to abnormal diastolic SR Ca^{2+} leak. CaMKII activation has been found to promote SR Ca^{2+} leak in various pathological models. Besides the enhanced intracellular Ca^{2+} concentration, intracellular ROS has also been indicated to promote CaMKII activation. Yet the underlying mechanisms of alcohol-promoted CaMKII activation are currently unknown. In addition to CaMKII-dependent SR Ca^{2+} leak, increased SR Ca^{2+} content has also been found to promote diastolic SR Ca^{2+} leak via enhanced RyR channel sensitivity. Therefore, I will test **the hypothesis that alcohol-evoked JNK activation leads to diastolic SR Ca^{2+} leak**

via JNK-dependent CaMKII activation (Aim 2) and SR Ca^{2+} overload (Aim 3).

Aim 1: Repeated binge alcohol-driven JNK activation promotes abnormal Ca^{2+} activities and atrial arrhythmias.

A mouse model of repeated binge alcohol exposure will be evaluated in this aim. JNK activation status and its contribution to burst pacing-induced atrial arrhythmia propensity, as well as aberrant diastolic SR Ca^{2+} waves will be assessed in intact hearts from repeated binge alcohol-exposed mice. To mimic alcohol-induced JNK activation, JNK activator-treated young mice will also be studied. To further dissect the role of JNK activation in repeated binge alcohol induced atrial arrhythmogenicity, a transgenic mouse strain with genetic overexpression of dominant negative JNK1 and JNK2 (JNK1/2dn) will be used for the proposed studies.

Aim 2: JNK-activated CaMKII underlies alcohol-induced diastolic SR Ca^{2+} mishandling

In this aim, I will study how alcohol-activated JNK prompts diastolic SR Ca^{2+} mishandling. CaMKII activation is known to prompt diastolic SR Ca^{2+} leak by phosphorylating RyR. I will test the novel hypothesis that **alcohol-evoked JNK activation leads to diastolic SR Ca^{2+} leak via CaMKII activation.** Activation of CaMKII will be assessed in the binge alcohol-treated mouse atria. A pharmacological CaMKII inhibitor (KN93) and a transgenic (Tg) mouse line with cardiac specific overexpression of the CaMKII inhibitor AIP will be used to assess the functional

contribution of the CaMKII on alcohol/JNK-driven SR Ca^{2+} mishandling. In addition, the mechanisms of binge alcohol induced CaMKII activation will be further explored using HEK293 cells overexpressing wildtype and mutated CaMKII.

Aim 3: SR Ca^{2+} overload as a potential underlying mechanism of alcohol-induced diastolic SR Ca^{2+} mishandling

Besides CaMKII-dependent RyR remodeling, increased SR Ca^{2+} load can also promote SR Ca^{2+} leak. I will further test the hypothesis that **alcohol-evoked JNK activation leads to diastolic SR Ca^{2+} leak via SR Ca^{2+} overload.** SR Ca^{2+} load will be assessed in alcohol-exposed and JNK-activated HL-1 myocytes, and the role of JNK/CaMKII activation in alcohol-induced SR Ca^{2+} load alteration with be further studied with specific pharmacological inhibitors. Moreover, a transgenic mouse line with loss-of-function mutated SR luminal Ca^{2+} sensor (RyR-E4872$Q^{+/-}$) will be used to further explore the role of SR Ca^{2+} overload in the occurrence of aberrant atrial Ca^{2+} activities.

Significance and Innovation

My proposed studies have the potential to contribute to the existing field of research in several ways. **First,** although it is known that JNK activation is critical in alcohol-induced organ/tissue injury, the role of JNK in alcohol-induced arrhythmias remains completely unknown to date. Specifically, the contribution of alcohol-promoted JNK activation in Ca-triggered atrial arrhythmias is completely unknown. This proposal tests a novel hypothesis that JNK activation is a predominant

regulator of alcohol-evoked SR Ca^{2+} leak. **Second,** this proposal is to explore a novel hypothesis that alcohol promoted SR Ca^{2+} leak is through JNK-regulated CaMKII activation and SR Ca^{2+} overload. **Third**, the results of JNK inhibition will shed light on modulating JNK activity as a novel therapeutic approach to treat and prevent alcohol-evoked AF genesis.

CHAPTER THREE

BINGE ALCOHOL ANIMAL MODEL

3. 1 Abstract

AF is one of the most commonly clinically diagnosed cardiac arrhythmia, and the most frequently observed form of binge alcohol-induced arrhythmia. Clinical evidence indicates that one-third of all new-onset AF cases are related to alcohol intoxication. Effective treatment strategies of binge alcohol-induced AF are currently not available due to the unknown pathological mechanisms. Here, I evaluated a repeated binge alcohol mouse model with drinking patterns that mimics that of humans during the holiday season with intervals of repeated binge alcohol episodes and recovery. In this mouse model, the cardiac function is not altered, similar to the clinical observation of HHS patients that usually present normal cardiac function. BAC measurement at the time frame of terminal studies showed no significantly increased blood alcohol level compared to sham controls, which provides a further opportunity to study the atrial arrhythmogenicity due to binge alcohol-induced arrhythmogenic substrate remodeling process rather than direct alcohol toxicity. Further, this model also agrees with the

clinical observations that patients tend to already recover from intoxication during the time frame with the highest frequency binge alcohol-induced arrhythmia.

3.2 Results

3.2.1 Blood Alcohol Concentration Measurement

The construction of our mouse model of repeated alcohol exposure was described in detail in Materials and Methods, Chapter Ten, page 183-184. In brief, mice (8-12 weeks of age) were treated with alcohol (2 g/kg BW, 12.67% v/v, I.P.) every other day for four injections (Figure 5A). Mouse BW was measured every time before the injection, and no significant BW alterations were found in mice receiving the alcohol injection (Figure 5B). Terminal study was performed within 24 hrs of the last dose of alcohol exposure. Echocardiography was performed right before the terminal study. Blood was collected during the sacrifice from the chest. No dramatical BW changes were found during the course of injection (Figure 5B).

A rabbit model of repeated binge alcohol exposure was constructed in similar manner. Young (6 months; n = 6) New Zealand White male rabbits were infused with alcohol (2 g/kg BW, 12.67% v/v, I.V.) every other day for totally four injections.

Mouse BAC was measured from serum extracted from the collected blood during sacrifice using an ethanol assay kit purchased from Abcam. No significant difference in BAC was found between alcohol-challenged mice and sham control ones at the time of sacrifice (0.27 ± 0.09 mg/dL vs. 0.25 ± 0.10 mg/dL, n = 5, 5, p = NS; Figure 5C), and the BAC level in alcohol-challenged mice were well below the

legal blood alcohol level for intoxication, which further suggests that the arrhythmia and arrhythmogenic events in alcohol-challenged mice are due to alcohol-induced atrial arrhythmogenic substrate remodeling rather than direct chemical insult from alcohol. This finding is comparable to the clinical findings that in the alcohol-caused death, the BAC is usually very low, suggesting that death happened during the post-alcohol recovery and withdraw (J. C. Clark, 1988; Denison, et al., 1994). Similar findings in animal studies have also shown that alcohol metabolism follows first degree elimination kinetics *in vivo* and BAC usually drops to sham level within hours (Greiffenstein, et al., 2007).

Figure 5

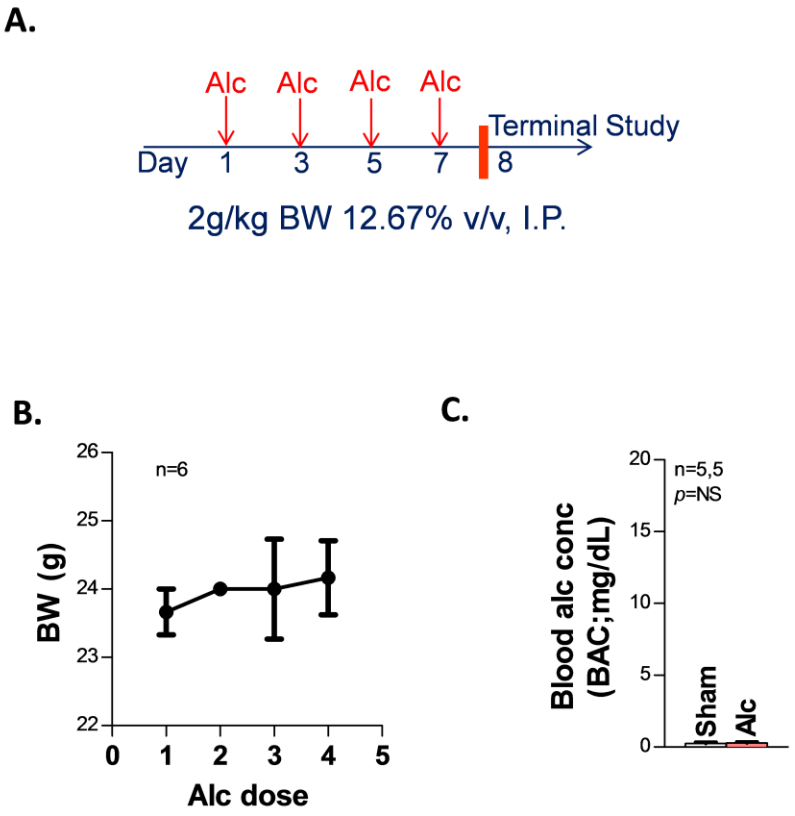

Figure 5. Mouse model of binge alcohol exposure. **A)** Schematic drawing showing the pattern of mouse alcohol exposure. Mice were exposed for alcohol (2 g/kg bodyweight, 12.67% v/v I.P.) every other day for totally 4 doses while terminal study was performed on the next day after the last dose of alcohol exposure. **B)** Unaltered bodyweight during the cause of alcohol exposure. **C)** Blood alcohol measurement showing unaltered blood alcohol concentration at the time of terminal study. Both alcohol-treated and sham control mice have only trace amount of alcohol compared to the legal limit of intoxication (80 mg/dL). All data were presented as Mean ± SEM. Unpaired Student *t*-test was used to compare the mean of the two groups in this study. *Courtesy to Dr.Xianlong Gao for BAC measurement.*

3.2.2 Cardiac Function Evaluation

M-mode echocardiography was performed on alcohol-challenged mice and sham controls within the time frame of terminal studies before the sacrifice as was previously described. Ejection fraction (EF) and factional shortening (FS) were calculated from the original recordings, and compared between alcohol-treated and sham groups (mice of 8-10 weeks of age, injected with 4 doses of saline in parallel time of the alcohol treatment). No significant difference was demonstrated in the EF between the two groups (64.4 ± 2.8% vs. 64.8 ± 3.1% in sham, n = 7, 4, *p* = NS; Figure 6A); similar findings were made in the FS measurements (34.7 ± 2.1% vs. 34.6 ± 2.3%, n = 7, 4, *p* = NS; Figure 6A). This result further indicates that no HF was induced by this repeated binge alcohol treatment, while the increased atrial arrhythmogenicity is due to the atrial arrhythmogenic substrate remodeling in the repeated binge alcohol-challenged mice rather than complications of HF.

3.2.3 Bodyweight and Heart Weight Measurements

Immediately following the heart and lung harvest and rinsing with cardioplegic solution, mouse heart weight and lung weight were measured. The HW/BW ratio (0.00452 ± 0.00024 g/kg BW vs. 0.00452 ± 0.00079 g/kg BW in sham, n = 6, 6, *p* = NS) and LW/BW ratio (0.00635 ± 0.00023 vs. 0.00562 ± 0.00050 in sham, n = 6, 6, *p* = NS) were not altered in alcohol-exposed mice compare to sham controls, further indicating the lack of cardiac hypertrophy or pulmonary congestion in this model (Figure 6B).

Figure 6

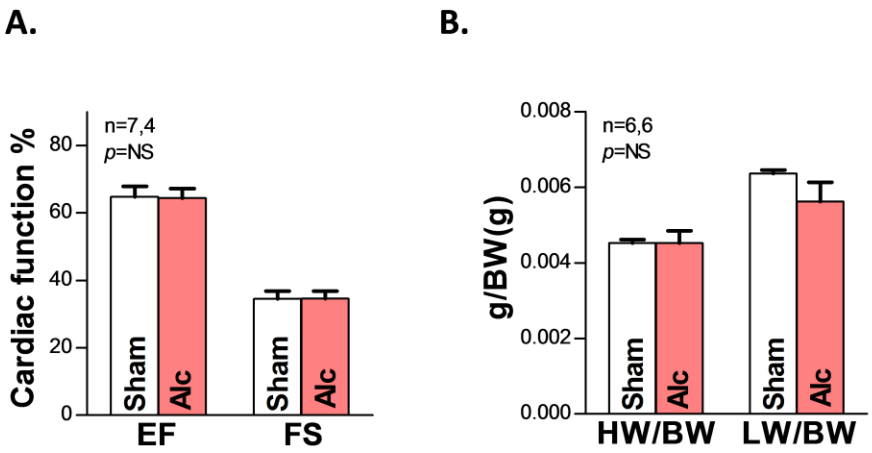

Figure 6. Unchanged cardiac function in repeated binge alcohol-exposed mice. **A)** Echocardiography shows unchanged ejection fraction (EF) and fractional shortening (FS) in binge alcohol exposed mice compared to sham controls. *Courtesy to Dr.Sarah Burris for performing the echocardiography.* **B)** Unaltered ratio of HW/BW and LW/BW in binge alcohol exposed mice compared to sham control. All data were presented as Mean ± SEM. Student *t*-test was used to compare the mean of the two groups in each study.

3.3 Discussion

AF is the most commonly observed form of HHS (Engel & Luck, 1983; Thornton, 1984). Emerging evidence suggest that one-third of all new-onset AF cases are related to alcohol intoxication (Hansson, et al., 2004; Lowenstein, et al., 1983; Maryniak, et al., 2006; Peter, et al., 1968; E. C. Rich, et al., 1985). Moreover, binge drinking-induced atrial arrhythmia affects even apparently healthy patients without clinical evidence of increased risk for cardiac arrhythmias (Ettinger, 1984; Ettinger, et al., 1978).

In the current study, I used a mouse model that mimics the holiday drinking pattern in which intervals of binge drinking and recovery alternate (Figure 5A). The terminal studies were performed within 24 hrs of the last dose of alcohol exposure, mimicking the time frame of the highest frequency of alcohol-induced arrhythmia in patients. At this time of the terminal study, BAC already recovered to baseline level. This finding is comparable to the results of previous studies in alcohol metabolism kinetics which showed that BAC usually returned to baseline level a few hours after exposure and the alcohol elimination in vivo follows first-order kinetics (Matyas, et al., 2016). This repeated binge alcohol model provides an opportunity to specifically investigate the binge alcohol-induced arrhythmogenic remodeling rather than the direct alcohol toxicity found in previously published animal models, where arrhythmia inducibility was examined in acute alcohol-infused animals where BAC was well above normal level (Anadon, et al., 1996; Y. Gao, et al., 2012).

In our model of binge alcohol-exposed mice, there is no significant difference in cardiac function (measured in EF and FS) compared to sham control ones. However, Kandadi et al. previously found that treating WT mice with alcohol (3 g/kg/d BW, I.P.) for 3 consecutive days resulted in decreased EF and FS (R. Guo & Ren, 2010, 2012; Kandadi, Hu, & Ren, 2013; Ma et al., 2010). These differences could be due to a higher dose of alcohol in these studies used compared to the binge model used for my thesis work (2 g/kg BW, 12.67% v/v, I.P., every other day for 4 doses). Also, it could be due to the different time frame of the terminal study. For instance, terminal studies in the Ren Lab were performed when the BAC was about 60 mg/dL which is significantly higher than control level (R. Guo & Ren, 2010), which is different from our situation where BAC is comparable to sham control level. In a long-term alcohol exposure mouse model (6 weeks) (R. H. Zhang, et al., 2013), EF is decreased compared to age-matched sham controls, similar to the findings in the Ai Lab on mice that were exposed to alcohol for 6 months. These results suggest that chronic alcohol exposures can affect cardiac function and induce alcoholic cardiomyopathy/HF, but binge alcohol exposures have no significant impact on the cardiac function at the time frame that BAC returns to normal level. In addition, the ratio of HW/BW was unaltered in our binge alcohol model, further suggesting the absence of cardiac hypertrophy, and pulmonary edema. Such evidence indicates that this binge model does not have HF as that was found in long term alcohol exposure animal models.

Although it is known that the first 24 hrs after binge drinking is the timeframe in which HHS most frequently appears, no clear statistics currently exist as to when the binge-alcohol arrhythmogenicity recovers to a normal level. Our future studies will explore the time frame, recovery of the arrhythmomgenic substrate, and molecular mechanisms that govern the recovery from alcohol-induced atrial arrhythmia.

Gender differences could also be a factor in binge alcohol induced atrial arrhythmia. Alcohol exposure has a differentiated impact on male and female subjects, with men having typically higher tolerance compared to women. For instance, in a long-term clinical study, it was found that more than 2 drinks per day promoted AF in women (Conen et al., 2008), but not in men (Mukamal, et al., 2005). Also, the hazard ratio of AF in women appears to be higher than in men for the same amount of alcohol intake (< 35 drinks per weeks); yet when taking the drinking habit into consideration, women tend to drink less than men (Mukamal, et al., 2005). For patients that developed AF after long-term alcohol drinking (4.9 yrs), the threshold of alcohol intake that leads to increased the risk thromboembolism or death is >27 drinks/week in men, which is much higher than that in women (20 drinks/week) (Overvad et al., 2013). Possible mechanisms of the gender difference effect of alcohol could be due to estrogen involvement in alcohol metabolism in the heart (el-Mas & Abdel-Rahman, 2012, 2014, 2015; Longnecker & Tseng, 1998).

While all the animals including both rabbits and mice were male, the gender difference of alcohol-induced arrhythmia is a necessary direction for future studies.

3.4 Conclusion

In this chapter, I evaluated a repeated binge alcohol mouse model that was recently developed in the Ai Lab. This binge alcohol model mimics the drinking patterns of humans during the holiday season with intervals of binge alcohol episodes and recovery. The cardiac function is not altered in this model, similar to the clinical observation of HHS patients. Also, no cardiac hypertrophy or lung edema was demonstrated. BAC measurements at the study end-point showed no significantly increased BAC compared to sham controls, which provides a model to study the binge alcohol-induced arrhythmogenic substrate-remodeling phenomena rather than direct alcohol toxicity.

CHAPTER FOUR

BINGE ALCOHOL PROMOTES JNK ACTIVATION THAT PROMOTES ATRIAL ARRHYTHMIAS

4.1 Abstract

Numerous clinical observations have established that binge alcohol can promote atrial arrhythmia in apparently healthy young patients. In this thesis, I have evaluated a repeated binge alcohol mouse model that mimics the drinking pattern of the holiday season with intervals of binge alcohol consumption and recovery. In this chapter, I used this mouse model together with a similarly-designed rabbit model of repeated binge alcohol exposure to test alcohol-induced atrial arrhythmia. Burst pacing protocols delivered on anaesthetized rabbits *in vivo*, and on Langendorff-perfused mouse heart *ex vivo* were used to challenge the cardiac tissue. The results showed increased burst pacing induced atrial arrhythmia in alcohol-treated rabbits and mice compared to sham controls.

The Ai Lab has previously discovered that JNK activation promotes atrial arrhythmia in rabbit aging models and in JNK activator (anisomycin) challenged

young rabbits. Others reported that alcohol exposure leads to JNK activation in non-atrial tissue; however, there is no report on whether binge alcohol exposure promotes JNK activation in the atria. In this section of the study, I found increased JNK activation in binge alcohol-exposed rabbits and mice compared to sham controls (Figure 7).

To further explore whether JNK activation plays a pivotal role in the atrial arrhythmogenicity, WT young mice were challenged with JNK activator anisomycin (20 mg/kg BW, I.P.) and assessed the inducibility of atrial arrhythmia. The results showed enhanced JNK activation in atrial tissue after alcohol treatment. To further understand whether JNK inhibition could be a potential therapeutic approach in preventing and managing binge alcohol-induced atrial arrhythmia, a mouse line overexpressing cardiac specific dominant negative JNK1 and JNK2 (JNK1/2dn) were subjected to the same repeated binge alcohol treatment, and atrial arrhythmia inducibility returned to sham control level.

In summary, this part of work strongly indicates that binge alcohol exposure promotes JNK activation in the atria that enhances the propensity of atrial arrhythmia (Figure 7).

Figure 7

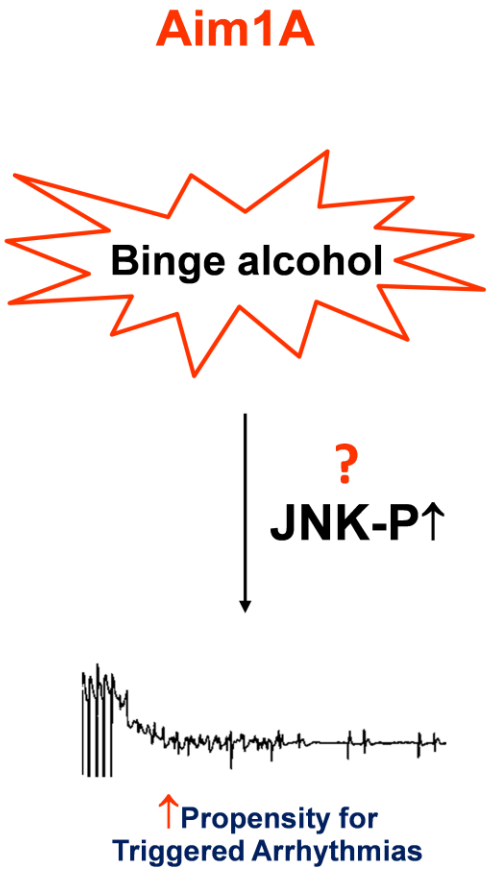

Figure 7. Schematic of the research focus for Aim1A. Binge alcohol consumption has been shown to promote atrial arrhythmia and the activation of stress-response kinase JNK, moreover, enhanced JNK activation can promote atrial arrhythmia. In this part of the thesis, I will explore whether binge alcohol promotes atrial arrhythmia in JNK-dependent mechanisms.

4.2 Results

4.2.1 Alcohol-induced Atrial Arrhythmia

To study atrial arrhythmogenicity after binge alcohol exposure compared to control, rabbit and mouse binge-alcohol models were constructed and evaluated previously. The protocols used to generate these models are described in detail in Materials and Methods, Chapter Ten, page 183. Young sham rabbits were challenged with alcohol and subjected to a series of atrial burst-pacing stimulations (Figure 8D) in open-chest procedure. The same burst pacing protocol (a full protocol contains multiple steps was applied to every animal. Details can be found in Materials and Methods, Chapter Ten, page 184-185). AF (Figure 8A) was induced in 6 out of 6 alcohol-challenged rabbits but in none of the 5 sham control ones (7.5 ± 2.1 incidences/temp/animal vs. 0.0 ± 0.0 incidences/temp/animal in sham, n = 6, 5; $p < 0.01$; Figure 8B). The average length of atrial tachycardia (AT)/AF induced was significantly increased in alcohol-challenged rabbits compared to sham control ones (29.8 ± 11.5 msec vs. 0.7 ± 0.1 msec in sham, n= 6, 5; $p < 0.05$; Figure 8C). All the detailed information about statistical analysis is provided in figure legends.

Figure 8

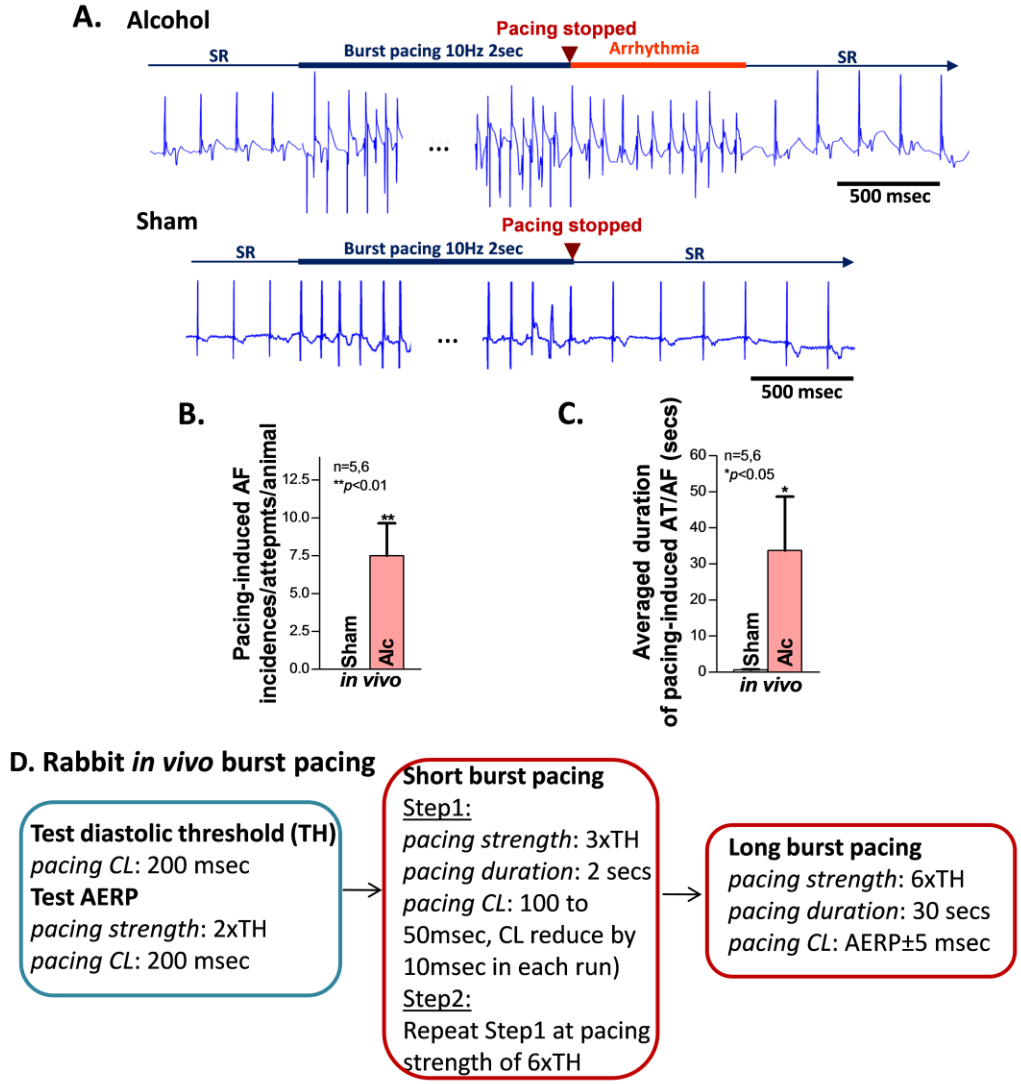

Figure 8. Binge alcohol exposure promotes burst pacing-induced atrial arrhythmia in rabbits. **A)** Representative electrograms (EG) of burst pacing (for 30 s at 6 x diastolic threshold, CL = 100 ms) induced AF followed by self-reversion to sinus rhythm (SR) in open-chest rabbits with pre-treatment of alcohol (2 g/kg BW, I.V. every other day for a total of 4 treatments), while the same burst pacing failed to induce atrial arrhythmia in sham control rabbit. **B)** Summarized data showing enhanced AF inducibility and **(C)** increased average duration of pacing-induced AT/AF in binge alcohol-exposed rabbits compared to that of sham controls. **B-C)** All data were presented as Mean ± SEM. Mann-Whitney test (non-parametric Student *t*-test for unpaired groups) was used to compare the difference in the mean for the two groups. *I would like to thank Drs. Cheryl Killingsworth and Qiang Zhang for assistance on in vivo rabbit studies.* Detailed *in vivo* burst pacing protocol in rabbits was described in **(D)**.

WT mice were treated with alcohol injections for a total of 4 doses. AF inducibility was measured on Langendorff-perfused intact mouse hearts with stable perfusion at 37°C from alcohol-challenged mice and sham controls (Figure 9A, 9B). The burst-pacing stimulations were delivered through a bipolar electrode located on the left atria while the electrograms were recorded simultaneously on the right atria (Figure 9B). The same burst pacing protocol was applied on every mouse heart among different experiment groups (Figure 9C). Representative images show that binge alcohol-exposed mice developed atrial arrhythmia after 30 seconds of pacing at 10Hz (3x diastolic threshold), while sham control mice returned to the sinus rhythm and no arrhythmias were induced (Figure 10A). Burst-pacing induced atrial arrhythmia was found in 4 out of 10 binge-alcohol exposed mice but none of the 7 sham control mice (Figure 10B). Also, the duration of burst-pacing induced atrial arrhythmias was longer in alcohol-treated mice compared to sham controls (2.23 ± 1.04 sec vs. 0.00 ± 0.00 sec in sham, n = 7, 10, $p < 0.01$; Figure 10C). Previously, our lab has found that JNK activation promotes the occurrence of atrial arrhythmia in rabbit model (Yan, et al., 2013) and JNK activation was implicated in alcohol exposure (Aroor, et al., 2010; C. H. Lang, et al., 2014; Lee, et al., 2002; S. Y. Li, et al., 2009; Masamune, et al., 2002; McCarroll, et al., 2003; Meriin, et al., 1999; Nishitani & Matsumoto, 2006). Thus, the next question to explore is whether JNK plays a significant role in binge alcohol-prompted atrial arrhythmia (Figure 7).

4.2.2 JNK Inhibition in JNK1/2 Dominant Negative Transgenic Mice Prevented Binge Alcohol-evoked Propensity of Atrial Arrhythmias

To test the functional role of binge alcohol in enhanced atrial arrhythmogenicity, mice with cardiac-specific expression of domininant-negative JNK1 and JNK2 (JNK1/2dn) mice were treated with the binge alcohol protocol. Atrial arrhythmogenesis was examined with burst pacing on Langendorff-perfused hearts as previously described. The results showed that after alcohol exposure, JNK1/2dn mice had similar pacing-induced AT/AF incidences compared to WT-sham mice (Figure 10B), meanwhile, the average duration of pacing-induced AT/AF in alcohol-treated JNK1/2dn mice were dramatically decreased compared to WT mice treated with alcohol (Figure 10C). These results strongly indicate that binge alcohol exposure promote atrial arrhythmia in JNK-dependent manner.

Figure 9

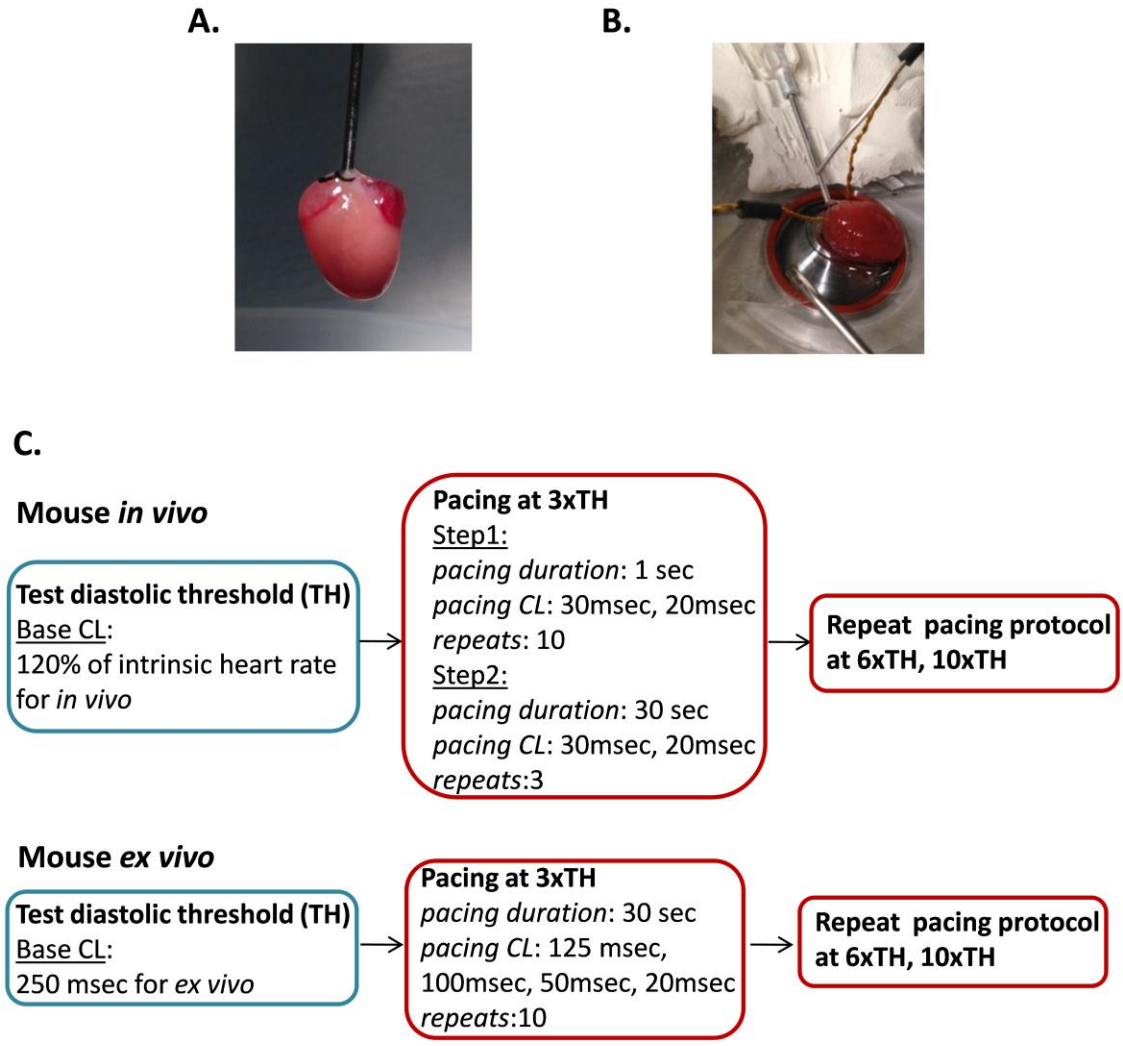

Figure 9. Langendorff-perfused mouse heart for dye loading and AF induction. **A)** An image of Langendorff-perfused mouse heart during dye loading. **B)** Langendorff-perfusion setup to measure burst pacing induced atrial arrhythmia; images showing a mouse heart being perfused through a custom-made catheter while pacing bipolar electrode located on the left atria and recording bipolar electrode located on the right atria. **C)** Burst pacing protocols for *in vivo* and *ex vivo* mouse atrial arrhythmia inducibility experiments were listed in detail.

Figure 10

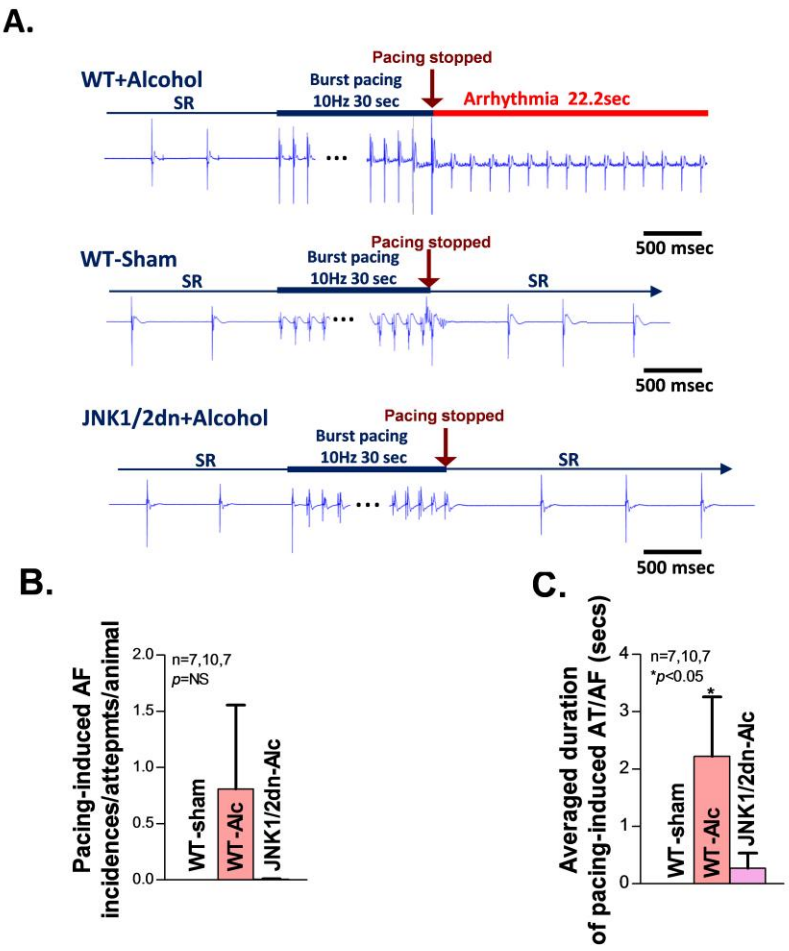

Figure 10. Binge alcohol exposure promotes burst pacing-induced atrial arrhythmia in mice. **A)** Representative electrograms showing burst-pacing induced AT in a Langendorff-perfused wildtype (WT) mouse heart with binge alcohol pre-exposure, and self-restored sinus rhythm (SR) after burst pacing in sham control mouse hearts as well as binge alcohol-exposed Jdn transgenic mouse hearts with overexpression of cardiac-specific inactivated dominant negative JNK. **B-C)** Summarized data of enhanced AF inducibility and **(C)** increased average duration of pacing-induced AT/AF in binge alcohol-exposed WT mouse hearts compared to that of sham controls, while JNK inhibition in JNK1/2dn mice prevented binge alcohol enhanced atrial arrhythmogenicity. **B-C)** All data were presented as Mean ± SEM. Kruskal-Wallis test (One-Way ANOVA on ranks) with post hoc test was used to compare the mean of selected groups .

4.2.3 Binge Alcohol Promotes JNK Activation in Mouse Heart

To further explore whether binge alcohol promotes JNK activation in the heart, phosphorylated JNK (JNK-P, activated JNK) was probed using immunoblotting in cardiac tissue from young rabbit and mice with or without binge alcohol exposure. The results suggest increased JNK phosphorylation in binge-alcohol exposed rabbits (142.4 ± 6.5% vs. 100.0 ± 9.9% in sham, n = 3, 3, $p < 0.05$; Figures 11A, 11B) and mouse hearts (136.3 ± 2.6% vs. 100.0 ± 4.2% in sham, n = 3, 3, $p < 0.05$; Figures 11C, 11D) compared to sham controls. On the other hand, the phosphorylation level of the other two major MAPKs, including ERK and p38, were not altered by repeated binge alcohol treatment (Figures 11E, 11F).

Indeed, the activity measurement of immunoprecipitated (IP-ed) cardiac JNK2 from binge alcohol-treated mice demonstrated a 37% increase compared to sham controls (30.3 ± 3.0 x 10^3 vs. 22.2 ± 2.6 x 10^3 relative luminescent unit (RLU) in sham, n = 8, 8, $p < 0.05$; Figure 12), while the activity level of JNK1 remains unaltered (26.4 ± 2.3 x 10^3 vs. 30.1 ± 1.4 x 10^3 RUL, n = 4, 5, p = NS; Figure 12). In summary, the immunoblotting data and kinase activity assay both indicate that binge alcohol exposure promotes the JNK activity, especially JNK2 activity in the atrial tissue.

Figure 11

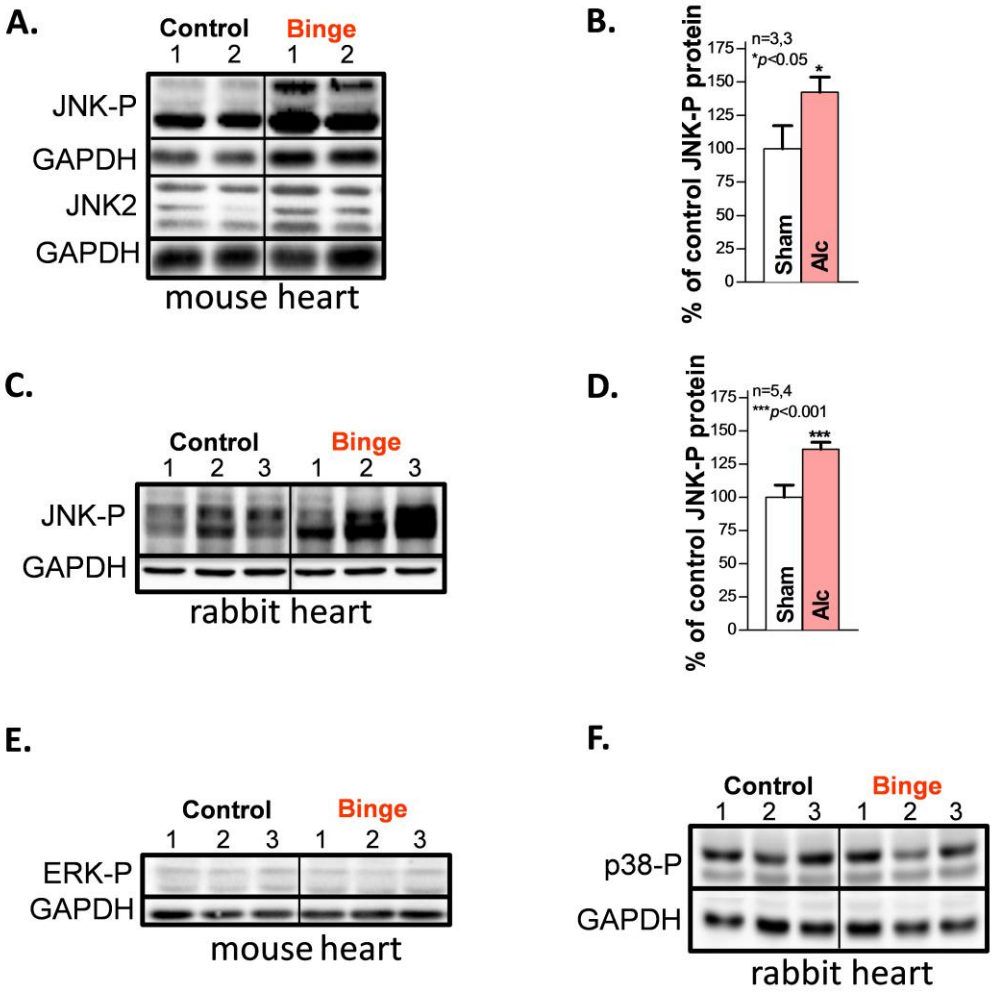

Figure 11. Alcohol exposure promotes JNK activation. **A-B)**. Immunoblotting images and summarized quantitative data showing increased phosphorylation of JNK (JNK-P, activated JNK) in binge alcohol-exposed mouse cardiac tissue. **C-D)**. Immunoblotting images and summarized quantitative data showing enhanced JNK activation binge alcohol-exposed rabbit cardiac tissue. **E-F)**. Immunoblotting images showed unaltered level of phosphorylated ERK or p38 in cardiac tissue after repeated binge alcohol treatment. **B-D)** All data were presented as Mean ± SEM. Unpaired Student *t*-test was used to compare the mean of the two groups in each study. *I would like to thank Ms.Weiwei Zhao for assistance on immunoblotting.*

Figure 12

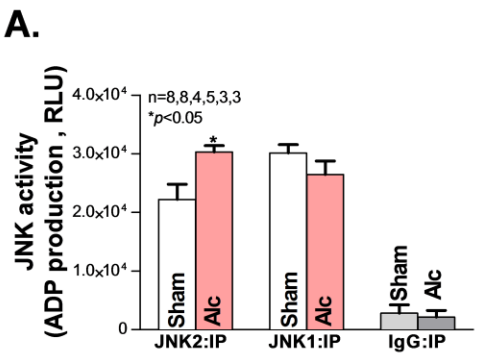

Figure 12. Summarized data showing enhanced ADP production from immunoprecipitated JNK2 kinase activity assay in binge alcohol exposed mouse hearts suggesting enhanced JNK2 activity, while the activity of JNK1 (the other major isoform of cardiac JNK) remains unchanged. Background signal from IgG pull-down control remained minimal. All data were presented as Mean ± SEM. One-Way ANOVA test with Tukey post hoc test was used to compare the mean of Alc vs. Sham in each set of IP using JNK2, JNK1, normal IgG, respectively. *Courtesy of Dr.Xianlong Gao for performing this experiment and training Jiajie Yan in the same process.*

4.2.4 JNK Activation Promotes Arrhythmia in the Absence Of Alcohol Challenge

To further confirm the role of JNK activation in atrial arrhythmia inducibility, mice were challenged with anisomycin to induce JNK activation. Atrial arrhythmia was induced with burst pacing delivered through an intracardiac catheter. Compared to sham control mice, anisomycin-challenged mice showed an average length of the induced arrhythmia (2.2 ± 0.9 sec vs. 0.0 ± 0.0 sec in sham, n = 5, 5, $p < 0.05$; Figure 13C). Anisomycin-challenged young rabbits (20 mg/kg BW, I.V.) also demonstrated enhanced burst pacing-induced atrial arrhythmia (29.8 ± 0.3 sec vs. 0.7 ± 0.1 sec in sham, n = 5, 6, $p < 0.05$; Figure 13A, 13B). Similarly, enhanced burst-pacing induced atrial arrhythmia was also observed in MKK7D mice, a mouse with induced activation of JNK upstream activator MKK7 (Figure 13A). These results further demonstrated the role of JNK activation in increased atrial arrhythmogenicity, thus further strengthen the previous findings that binge alcohol promotes burst pacing-induced atrial arrhythmia in JNK-dependent pathways.

Figure 13

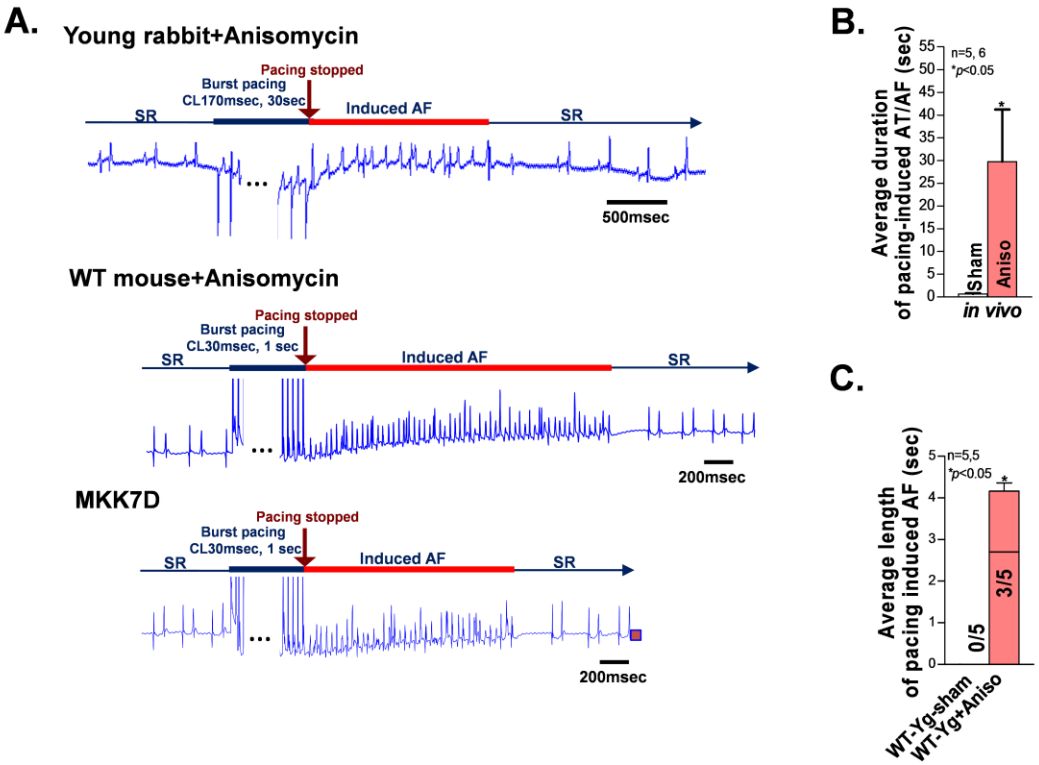

Figure 13. JNK activation (without alcohol exposure) promotes atrial arrhythmia. **A)** Representative electrograms (EG) of burst pacing induced AT/AF followed by self-reversion to sinus rhythm three JNK activation animal models including 1) young rabbit treated with anisomycin (20mg/kg BW I.V.; EMD; every other day for a total of 4 doses. Yan et al. *Cardiovasc Res*. 2013;97(3):589-97); 2) WT young mice treated with anisomycin (20mg/kg BW I.P.; EMD; every other day for a total of 4 doses) where burst pacing was delivered through anintra-cardiac octopolar catheter with simultaneous recording function; 3) MKK7D mice treated with tamoxifen to induce overexpression of MKK7D (1 mg/day I.P. for 4 days, Andersson et al. JMCC(2009) 47:180-187) where JNK is constituitively actived. **B)** Summarized data showing increased average duration of pacing-induced AT/AF in anisomycin-treated rabbits compared to sham controls. **C)** Summarized data showing increased average duration of burst pacing-induced AT/AF in anisomycin-treated young WT mice compared to sham controls. All data were presented as Mean ± SEM. Mann-Whitney test (non-parametric Student *t*-test for unpaired groups) was used to compare the difference in mean between the two groups in each study.

4.3 Discussion

Accumulating clinical data have established that binge alcohol promotes cardiac arrhythmia, while AF is the most commonly diagnosed type of arrhythmia in HHS. In *in vivo* and *ex vivo* atrial arrhythmia induction studies using our rabbit and mouse binge alcohol models, I found increased burst pacing-induced AT/AF in binge alcohol-exposed animals compared to sham controls. These results were consistent with the clinical findings. And as emphasized before, atrial arrhythmia was probed when BAC drops to normal level in our mouse model, thus allowing us to focus on the arrhythmogenic remodeling in the atrial tissue after binge alcohol treatment.

In this *ex vivo* AT/AF inducibility study, I used atrial burst pacing challenge on alcohol-exposed versus sham control mouse heart when the hearts. I also studied hearts from alcohol-exposed JNK1/2dn in the same experimental setting. The results showed that alcohol promotes burst pacing induced atrial arrhythmia in JNK-dependent manner. Moreover, JNK activation *per se* promotes atrial arrhythmogenesis in both mouse and rabbit hearts.

In these experiments, all mouse heart preparations were Langendorff-perfused to minimize the impact of adrenergic effect. However, this experimental design eliminated the potential contribution of neurohormonal response (for instance the adrenergic response and vagal nerves) in alcohol-induced atrial arrhythmia. In the future studies, it would be valuable to use the telemetry approach on conscious mice to monitor the atrial arrhythmia *in vivo* while the innate

neural/hormonal regulations are present. This will be discussed in detail in the Chapter Eight Future Directions.

JNK activation has been observed in various pathological conditions and is involved in the development of cancer, diabetes, and arthritis (Davis, 2000; Kamata, et al., 2005; Rose, Force, & Wang), and JNK inhibition has been explored as a possible anti-cancer and arthritis therapeutic target in clinical trials (Bogoyevitch & Kobe, 2006; Kamata, et al., 2005). In the field of cardiac pathology, JNK activation has been shown in various cardiac diseases including HF of both ischemic and non-ischemic etiology (Haq et al., 2001) and in ischemic-reperfusion injury models, in which the heart is prone to arrhythmia (Bogoyevitch, et al., 1996; Siow, et al., 2000). Moreover, the human atria shows enhanced propensity for AF during aging (Benjamin, et al., 1994; Go, et al., 2001; M. W. Rich, 2009), and our lab discovered that the increased JNK activation was positively correlated with increased AT/AF in animal models (manuscript in submission)(Yan, et al., 2013). Interestingly, emerging evidence suggests that JNK activation is enhanced after alcohol exposure (Aroor, et al., 2010; C. H. Lang, et al., 2014; Lee, et al., 2002; S. Y. Li, et al., 2009; Masamune, et al., 2002; McCarroll, et al., 2003; Meriin, et al., 1999; Nishitani & Matsumoto, 2006). Indeed, in this thesis, the JNK2 specific activity measured from IP-ed JNK2 proteins from binge-alcohol treated mouse cardiac tissue was significantly increased, while the activity level of JNK1 remains unaltered compared to sham controls. This enhanced JNK2 activity was also confirmed in immunoblotting studies using a phospho-specific JNK antibody. Also,

my results in alcohol-challenged JNK1/2dn mice for the first time identified that JNK plays a pivotal role in binge alcohol induced arrhythmia. JNK associated arrhythmogenesis has also been observed in mice treated with repeated binge alcohol or JNK activator anisomycin, which provided further evidence suggesting a critical role of JNK activation in atrial arrhythmogenesis. These results were consistent with our recently reported findings in an aged rabbit model (Yan, et al., 2013). Strikingly, JNK inhibition in JNK1/2dn mice prevented alcohol-induced arrhythmia. This further supports that JNK activation plays a predominant role in alcohol-induced arrhythmia.

JNK1 and JNK2 are the two major cardiac isoforms of JNK, that are thought to have distinct functions (Bogoyevitch & Kobe, 2006). In the heart, JNK1 appears to help preserve cardiac function and promote apoptosis in ischemia-reperfusion hearts (Bogoyevitch & Kobe, 2006). However, to our knowledge, the role of JNK2 in normal or pathological heart function has not been identified to date. In my studies, JNK2KO abolished the anisomycin-induced atrial arrhythmia suggesting that JNK2 is the primary isoform involving atrial arrhythmogenesis. I also used JNK1/2dn mice to further block the potential JNK compensation between different isoforms. In this mouse model, JNK1/2dn overexpression is cardiac-specific, which enhances the accuracy of the manipulation of JNK signaling in the heart by avoiding potential systemic impacts, and the background JNK signaling in the dominant negative

expression model is beneficial for the development and survival of the animal (Rose, et al., 2010).

For each of the JNK isoforms, the gene gives rise to several splice variants encoding short or long proteins which can be distributed to different cellular domain in multiple organs (Casanova, Callejo, Calvo, & Chinchetru, 2000; Yang et al., 2007). JNK alternative splicing is currently a very active field of research, yet the cause of alternative splicing and the function of short and long JNK protein haven't been revealed. There is a previous report showing that multiple isoforms of JNK arise from alternative splicing of the three genes that encode JNK1, JNK2 and JNK3 (Gupta et al., 1996). The JNK isoforms resulted from alternative splicing usually carry variations on the C-terminus which may facilitate the JNK localization in different cellular domains (Casanova, et al., 2000; Yang, et al., 2007), while the sequence (Thr-Pro-Tyr) recognized by upstream kinases (MKK4 and MKK7) remains conservative (Barr & Bogoyevitch, 2001). Although this topic is not explored in the current thesis, the different possibility and functional implications of JNK2 splicing is one of the future directions for this research.

Increased cellular ROS has been proven to promote JNK activation, and alcohol exposure has been shown to increase intracellular ROS (R. H. Zhang, et al., 2013). The current study focuses on the alcohol-activated JNK and its downstream functional consequences aiming at exploring the possibility of using JNK as a therapeutic target. It is also valuable to understand the mechanism of how JNK is

activated in alcohol-exposed hearts, for instance, whether JNK activation is via the canonical pathway of JNK activation or via direct oxidation of certain amino acids of the JNK molecule. In future studies, approaches of mutagenesis, bioinformatics and molecular modeling will be used combined with molecular biology approaches to assess the potential amino acid site of JNK that is vulnerable to oxidation.

Other than JNK, important members of stress-responsive the MAPKs also include MAPK p38 and ERK (Rose, et al., 2010). *Ex vivo* acute alcohol treatment (5mM) on cardiac tissue promoted the activation of p38 (Umoh, Walker, Al-Rubaiee, Jeffress, & Haddad, 2014b). Five weeks of binge drinking exposure also increased p38 activation in rat ventricles (Gu, Fink, Chowdhury, Geenen, & Piano, 2013). Aroor et al. also found that ERK is activated in multiple non-cardiac tissue (Aroor & Shukla, 2004). However, in our current repeated binge alcohol exposure model, the data suggest no significant alterations in the level of p38 and ERK activation (Figures 11E, 11F). Thus the differences in findings could be due to different alcohol-exposure model and different organs and tissue types.

4.4 Conclusion

This part of my thesis work shows that repeated binge alcohol promotes enhanced JNK activation, and atrial arrhythmia inducibility in both mouse and rabbit models of binge alcohol exposure. In addition, activating JNK without alcohol exposure also promotes atrial arrhythmia inducibility in mice. Abolishing the JNK pathway with

dominant negative overexpression of JNK attenuated the alcohol-promoted atrial arrhythmia during burst pacing.

In summary, this part of the work strongly indicates that binge alcohol exposure enhances JNK activation that in turn enhances propensity of atrial arrhythmia (Figure 7). In the next chapter, I will further explore the electrophysiological mechanism of JNK promoted atrial arrhythmia.

CHAPTER FIVE

BINGE ALCOHOL PROMOTES ATRIAL CALCIUM MISHANDLING

5.1 Abstract

Triggered activity has long been established as a pivotal mechanism of arrhythmogenesis. Extensive studies indicate that increased Ca^{2+} wave frequency contributes to arrhythmia initiation. In the previous chapter of this thesis, I have discovered that repeated binge alcohol exposure promotes burst pacing-induced atrial arrhythmogenesis in mouse in a JNK-dependent manner.

In this section, I aim at exploring whether Ca^{2+} mishandling, especially Ca^{2+} waves, in repeated binge alcohol-induced atrial arrhythmia (Figure 14). To compare the frequency of Ca^{2+} waves between binge alcohol-exposed hearts versus that of the sham controls, confocal Ca^{2+} imaging on the atria was performed on intracellular Ca^{2+} sensitive dye-loaded intact heart. The results showed increased spontaneous and burst pacing-induced Ca^{2+} waves in repeated binge alcohol-exposed hearts. To further explore whether abolishing the JNK pathway can attenuate alcohol-induced Ca^{2+} wave in the atria, JNK1/2dn mice with cardiac-specific overexpression of dominant negative JNK were treated with repeated binge alcohol and studied using

confocal Ca^{2+} microscopy. The results showed decreased Ca^{2+} wave frequency in JNK1/2dn mice after binge alcohol exposure compared to WT mice treated with alcohol, further indicating that JNK activation plays a pivotal role in binge alcohol-enhanced arrhythmogenic Ca^{2+} waves in the atria. To further understand the contribution of JNK activation in atrial Ca^{2+} waves, JNK activator anisomycin-challenged WT and JNK2KO (JNK2 knock-out) mice were also studied using confocal Ca^{2+} imaging method. The results showed that knocking out JNK2 effectively prevented anisomycin treatment-promoted Ca^{2+} waves, further suggesting the specific role of JNK2 activation in the generation of atrial Ca^{2+} waves.

Figure 14

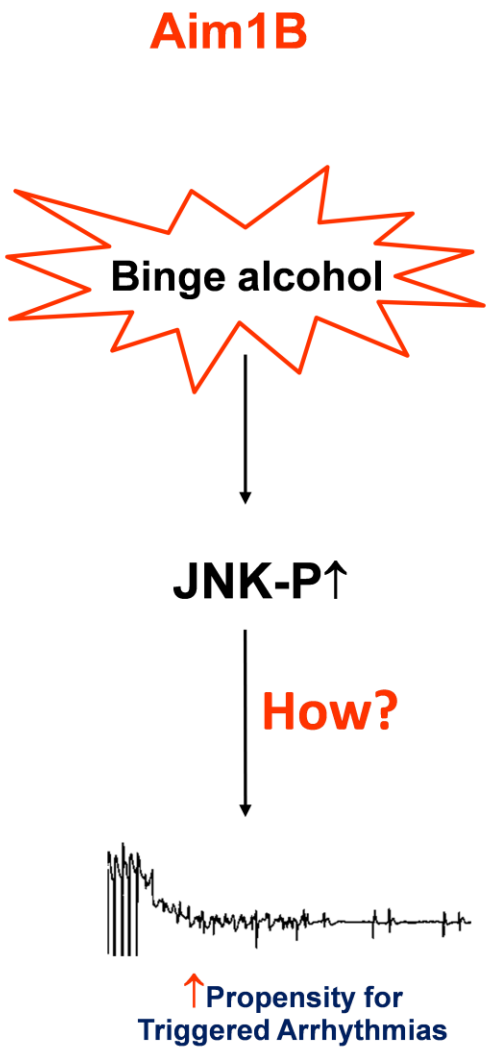

Figure 14. Schematic of the research focus for Aim1B. The previous chapter of the thesis indicated that binge alcohol consumption promotes atrial arrhythmia in JNK-dependent mechanisms. Aberrant Ca^{2+} mishandling has been shown to promote the initiation of arrhythmia. In this part of the thesis, I will explore whether Ca^{2+} mishandling, especially diastolic Ca^{2+} waves, underlie the binge alcohol-prompted JNK-dependent atrial arrhythmia.

5.2 Results

5.2.1 Binge Alcohol Promotes Atrial Ca^{2+} Waves via JNK Activation

Previous sections have shown that binge alcohol increased atrial arrhythmogenicity through activated JNK. Ca^{2+} mishandling is known to be pivotal for enhanced Ca^{2+} triggered arrhythmic activities. In this section, Ca^{2+} sensitive dye Rhod2-loaded intact mouse heart from sham and repeated alcohol-exposed mice were used in ex vivo confocal imaging to examine Ca^{2+} mishandling in the atrial tissue.

Langendorff-perfusion setting for dye loading is shown in Figure 9A, and the perfusion chamber on a confocal microscope with a mouse heart stabilized is shown in Figure 9B. During the entire imaging experiment, the mouse heart was continuously perfused with 37°C-oxygenated normal Tyrodes' solution to preserve viability. Electro-mechanical uncouplers of combined BDM (5 mM) and blebbistatin (3 μM) were used control the movement of the cardiac muscle and stabilized the heart for confocal imaging (B. Chen et al., 2012; W. Chen, et al., 2014; J. Zhang et al., 2014b; J. Zhang, et al., 2015). Burst pacing was delivered via a bipolar electrode located on the atrium that was not being examined with confocal imaging to avoid potential pacing-related artifacts. Sinus and post-burst pacing Ca^{2+} responses were recorded from both left atria and right atria for each heart. The methods used in this section are described in detailed in Materials and Methods, Chapter Ten, page 188-189.

Example recording traces show enhanced spontaneous and 10Hz burst pacing-induced Ca^{2+} waves in atria from binge alcohol-exposed mice compared to sham controls (Figures 15B) and summarized data from repeated experiments confirmed the repeated binge alcohol-promoted atrial Ca^{2+} waves both during sinus rhythm (4.35 ± 1.83 incidences/mm*sec vs. 0.02 ± 0.02 incidences/mm*sec in sham, n = 7, 7, $p < 0.01$; Figure 16A) and after 10Hz burst pacing (4.19 ± 1.62 incidences/mm*sec vs. 0.76 ± 0.57 incidences/mm*sec in sham, n = 7, 7, $p < 0.05$; Figure 16A). Previous findings from multiple sources suggest that Ca^{2+} waves stem from diastolic SR Ca^{2+} leak (D. A. Eisner, Kashimura, O'Neill, et al., 2009; D. A. Eisner, Kashimura, Venetucci, et al., 2009). The intracellular Ca^{2+} decay constant τ was also prolonged in binge alcohol-exposed mice compared to sham controls (37.81 ± 1.26 msec vs. 31.02 ± 0.96 msec in sham, n = 7, 7, $p < 0.05$; Figure 16B), further suggesting the presence of diastolic SR Ca^{2+} leak in alcohol-exposed mice.

To further understand the role of JNK signaling in binge alcohol-promoted atrial Ca^{2+} waves, JNK1/2dn mice with JNK signaling ablated by overexpression of cardiac-specific dominant negative JNK (both JNK1 and JNK2) were also challenged with repeated binge alcohol exposure. Sample confocal Ca^{2+} imaging recording showed that after burst pacing, sinus rhythm resumed without the eruption of Ca^{2+} waves in alcohol-challenged JNK1/2dn hearts (Figure 15C). The summarized data further showed that alcohol-treated JNK1/2dn mice have significantly lower frequency of Ca^{2+} waves compared to repeated binge alcohol-treated WT mice

during sinus rhythm (0.14 ± 0.09 incidences/mm*sec in JNK1/2dn vs. 4.34 ± 1.83 incidences/mm*sec in binge alcohol-treated WT, n = 6, 7, $p < 0.01$; Figure 16A) and post 10Hz burst pacing (0.75 ± 0.32 incidences/mm*sec in JNK1/2dn vs. 4.19 ± 1.62 incidences/mm*sec in binge alcohol-treated WT, n = 6, 7, $p < 0.05$; Figure 16A). Meanwhile, the repeated binge alcohol-expose JNK1/2dn mice showed comparable level of Ca^{2+} wave frequency with sham WT (0.02 ± 0.02 incidences/mm*sec during sinus rhythm, n = 7, *p* = NS; 0.76 ± 0.57 incidences/mm*sec post burst pacing, n = 7, *p* = NS; Figure 16A) mice and sham JNK1/2 mice (0.07 ± 0.07 incidences/mm*sec during sinus rhythm, n = 3, *p* = NS; 1.11 ± 0.65 incidences/mm*sec post burst pacing, n = 3, *p* = NS; Figure 16A).

Similarly, the intracellular Ca^{2+} decay time constant in binge alcohol-treated JNK1/2dn mice (31.14 ± 2.96 msec, n = 6; Figure 16B) was at similar level to sham JNK1/2dn mice (32.00 ± 0.21 msec, n = 3, *p* = NS; Figure 16B) and sham WT mice (31.02 ± 0.96 msec in WT, n = 7, *p* = NS; Figure 16B). In addition, there is no significant difference demonstrated between binge alcohol treated WT mice (22.80 ± 0.85 msec vs. 22.07 ± 0.61 msec, n = 7, 7, *p* = NS; Figure 16C) or JNK1/2dn mice (22.30 ± 0.57 msec vs. 22.20 ± 0.50 msec, n = 6, 2, *p* = NS; Figure 16C). In summary, these results provide further evidence that alcohol-evoked JNK activation plays an essential role in abnormal Ca^{2+} activities.

Figure 15

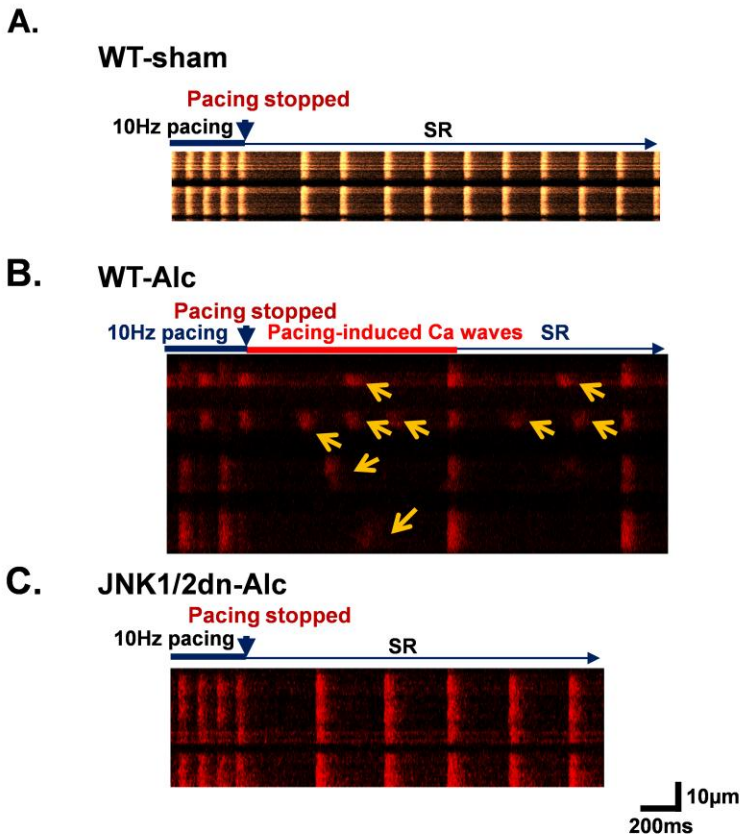

Figure 15. Binge alcohol exposure increases atrial Ca^{2+} wave frequency in JNK-dependent manner. **A-B)**. Representative confocal images showing increased frequency of Ca^{2+} waves (labeled with yellow arrow) in binge alcohol-exposed WT mouse atria after 10Hz burst pacing compared to that of sham controls, while JNK inhibition in JNK1/2dn Tg mice **(C)** with overexpression of cardiac-specific inactivated dominant negative JNK prevented binge alcohol-driven abnormal Ca^{2+} waves.

Figure 16

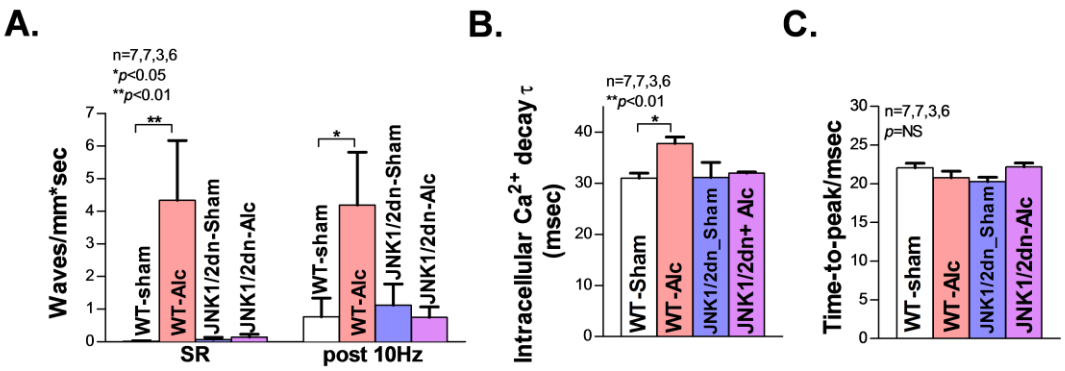

Figure 16. Binge alcohol exposure increases atrial Ca^{2+} wave frequency and intracellular Ca^{2+} decay constant τ in JNK-dependent manner. **A)**. Summarized data showing increased frequency of Ca^{2+} waves in binge alcohol-exposed WT mouse atria during sinus rhythm and after 10Hz burst pacing compared to that of sham controls, while JNK pathway inhibition in JNK1/2dn mice prevented binge alcohol-driven abnormal Ca^{2+} waves. Similar level of Ca^{2+} wave frequency was demonstrated between sham JNK1/2dn mice and alcohol-treated JNK1/2 mice, further indicating the role of JNK in alcohol-induced SR Ca^{2+} waves. Moreover, JNK1/2dn mice showed comparable level of Ca^{2+} wave frequency both during sinus rhythm and after 10Hz burst pacing, suggesting similar response in SR Ca^{2+} handling in the genetically modified mice compared to WT sham controls. **B)**. Atrial intracllular Ca^{2+} decay constant τ was increased in binge alcohol-exposed WT mice, but binge alcohol-exposed JNK1/2dn mice exhibited unchanged τ of Ca^{2+} decay compared to that of sham controls. Similar level of intracellular Ca^{2+} decay constant was shown between sham JNK1/2dn mice and alcohol-treated JNK1/2dn mice, further indicating the role of JNK in the prolongation of Ca^{2+} decay constant τ. **C)**. Pooled data of unchanged time to peak of Ca^{2+} transients in binge alcohol-treated WT or JNK1/2dn mice compared to that of sham controls and sham JNK1/2dn mice. All data were presented as Mean ± SEM. For **A)**, Kruskal-Wallis test (One-Way ANOVA on ranks) with post hoc test was used to compare the mean of WT-sham vs WT-Act and JNK1/2dn-sham vs JNK1/2dn-Alc for Ca^{2+} wave frequency during sinus (SR) and post 10Hz pacing. One-Way ANOVA test with Tukey post hoc test was used to compare the mean of alcohol-treated versus sham in both WT mice and JNK1/2dn mice in **B)** intracellular Ca^{2+} decay constant τ, and **C)** time-to-peak for Ca^{2+} transient.

5.2.2 Pharmacological JNK Activation Also Promotes Atrial Ca^{2+} Waves

To further confirm the role of JNK activation in atrial Ca^{2+} mishandling, WT mice were directly challenged with 4 doses of anisomycin injection to induce JNK activation. Confocal imaging on the atria was performed on Langendorff-perfused intact hearts. An example recording showed large amount of atrial Ca^{2+} waves after burst pacing (Figure 17A) in anisomycin-treated WT mice.

My results suggest that anisomycin failed to elicit atrial Ca^{2+} waves after burst pacing in JNK2KO mice (Figure 17A). Summarized data further confirm that Ca^{2+} wave frequency was showed a trend of increase in anisomycin-challenged WT mice compared to JNK2KO mice both during sinus rhythm (7.11 ± 3.68 incidences/mm*sec vs. 2.86 ± 2.86 incidences/mm*sec in anisomycin-treated JNK2KO; n = 9, 5, p = NS ; Figure 17B) and a significant increase after 10Hz burst pacing (29.06 ± 12.16 incidences/mm*sec vs. 2.28 ± 2.28 incidences/mm*sec in anisomycin-treated JNK2KO; n = 9, 5, $p < 0.05$; Figure 17B). Also, the intracellular Ca^{2+} decay prolongation during both sinus rhythm and post-10Hz pacing was observed in anisomycin-challenged WT mice but not in JNK2KO mice (38.70 ± 2.76 msec vs. 29.23 ± 0.37 msec in JNK2KO-aniso during sinus rhythm, n = 9, 5, $p < 0.05$; 46.76 ± 3.50 msec vs. 33.55 ± 3.68 msec in JNK2KO-aniso post 10 Hz pacing, n = 9, 5, $p < 0.05$; Figure 17C). On the other hand, no significance difference was demonstrated in intracellular Ca^{2+} decay constant between during sinus rhythm and post-10Hz burst pacing in either anisomycin-treated WT (46.76 ± 3.50 msec post

10Hz vs. 38.70 ± 2.76 msec SR, n = 9, 9, *p* = NS, Figure 17C) or anisomycin-treated JNK2KO mice (33.55 ± 3.68 msec post 10Hz vs. 29.23 ± 0.37 msec SR, n = 5, 5, *p* = NS, Figure 17C). Such results further confirm the role of JNK2 in atrial Ca^{2+} mishandling, yet, the underlying mechanisms of the increased Ca^{2+} waves and the prolongation of intracellular Ca^{2+} decay constant τ demands more thorough investigation which I studied in the following section of this thesis. I address this issue in the following section of this thesis.

Figure 17

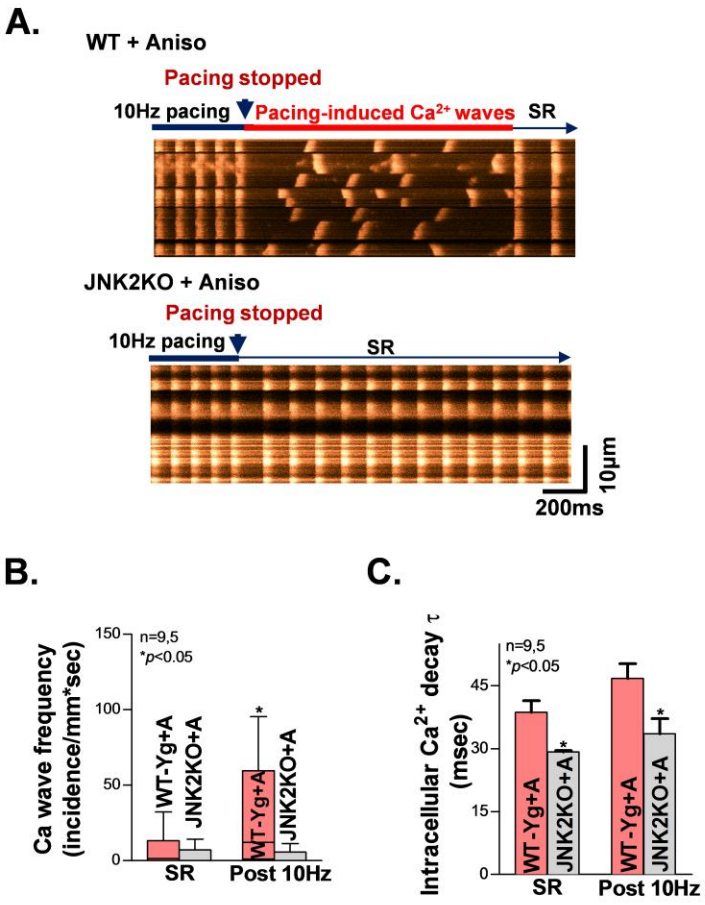

Figure 17. JNK activation promotes atrial Ca^{2+} waves without binge alcohol. **A)**. *ex vivo* confocal imaging shows enhanced atrial Ca^{2+} waves in JNK activator anisomycin-treated young mouse; while knocking out JNK2 prevented anisomycin-treatment induced atrial Ca^{2+} waves. **B)**. Summarized data showing increased atrial Ca^{2+} waves in anisomycin-treated WT control mice compared to anisomycin-treated JNK2KO mice during both sinus rhythm (SR) and post 10Hz burst pacing. Kruskal-Wallis test (One-Way ANOVA on ranks) with post hoc test was used to compare the mean of selected groups. **C)**. Summarized data shows increased Ca^{2+} decay constant τ anisomycin-treated WT mice compared to anisomycin-treated JNK2KO both during sinus rhythm and after 10Hz burst pacing. All data were presented as Mean ± SEM. One-Way ANOVA test with Tukey post hoc test was used to compare the mean of the pairs of interest.

5.3 Discussion

5.3.1 SR Ca^{2+} Mishandling in Repeated Binge Alcohol-exposed Mice Promotes Atrial Arrhythmia

Ca^{2+}-triggered activities are known as a pivotal mechanism of arrhythmogenesis. In the non-reentrant mechanism, the arising Ca^{2+} waves increase the local intracellular Ca^{2+} concentration and promote the reverse activation of NCX, which leads to inward transient current that depolarizes the plasma membrane and causes DADs. Extensive studies indicate that increased Ca^{2+} wave frequency contributes to arrhythmia initiation (Bers, 2014; W. Chen, et al., 2014; Ferrier, 1977; Gonano, et al., 2011; Kass, Lederer, et al., 1978; Kass, Tsien, et al., 1978; Lederer & Tsien, 1976; Xie, et al., 2013), and this mechanism has been reviewed by many authors (Bers, 2014; Boyden, Dun, & Stuyvers, 2015; Pogwizd & Bers, 2004). In the current studies, I found dramatically increased atrial Ca^{2+} wave frequency in binge alcohol-exposed mice which are essential for the rise of triggered activity. Moreover, results from JNK1/2dn mice further showed that the repeated binge alcohol induced abnormal Ca^{2+} waves are JNK-dependent.

On the other hand, elimination of Ca^{2+} waves alleviates triggered arrhythmias in animal models (Bai et al., 2013; J. Zhang, et al., 2015), which further demonstrates the critical contribution of Ca^{2+} waves in arrhythmogenicity. Although it was previously observed that Ca^{2+} wave-induced-V_m oscillation in one cell may not be sufficient to reach the threshold that opens Na^+ channels to trigger an ectopic action

potential, and Ca^{2+} waves do not necessarily trigger arrhythmic electrical activities at a 1:1 ratio (Haghighi, et al., 2015; B. Liu et al., 2015; Maruyama, et al., 2010), it is also observed in our and other labs (Xie, et al., 2013; J. Zhang, et al., 2014b; J. Zhang, et al., 2015) that during simultaneous confocal Ca^{2+} imaging in which Ca^{2+} transients/Ca^{2+} waves within a scale of several adjacent myocytes and atrial electrogram recordings in which electrical signals from the intact atrial tissue on Langendorff-perfused mouse hearts, large amount of Ca^{2+} waves proceeded atrial arrhythmia.

Although alcohol-induced cardiac Ca^{2+} mishandling has been studied, previous research primarily focused on the response of cardiac myocytes isolated from alcohol-treated animals, or cells subjected to transiently alcohol exposure (Aistrup, et al., 2006). To the best of my knowledge, this study is the first to reveal alcohol-caused Ca^{2+} waves in intact atrial tissue where the cells being imaged were still located in the native tissue environment. In this section, I found that alcohol-exposed mice have increased frequency for atrial Ca^{2+} waves. The striking rescue by ablating JNK pathway in JNK1/2dn mice further suggests that JNK plays a key role in defining AF propensity in the binge-alcohol exposed atria and that JNK activation drives arrhythmogenic diastolic Ca^{2+} handling dysfunction. To further dissect the role of JNK activation in the arising of arrhythmogenic Ca^{2+} waves without alcohol exposure, I used JNK activator anisomycin to treat WT and JNK2KO mice (lacking

JNK2 pathway) and found an increased frequency of Ca^{2+} waves in WT mice compared to the JNK2KO mice undergoing the same treatment.

Moreover, alcohol-treated and JNK-activated mice exhibited prolonged atrial intracellular Ca^{2+} decay constant τ compared to the sham control ones, which further suggest the existence of increased SR Ca^{2+} leak, a common contributor of Ca^{2+} waves. It is known that besides increased SR Ca^{2+} leak, there are additional factors that can contribute to prolongation of intracellular Ca^{2+} decay. Such factors include decreased SR Ca^{2+} uptake, decreased NCX activity and decreased Ca^{2+} extrusion from sarcolemmal Ca^{2+} pumps (J. W. Bassani, R. A. Bassani, & D. M. Bers, 1994; R. A. Bassani, J. W. Bassani, & D. M. Bers, 1994). However, in this thesis, I showed in Chapter Seven that SERCA activity is increased by alcohol treatment, which suggests that decreased SR Ca^{2+} uptake is not likely a contributing factor to the prolongation of intracellular Ca^{2+} decay constant. Moreover, data from the Ai Lab has also that the time constant for NCX Ca^{2+} extrusion is not altered by JNK activation (via anisomycin treatment) measured in HL-1 cells, which further suggest that JNK activation is not likely to contribute to altered NCX function. To date, it is still not clear whether alcohol or JNK activation affects sarcolemmal Ca^{2+} pumps.

However, data from the Fill Lab showed that in RyR single channel recording experiments, CaMKII inhibition reversed both anisomycin treatment or alcohol treatment induced increase in RyR single channel opening probability. Also, I showed that inhibiting CaMKII precluded both the increased SR Ca^{2+} leak in HL-1

(Figure 22A) and the increased intracellular Ca^{2+} decay constant in mouse atria (Figure 23B). These evidences suggest that CaMKII inhibition prevents alcohol treatment/JNK activation-induced diastolic SR Ca^{2+} leak induced by precluding CaMKII-dependent increase in RyR single channel activity. Moreover, CaMKII inhibition with KN93 reversed the binge alcohol-induced intracellular Ca^{2+} decay prolongation in mouse atria. Taken together, this evidence lends further support that the CaMKII-dependent increased SR Ca^{2+} leak plays a major role in intracellular Ca^{2+} decay prolongation.

Although Ca^{2+} waves can increase the intracellular Ca^{2+} concentration, intracellular Ca^{2+} buffers also plays an important role in determining the propagation of Ca^{2+} waves. Previous research suggests that increasing intracellular buffer decreases the frequency and kinetics of Ca^{2+} waves in multiple cells types such as olfactory ensheathing cells (a specific type of glia cell) (Stavermann et al., 2015), HeLa cells (Rintoul & Baimbridge, 2003), and cardiac myocytes (M. Chen, Wang, & Qu, 2010; Sugai, et al., 2009). Myofilaments act as one of the prominent Ca^{2+} buffer systems in myocytes since the activation of contractile machinery directly depends on the binding of large amount of Ca^{2+} released from SR to troponin C (about 50% of SR Ca^{2+} release in each cardiac cycle)(Shannon, Ginsburg, & Bers, 2000). When myofilament Ca^{2+} sensitivity is increased, the steady-state force-Ca^{2+} relationship curve shift towards the left and the contractile machinery is activated at lower intracellular Ca^{2+} concentration (Huke & Knollmann, 2010), as a

result, the un-buffered Ca^{2+} in the cytosol tends to increase. Increased myofilament Ca^{2+} sensitivity has been observed in human arrhythmogenic diseases such as dilated cardiomyopathy (Wolff, Buck, Stoker, Greaser, & Mentzer, 1996), and was shown to promote arrhythmogenicity in mice with mutated troponin (Knollmann et al., 2003; Puglisi, Yuan, Bassani, & Bers, 1999). On the other hand, there are controversial results on whether intracellular Ca^{2+} buffer plays a key role in Ca^{2+} wave initiation and propagation which is essential for increasing local intracellular Ca^{2+} concentration (W. Chen, Aistrup, Wasserstrom, & Shiferaw, 2011). For instance, increased myofilament Ca^{2+} sensitivity via Pak1 overexpression significantly decreased Ca^{2+} spark amplitude (Sheehan, Ke, Wolska, & Solaro, 2009). Also, Mira et al. showed that myofilament sensitivity does not play an important role in regulating Ca^{2+} wave propagation speed measured in ventricular trabeculae during stretch (Miura et al., 2015), similar to findings made in a computational modeling study (X. Chen et al., 2014). In the current study, I focused on the contribution of alcohol-induced diastolic SR Ca^{2+} release properties rather than the Ca^{2+} buffers properties of the cell. The Ca^{2+} buffers such as myofilament Ca^{2+} sensitivity or other intracellular Ca^{2+} buffer systems could be a future direction for this study, which will be discussed in Chapter Nine Future Directions.

Increased diastolic RyR opening probability can give rise to both Ca^{2+} waves and Ca^{2+} sparks in myocytes. The frequency of Ca^{2+} waves and Ca^{2+} sparks are both used to quantify the RyR diastolic opening in cardiac diseases. For instance,

Wasserstrom's group measured Ca^{2+} spark frequency in alcohol-treated cells as a parameter for quantifying Ca^{2+} mishandling (Aistrup, et al., 2006). Yet in the current study, the frequency of Ca^{2+} sparks was not used as an indicator of arrhythmogenic Ca^{2+} mishandling. This is due to the technical challenge in *ex vivo* confocal imaging of tissue, where Ca^{2+} imaging background is typically higher than that inside isolated single cells. However, Ca^{2+} spark imaging is worthy of exploration considering its ability to detect local SR Ca^{2+} release events. This work could be accomplished in circumstances where the dye loading conditions or microscopy are further improved after extensive experimental optimization.

5.3.2 Limitations in Quantifying SR Ca^{2+} Mishandling in Mouse Atria

It is common practice to use electro-mechanical uncouplers to stabilize the heart during confocal Ca^{2+} imaging since even the slightest motion can cause significantly disturbance on the scanned images acquired with a 40x objective. In my thesis, combined blebbistatin (3 μM) and BDM (5 mM) was used. This is a very low dose that is efficient to stabilize the heart for imaging reported by other labs and was tested in the Ai Lab (B. Chen, et al., 2012; W. Chen, et al., 2014; J. Zhang, et al., 2014b; J. Zhang, et al., 2015).

However, the usage of blebbistatin raises concerns that blebbistatin (an ATP-inhibiting agent specific for myosin II) may alter the intracellular Ca^{2+} handling for myocytes. For instance, findings from Dr. de Tombe's lab has shown that blebbistatin (0.5 μM, 1 hr) increases apparent diastolic emission (Ca^{2+} background

signal) (Farman et al., 2008), similar to the findings in the Efimov Lab using 10 μM blebbistatin in Langendorff-perfused rabbit hearts (Fedorov et al., 2007).

At the same time, findings from the Dr. de Tombe's lab have shown that 0.5 μM blebbistatin causes no significant changes Ca^{2+} transient amplitude during pacing or the rate of Ca^{2+} relaxation (intracellular Ca^{2+} decay rate) (Farman, et al., 2008). Similarly, the Efimov Lab provided further evidence showing that 10μM blebbistatin did not alter Ca^{2+} transient morphology in Langendorff-perfused rabbit hearts (Fedorov, et al., 2007). In this light, the two parameters used in my thesis to quantify SR Ca^{2+} mishandling in mouse atria, intracellular Ca^{2+} decay constant and Ca^{2+} wave frequency, are not affected by the usage of blebbistatin.

Moreover, findings from the Efimov Lab further suggest that 10 μM blebbistatin has no effect of action potential/Ca^{2+} transient morphology, atrial and ventricular effective refractory period and atrial and ventricular activation pattern. These results further suggest 10 μM blebbistatin is not likely to alter arrhythmogenicity in Langendorff-perfused hearts. In my thesis, all the experiments were carried out under the same conditions with the same uncoupler application in my thesis studies. Thus even if there are any blebbistatin-induced intracellular Ca^{2+} mishandling in the treated group, a proper control was used to show the background signal in this thesis.

5.3.3 Other Potential Contributors to the Initiation and Maintaneous of AF

Multiple cellular and tissue factors are also known to contribute to AF, such as abnormal function of surface membrane channels (S. Wagner et al., 2011) and tissue level electrical reentry, as previously reported (Bai, et al., 2013). In the binge alcohol mouse model, my preliminary data suggest unaltered action potential conduction velocity in repeated binge alcohol-exposed mice. Slow cardiac tissue conduction is usually found in pathological conditions where major gap junction remodeling is enhanced (Jones & Lancaster, 2015; Yan, et al., 2013), or fibrosis formation is increased (Ohkubo, et al., 2010; Stevenson, et al., 1989). My observations on atrial tissue conduction velocity in repeated alcohol-exposed mice is consistent with immunoblotting data obtained by the Ai Lab that showed unchanged expression level of Cx43 in binge alcohol-exposed mouse atria (Figures. 2A,2B). These results suggest that reentrant arrhythmogenic substrate may not serve as a predominant factor for the binge alcohol-promoted atrial arrhythmia. In addition, enhanced heterogeneity between simultaneously recorded atrial action potential maps and Ca^{2+} transient maps further indicate the existence of ectopic Ca^{2+} activities.

While gap junction proteins provide channels for conjuncted cells to be electrically and metabolically coupled, fibrosis can form non-conductive zones that block the action potential propagation and promote reentry. Long term alcohol exposure was found to increase fibrosis formation in human (Sokolova, 2016) and

in various animal models (W. Liu, et al., 2011; Steiner, et al., 2015; Vasdev, et al., 1975). In a mouse model of chronic alcohol feeding (24weeks), upregulation of collagenα I-V was found in both mRNA and protein level in the heart (Steiner, et al., 2015). On the contrary, Matyas et al. found that neither chronic nor binge drinking is associated with increased cardiac fibrosis using picro-sirius red staining (Matyas, et al., 2016). Meanwhile, it is found that low doses of alcohol exposure protect the heart from fibrosis (Yu et al., 2016). Previous research suggest that cardiac interstitial formation relies on the activation of inflammatory cytokines, which further activates the cardiac fibroblast; and ECM remodeling typically happens in the time scale of weeks after the tissue insult (Czubryt, 2012). Thus, it is reasonable to assume that in the current model of binge drinking, fibrosis formation is not likely a significant contributor of atrial arrhythmia; however, this would need to be tested in future experiments for confirmation. It would also be an interesting future study to closely monitor the interaction of inflammation of cytokines and the multiple cell types involved in fibrosis formation in long-term alcohol exposure, which will be discussed in Chapter Eight Future Directions.

It is well accepted that triggered activity and reentry substrates interact because triggered premature beats are required to engage reentry to form full-scale arrhythmia (Ai, 2015; Pogwizd et al., 1992). Therefore, I acknowledge the complexity and feedback interactions between reentry and Ca^{2+} handling remodeling that eventually cause arrhythmogenesis. In the current studies, I

discovered a previously unrecognized causal link between JNK2 activation and alcohol-induced Ca^{2+} mishandling and enhancement of AF propensity. Investigations of the contribution of alcohol on tissue reentry mechanisms are valuable yet realistically beyond the scope of the current studies.

5.4 Conclusion

In this chapter of the study, I explored the underlying electrophysiological mechanisms of JNK-evoked atrial arrhythmia in repeated binge alcohol exposed mouse hearts by assessing the cardiac Ca^{2+} activities in intact atria. Confocal Ca^{2+} imaging on the atria showed increased spontaneous and burst pacing-induced Ca^{2+} waves and prolonged intracellular Ca^{2+} decay constant τ in repeated binge alcohol exposed hearts compared to sham controls. Pharmacological challenge with JNK activator anisomycin also promotes abnormal Ca^{2+} sparks and waves. In contrast, JNK2 knockout prevented anisomycin-induced increase in Ca^{2+} spark and wave frequency compared to sham control level. Moreover, JNK inhibition with cardiac-specific overexpression of dominant negative JNK precluded the binge alcohol promoted Ca^{2+} waves and prolongation of intracellular Ca^{2+} decay constant. These results strongly suggest that JNK activation underlies binge alcohol-promoted atrial Ca^{2+} mishandling and atrial arrhythmia.

CHAPTER SIX

BINGE ALCOHOL PROMOTES DIASTOLIC SARCOPLASMIC RETICULUM CALCIUM MISHANDLING VIA ENHANCING CaMKII-DEPENDENT RyR PHOSPHORYLATION

6.1 Abstract

Accumulating evidence suggests that increased diastolic SR Ca^{2+} leak is an important cellular mechanism of enhanced Ca^{2+} waves in cardiac myocytes. In the previous chapter, I have demonstrated the enhanced frequency of atrial Ca^{2+} waves in binge alcohol treated mice compared to controls in JNK-dependent manner. Interestingly, binge alcohol treatment also increased the intracellular Ca^{2+} decay constant τ, which suggests enhanced diastolic SR Ca^{2+} leak or altered SR Ca^{2+} uptake. Previous studies have shown that the activation of CaMKII, a prominent pro-arrhythmogenic molecule, promotes SR Ca^{2+} mishandling via phosphorylating RyR.

In this part of my thesis study, I aim to explore the underlying mechanisms of alcohol-JNK promoted SR Ca^{2+} waves by examining the contribution of diastolic SR Ca^{2+} leak (Figure 18). Increased diastolic SR Ca^{2+} leak was found in alcohol-treated (50 mM, 24 hrs) and JNK-activated (treated with 0.2 μM anisomycin, 24 hrs) atrial myocytes via tetracaine-perfusion protocol using confocal Ca^{2+} imaging. Pretreating the cells with JNK inhibitor (JNK2I, 170 nM; EMD) or CaMKII inhibitor (KN93, 0.2

μM;EMD) before alcohol exposure precluded the anisomycin or alcohol-induced increase in SR Ca^{2+} leak, further demonstrating that either JNK or CaMKII inhibition was sufficient to return the SR Ca^{2+} leak to sham control level. Enhanced CaMKII phosphorylation and CaMKII-dependent RyR phosphorylation were also found after repeated binge alcohol treatment in both atrial cells and mice. Similar effects were also found in anisomycin-treated atrial cells and mice. Moreover, a single dose of KN93 injection (7.2 mg/kg, BW, I.P.) after repeated binge alcohol-exposed mice decreased atrial Ca^{2+} wave frequency and atrial arrhythmogenicity, further suggesting the potential therapeutic role of CaMKII inhibition. Similarly, challenging AC3I mice (a mouse line that overexpresses the CaMKII inhibitor AIP) with JNK activator anisomycin (500 mg/kg BW, I.P.) failed to increase atrial Ca^{2+} arrhythmogenic events and atrial arrhythmia. In sum, these results indicate that alcohol/JNK activation promotes diastolic SR Ca^{2+} leak via enhancing CaMKII activation, and CaMKII inhibition attenuates the binge alcohol-induced atrial arrhythmia.

To further explore the mechanisms of alcohol-promoted CaMKII activation, WT and mutant CaMKII (vv-CaMKII, lacking ROS-sensitive amino acid site Met280/281 due to Met-to-Val, a loss of function mutation) were overexpressed in HEK293 cells. Alcohol treatment promotes CaMKII activation in overexpressed wt CaMKII and vv-CaMKII to a similar extent, suggesting that alcohol-induced CaMKII activation is JNK-dependent but does not rely on the direct CaMKII oxidation on

Met280/281. Moreover, this alcohol-induced CaMKII activation was suppressed by pretreatment with JNK2 inhibitor (JNK2I, 170nM). Overall, these results suggest that alcohol exposure-induced CaMKII activation is dependent upon JNK activation but not of a direct effect of CaMKII oxidation on CaMKII-Met280/281.

Figure 18

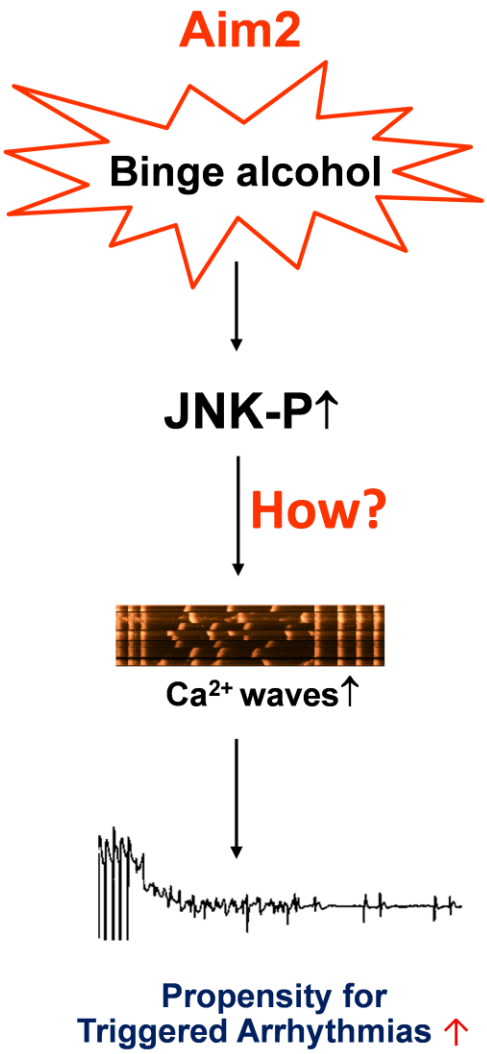

Figure 18. Schematic of the research focus for Aim2. The previous chapter of the thesis indicated that aberrant Ca^{2+} mishandling underlies alcohol/JNK activation prompted atrial arrhythmias. CaMKII activation has been shown to promote diastolic SR Ca^{2+} leak via phosphorylating RyR. In this part of the thesis, I will further explore whether alcohol/JNK activation promotes Ca^{2+} mishandling via CaMKII-dependent RyR hyperphosphorylation.

6.2 Results

6.2.1 Alcohol/JNK Activation Promotes SR Ca^{2+} Mishandling via CaMKII Activation

6.2.1.1 Alcohol treatment promotes SR Ca^{2+} leak in a JNK-dependent manner. In order to explore the underlying mechanisms of enhanced SR Ca^{2+} wave and prolonged intracellular Ca^{2+} decay constant observed in binge alcohol exposed mouse atria, diastolic SR Ca^{2+} leak was measured from HL-1 cells (an atrial cell line) (Claycomb, et al., 1998; S. M. White, et al., 2004), grown to confluence on glass monolayers that were treated with alcohol (50 mM, 24 hrs). The remaining alcohol concentration in the cell culture medium was measured using the alcohol concentration measurement kit used to measure BAC in our repeated alcohol-exposed mouse model. After 24hrs incubation, cell culture medium alcohol concentration dropped significantly compared to the alcohol concentration when treatment was initiated, however, it is still significantly higher than sham control level (15.79 ± 0.42 mg/dL vs. 0.29 ± 0.23 mg/dL in sham, n= 5, 4, N = 2, $p < 0.001$; Figure 19B).

The diastolic SR Ca^{2+} leak was measured using the tetracaine perfusion protocol (Figure 20E) that is widely used in many previous studies (Ai, et al., 2005; Santiago et al., 2010; Shannon, Ginsburg, & Bers, 2002; Shannon, et al., 2003). The summarized results showed increased SR Ca^{2+} leak in alcohol-treated cells compared to sham controls (0.15 ± 0.02 $\Delta F/F_0$ vs. 0.05 ± 0.01 $\Delta F/F_0$ in sham, n = 12,

11, N = 4, $p < 0.001$; Figure 19A), while pretreatment with JNK2 inhibitor (JNK2I, 170nM) reversed the SR Ca^{2+} leak back to sham control level (0.05 ± 0.01 $\Delta F/F_0$ vs. 0.05 ± 0.01 $\Delta F/F_0$ in sham, n = 8, 11, N = 4, p = NS; Figure 19A).

Figure 19

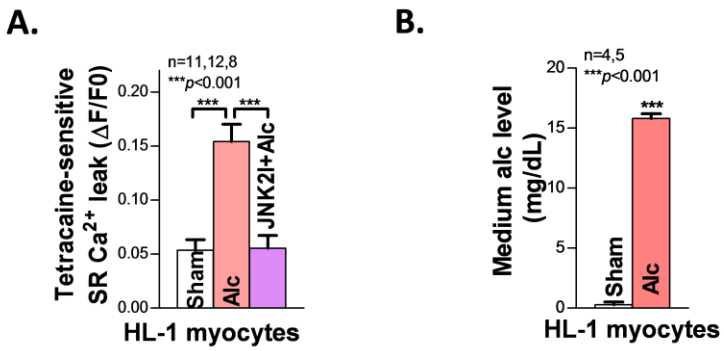

Figure 19. Alcohol exposure promotes SR Ca^{2+} leak in JNK-dependent manner **A)**. Summarized data showing increased tetracaine-sensitive diastolic SR Ca^{2+} leak in alcohol-treated HL-1 myocytes; JNK2 inhibitor JNK2I-IX (JNK2I) treatment completely prevented these alcohol actions. Measurements were carried out in 4 batches of cells (N=4) that totally included 11 sham monlayers (preparation), 12 alcohol-treated monolayers and 8 alcohol-treated monolayers with JNK2I pretreatment (n=11,12, 8). One-Way ANOVA test with Tukey post hoc test was used to compare the mean of selected groups. **B)**. Summarized data showing medium alcohol concentration after treatment for 24hrs, which is significantly higher than sham, yet below the legal limit of 80mg/dL. Measurements were carried out in the medium of 2 batches of cells (N=2) that totally included 4 sham monlayers and 5 alcohol-treated monolayers. Unpaired Student *t*-test was used to compare the mean of two groups in this study.

To further test that JNK activation *per se* can increase SR Ca^{2+} leak, HL-1 monolayers were treated with anisomycin (0.2 μM, 24 hrs) and subjected to the same SR Ca^{2+} leak measurement protocol. The results showed increased tetracaine-sensitive SR Ca^{2+} leak in anisomycin-treated cells compared to sham controls (0.18 ± 0.02 vs. 0.09 ± 0.01 $\Delta F/F_0$ in sham, n= 14, 10, N = 7, $p < 0.01$; Figure 20A), which could be reversed by pretreatment of JNK2 inhibitor (0.09 ± 0.02 vs. 0.09 ± 0.01 $\Delta F/F_0$ in sham, n = 11,10, N = 7, p = NS; Figure 20A). It is previously shown that HL-1 cell retain genetical and physiological phenotypes of adult atrial myocytes (Claycomb, et al., 1998; S. M. White, et al., 2004). To further confirm this result is not dependent on cell type, the same experiments were repeated in freshly-isolated rabbit atrial myocytes, and similar results were found in the rabbit atrial cells: anisomycin treatment significantly increased SR Ca^{2+} leak (0.09 ± 0.02 $\Delta F/F_0$ vs. 0.03 ± 0.01 $\Delta F/F_0$ in sham, n = 7, 9, N = 3, $p < 0.01$; Figure 20B), while JNK2 inhibitor pre-treatment returned the SR Ca^{2+} leak level to sham control level (0.01± 0.00, n=6, N = 3, p = NS compared to sham; Figure 20B). Meanwhile, intracellular Ca^{2+} decay constant τ was significantly prolonged after anisomycin challenge in HL-1 cells (187.2 ± 18.5 msec vs. 101.0 ± 11.3 msec in sham, n = 8, 6, N = 3, $p < 0.001$; Figure 20C), which was also reversed by JNK2 inhibition (111.6 ± 7.2 msec vs. 101.0 ± 11.3 msec in sham, n = 11, 6, N = 3, $p < 0.001$; Figure 20C), further suggesting the role of JNK activation in enhancing diastolic SR Ca^{2+} leak.

To further assess the specific role of JNK2 versus JNK1 in JNK activation-induced increase in diastolic SR Ca^{2+} leak, JNK1 (JNK1dn) or JNK2 (JNK2dn) dominant negative proteins were overexpressed in HL-1 before the anisomycin treatment. JNK2dn-overexpressing cells showed significantly lower level of SR Ca^{2+} leak compared to JNK1dn-overexpressing cells after the same anisomycin challenge ($0.06 \pm 0.02\ \Delta F/F_0$ in JNK2dn vs. $0.15 \pm 0.02\ \Delta F/F_0$ in JNK1dn, n = 7, 6, N = 3, $p < 0.05$; Figure 20D), further suggesting JNK2 is the primary JNK protein that is involved in regulating SR Ca^{2+} leak.

Figure 20

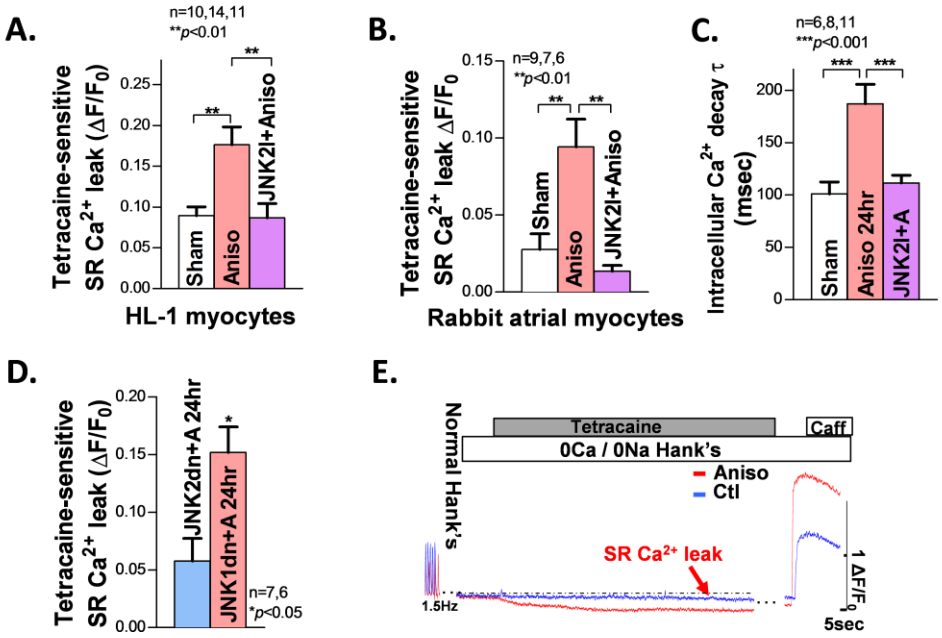

Figure 20. JNK activation promotes SR Ca^{2+} leak in without alcohol challenge. **A)**. Summarized data showing increased tetracaine-sensitive diastolic SR Ca^{2+} leak in anisomycin-treated (Aniso) HL-1 myocytes; JNK2 inhibitor JNK2I-IX (JNK2I) treatment completely prevented these alcohol actions. Measurements were carried out in 7 batches of cells (N=7) that totally included 10 sham monlayers (preparation), 14 anisomycin-treated monolayers and 11 anisomycin-treated monolayers with JNK2I pretreatment (n=10,14,11). **B)**. Summarized data showing anisomycin increased tetracaine-sensitive SR Ca^{2+} leak in rabbit atrial myocytes as in HL-1 myocytes. Measurements were carried out in 3 batches of cells (N=3) that totally included 9 sham preparation, 7 anisomycin-treated preparation and 6 anisomycin-treated monolayers with JNK2I pretreatment (n=9,7,6). **C)**. Prolonged τ for intracellular Ca^{2+} decay in anisomycin-treated atrial myocytes compared to sham-controls; JNK2 inhibitor JNK2I treatment completely prevented these anisomycin actions. Measurements were carried out in 3 batches of cells (N=3) that totally included 6 sham monlayers, 8 anisomycin-treated monolayers and 11 anisomycin-treated monolayers with JNK2I pretreatment (n=10,14,11). All data were presented as Mean ± SEM. For **A-C)**, One-Way ANOVA test with Tukey post hoc test was used to compare the mean of selected groups. **D**). Summarized data suggest that overexpression of inactivated JNK2dn proteins attenuates anisomycin-induced SR diastolic Ca^{2+} leak, while inactivated JNK1dn has no such rescue effects. Measurements were carried out in 3 batches of cells (N=3) that totally included 7 anisomycin-treated JNK2dn-overexpressing monlayers and 6 anisomycin-treated JNK1dn-overexpressing monolayers (n=7,6). Unpaired Student *t*-test was used to compare the mean of the two groups in this study. **E**). Example traces of aniso-treated versus sham control of the tetracaine-sensitive leak measurement protocol using confocal microscope.

6.2.1.2 CaMKII activation and CaMKII-dependent RyR phosphorylation in alcohol treatment. RyR is a phospho-protein and its phosphorylation has been reported to increase opening probability (D. A. Eisner, Kashimura, O'Neill, et al., 2009; D. A. Eisner, Kashimura, Venetucci, et al., 2009; Houser, 2014; O'Brien, et al., 2015). CaMKII is one of the primary kinases that have been reported to phosphorylate RyR. To explore whether the increased diastolic SR Ca^{2+} leak is related to CaMKII-dependent RyR phosphorylation, we used phosphorylation-specific CaMKII antibody to probe the CaMKII activation status in cardiac tissue from alcohol-exposed mice versus sham controls. The results showed that CaMKII activation was enhanced in alcohol-treated mouse cardiac tissue (147.6 ± 10.3 % vs. 100.0 ± 11.1% in sham, n = 3, 3, $p < 0.05$; Figure 21A), while the expression level of CaMKII-δ was comparable between alcohol-treated mouse cardiac tissue and sham controls (86.5 ± 3.0 % vs. 100.0 ± 5.7% in sham, n=3, 3, *p*=NS; Figure 21A). Subsequently, CaMKII-dependent RyR phosphorylation was also increased compared to sham controls (169.5 ± 19.8% vs. 100.0 ± 10.7%, n = 3, 3, $p < 0.05$; Figure 21B). In contrast, PKA-dependent RyR phosphorylation was unaltered (Figure 21B), suggesting that CaMKII-dependent rather than PKA-dependent RyR phosphorylation plays an essential roles in alcohol-induced diastolic SR Ca^{2+} leak.

Figure 21

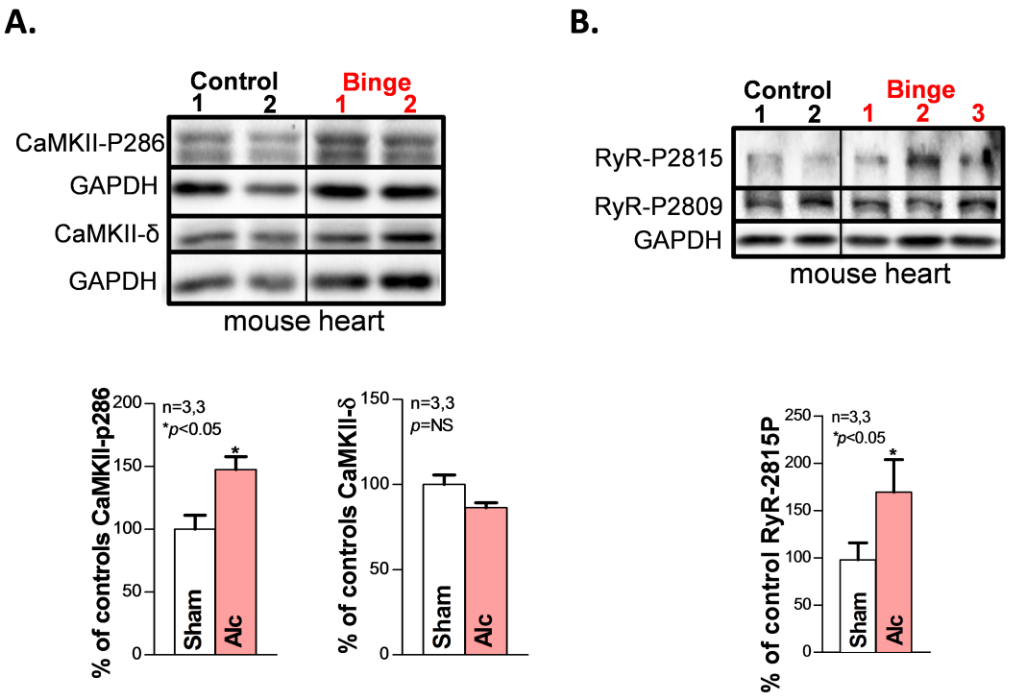

Figure. 21 Alcohol exposure promotes CaMKII activation and CaMKII-dependent phosphorylation of RyR. **A)**. Immunoblotting images and summarized data showing enhanced phosphorylation of CaMKII (CaMKII-P286) in cardiac tissue from alcohol-exposed mice while the expression level of CaMKIIδ remain unaltered between experiment groups. **B)**. Immunoblotting images and summarized data showing enhanced CaMKII-dependent RyR-phosphorylation (RyR-2815), but not PKA-dependent phosphorylation of RyR-2809 in binge alcohol-exposed mouse hearts. All data were presented as Mean ± SEM. Unpaired Student *t*-test was used to compare the mean of two groups in each study. *I would like to thank Ms.Weiwei Zhao for assistance on immunoblotting.*

6.2.1.3 CaMKII inhibition rescues alcohol-induced and anisomycin-induced JNK activation that in turn evokes diastolic SR Ca^{2+} leak. To further understand the functional consequence of CaMKII activation in alcohol-induced SR Ca^{2+} leak, HL-1 cell monolayers were pre-treated with CaMKII inhibitor KN93 or its inactive analogue KN92 before being exposed to alcohol or the JNK activator anisomycin. SR Ca^{2+} leak was measured using the tetracaine perfusion method (Figure 20E) described together with the cell treatment conditions in detail in Methods and Methods, Chapter Ten, page 191-193. The summarized data showed that KN93 pretreatment in HL-1 cells before alcohol exposure abolished the alcohol-promoted SR Ca^{2+} leak and return the SR Ca^{2+} leak level to sham control (0.06 ± 0.02 vs. 0.05 ± 0.00 $\Delta F/F_0$ in sham, n=9, 11, p = NS; Figure 22A). Also, KN93 pretreatment abolished the increase in tetracaine-sensitive SR Ca^{2+} leak in anisomycin-treated HL-1 cells (0.11 ± 0.02 vs. 0.09 ± 0.01 $\Delta F/F_0$ in sham, n = 10, 10, N = 4, p = NS; Figure 22B). On the contrary, KN92 pre-treatment has no effect in decreasing alcohol (0.15 ± 0.02 vs. 0.05 ± 0.00 $\Delta F/F_0$ in sham, n = 11, 11, N = 4, $p < 0.001$; Figures 22A) or anisomycin induced increase in diastolic SR Ca^{2+} leak (0.19 ± 0.02 vs. 0.09 ± 0.01 $\Delta F/F_0$ in sham, n = 10, 10, $p < 0.01$; Figure 22B). These results further suggest that alcohol promotes diastolic SR Ca^{2+} leak via subsequent CaMKII activation.

Figure 22

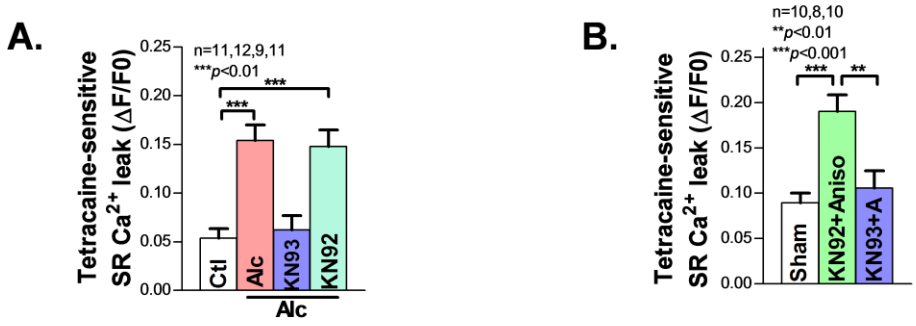

Figure 22. CaMKII inhibition prevents alcohol-treatment or JNK activation-promoted diastolic SR Ca^{2+} leak. **A)**. CaMKII inhibition with KN93, but not the inactive analogue KN92, reversed the alcohol-induced increase in tetracaine-sensitive SR Ca^{2+} leak. Measurements were carried out in 4 batches of cells (N=4) that totally included 11 sham monlayers (preparation), 12 alcohol-treated monolayers, 9 alcohol-treated monolayers with KN93 pretreatment, and 11 alcohol-treated monolayers with KN92 pretreatment (n=11,12,9,11). **B)**. Similarly, KN93 but not KN92 prevented anisomycin, the JNK activator, induced increase in tetracaine-sensitive diastolic SR Ca^{2+} leak. Measurements were carried out in 7 batches of cells (N=7) that totally included 10 sham monlayers, 8 anisomycin-treated monolayers with KN92 pretreatment and 10 anisomycin-treated monolayers with KN93 pretreatment (n=10,8,10). All data were presented as Mean ± SEM. One-Way ANOVA test with Tukey post hoc test was used to compare the mean of selected groups.

6.2.1.4 CaMKII inhibition decreased the Ca^{2+} mishandling *in vivo*. With these encouraging *in vitro* results, it is beneficial to explore whether CaMKII activation could be a therapeutic target to treat binge alcohol-enhanced SR Ca^{2+} mishandling. Indeed, a single injection of KN93 (7.2 mg/kg BW) but not KN92 (7.2 mg/kg BW) after the binge alcohol exposure significantly decreased the binge alcohol-boosted SR Ca^{2+} waves. Summarized data showed that after repeated binge alcohol exposure, KN93 treatment showed a tendency of lowering Ca2+ wave frequency during sinus rhythm compared to KN92 treatment (0.05 ± 0.05 incidences/mm*sec vs. 4.91 ± 4.33 incidences/mm*sec, n = 4, 5, p = NS; Figure 23A), and demonstrated significant decrease in Ca^{2+} wave frequency post 10Hz burst pacing compared to KN92 treatment (0.60 ± 0.36 incidences/mm*sec vs. 7.78 ± 5.33 incidences/mm*sec, n = 4, 5, $p < 0.05$; Figure 23A). Moreover, KN93 treatment reversed the intracellular Ca^{2+} decay constant τ to sham control level, which is significant decreased than that in KN92-treated repeated binge alcohol exposed mice (28.99 ± 1.26 msec vs. 42.35 ± 4.62 msec in KN92-treated; n = 4, 5, $p < 0.05$; Figure 23B).

Figure 23

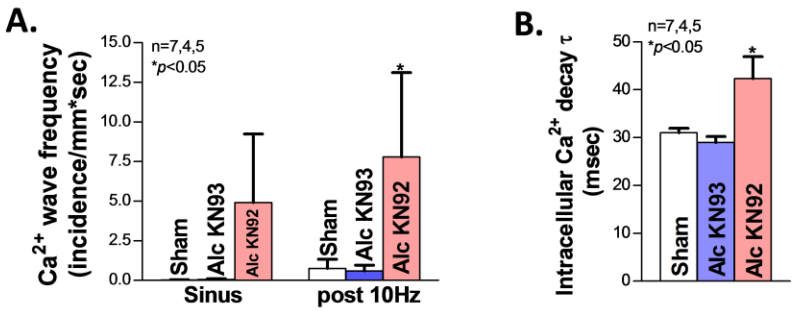

Figure 23. CaMKII inhibition reversed binge alcohol-induced Ca^{2+} mishandling *in vivo*. **A)** Single-dose KN93 (7.2mg/kg BW, I.P., 3 hrs prior to terminal study) treatment (but not KN92 treatment, 7.2mg/kg BW, I.P., 3hrs prior to terminal study) in binge alcohol-exposed mice reversed alcohol actions on atrial Ca^{2+} waves and Ca^{2+} decay constant τ prolongation **(B)**. All data were presented as Mean ± SEM. For **A)**, Kruskal-Wallis test (One-Way ANOVA on ranks) with post hoc test was used to compare the mean of selected groups. For **B)**, One-Way ANOVA test with Tukey post hoc test was used to compare the mean of selected groups. The sham control group was the same data presented in Figure 12A and Figure 12B.

These results were supported by further experiments in AC3I mice, which overexpress the cardiac-specific CaMKII-inhibiting peptide AIP. Treatment of AC3I mice with anisomycin did not increase Ca^{2+} waves or prolong the intracellular Ca^{2+} decay constant τ (35.03 ± 2.17 msec vs. 33.36 ± 3.14 msec in sham AC3I mice, n=5,4, *p* = NS; Figure 24B). Meanwhile, anisomycin-treated AC3I mice (n=6) did not show burst pacing-induced atrial arrhythmia *in vivo* (burst pacing protocol described in detail in Figure 9C), comparable to findings in sham AC3I mice (treated with 4 doses of saline at the same, n = 6). Figure 24A showed a pair of *ex vivo* atrial electrogram recording from anisomycin-treated versus sham AC3I mice in which sinus rhythm resumed after burst pacing. Mild ECG baseline drift was observed in Figure 24A in both sham and anisomycin-treated AC3I animals. This type of noise could be due to respiration, motion of the animal, changes in electrode impedance, and ECG amplifier automatic re-adjustment after the burst pacing stimuli, which is commonly observed in ECG/electrogram recordings and can usually be eliminated with low-frequency filter during off-line data process (Y. Luo et al., 2013). Such results strongly suggest that CaMKII activation mediates JNK activation-enhanced atrial arrhythmogenicity. Moreover, alcohol-prompted JNK activation promoted the incidence of SR Ca^{2+} waves in CaMKII-dependent manner.

Figure 24

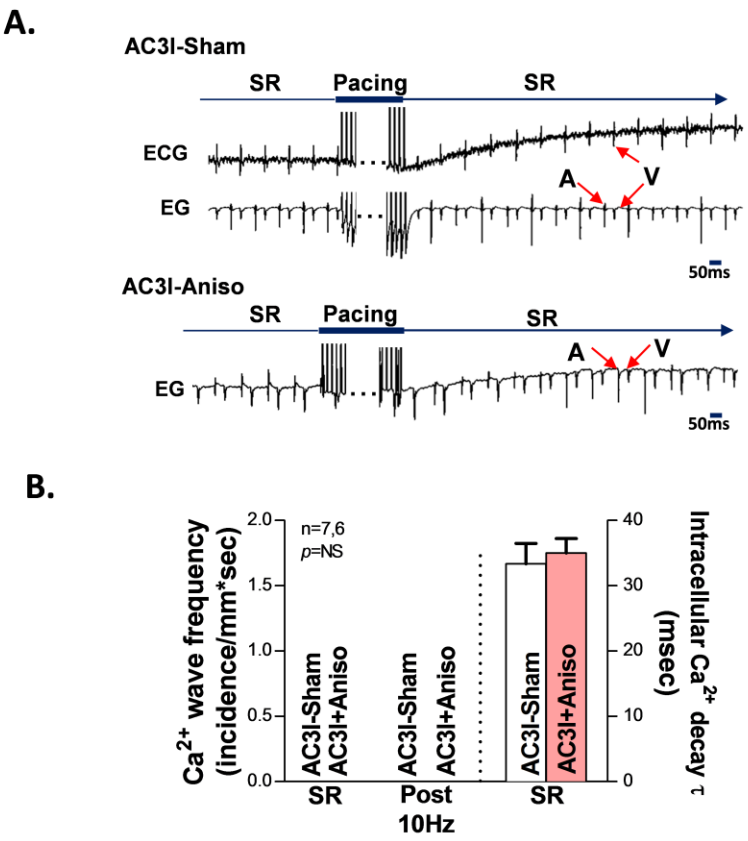

Figure 24. CaMKII inhibition reversed JNK activation-induced atrial arrhythmia and Ca^{2+} mishandling *in vivo*. **A)** Representative electrograms of burst-pacing followed by self-reversion to sinus rhythm (no arrhythmia induced) in anisomycin-treated AC3I mice and AC3I-sham control mice (n=0/6, 0/6). 'A' labels atrial signal, 'V' labels ventricular signal. **B)** Summarized data suggest that CaMKII inhibition in AC3I mice completely abolished anisomycin-induced aberrant atrial Ca^{2+} waves and prolonged tau of Ca^{2+} decay. The results suggest that CaMKII inhibition in AC3I mice prevents anisomycin-induced atrial arrhythmias. All data were presented as Mean ± SEM. For B), unpaired Student *t*-test was used to compare the mean of two groups for Intracellular Ca^{2+} decay constant data

6.2.2 The mechanisms of JNK promoted CaMKII activation

In the previous sections, I have shown that alcohol-treatment can induce JNK activation and CaMKII activation. Inhibiting JNK or CaMKII both prevented alcohol-induced increase in tetracaine-sensitive diastolic SR Ca^{2+} leak. However, what is the underlying mechanism of alcohol-induced CaMKII activation?

6.2.2.1 JNK inhibition prevents alcohol-induced CaMKII activation. To understand whether alcohol-induced CaMKII activation is dependent upon JNK activation, HL-1 myocytes were pretreated with JNK2 inhibitor (170nM, JNK inhibitor IX, EMD) before exposure to alcohol treatment. Representative image and summarized data of immunoblotting showed alcohol treatment (n = 5) increases the CaMKII phosphorylation by 43.4% compared to sham (n = 6, $p < 0.05$; Figure 25A), while JNK2 inhibition (n = 5) alleviated alcohol-induced CaMKII activation and returned the CaMKII activation to sham control level (81.24 ± 4.25% vs. 100.00 ± 7.12% in sham, n = 5, 2, p = NS; Figure 25A). Based on the information that CaMKII phosphorylation increased by alcohol-treatment and is reversed to control level by JNK2 inhibition, it is of great importance to confirm the CaMKII function after alcohol treatment. HA-tagged wild-type CaMKII (wt-CaMKII) was overexpressed in HEK293 cells before alcohol treatment with or without JNK2 inhibitor pre-treatment. CaMKII that was pulled down from sham, alcohol-treated, and JNK2I/alcohol-treated HEK293 cell homogenates and CaMKII activity was measured with ADP Glo™ experiments (detailed information provided in Materials and

Methods, Chapter Ten, page 193-194). Summarized results suggest that CaMKII activity is significantly increased by alcohol treatment (136.2 ± 8.9% vs. 107.7 ± 4.6 % in sham, $p < 0.05$, n = 7, 10; Figure 25B), while pre-treating cells with JNK2 inhibitor (170nM; JNK inhibitor IX, EMD) before adding alcohol reverses CaMKII activity back to sham control level (117.30 ± 8.93% vs. 107.70 ± 4.62 % in sham, n = 5, 10, p = NS; Figure 25B). Such evidence strongly suggests that JNK activation contributes to CaMKII activation in binge alcohol-exposed cells.

6.2.2.2 Direct oxidation of CaMKII is not predominant in alcohol-promoted CaMKII activation. ROS has been implicated in promoting CaMKII activation in various experimental models (Anderson, 2015), and alcohol treatment has been shown to enhance cellular ROS (Mansouri, Demeilliers, Amsellem, Pessayre, & Fromenty, 2001) (Ojeda, Barrero, Nogales, Murillo, & Carreras, 2012). JNK activation and ROS production has also been found in an augmentation signaling loop in alcohol-exposed tissue (Jin, et al., 2013). In order to understand the mechanisms of CaMKII activation in alcohol-exposed cells, it is of vital importance to determine whether CaMKII activation is dependent upon cellular ROS. To examine whether ROS directly activates CaMKII, a HA-tagged mutant CaMKII was overexpressed before alcohol treatment. This mutant CaMKII (vv-CaMKII) carries a mutation Met280/281Val, which renders it nonresponsive to intracellular ROS, yet still able to be phosphorylated on the autophosphorylation site and still capable of catalyzing the reaction with CaMKII substrate (Erickson,

Patel, Ferguson, Bossuyt, & Bers, 2011) This construct was made according to previous publications (Erickson, 2014; Erickson, He, et al., 2011; Luczak & Anderson, 2014). My results show that after alcohol exposure, vv-CaMKII kinase activity is significantly increased compared to sham controls (135.80 ± 9.56% vs. 100.00 ± 6.06% in sham, n = 7, 4, $p < 0.05$; Figure 25C), which further suggests that CaMKII oxidation on the oxidation-responsive amino acid residues does not play a direct role in CaMKII activation in alcohol-exposed cells.

Figure 25

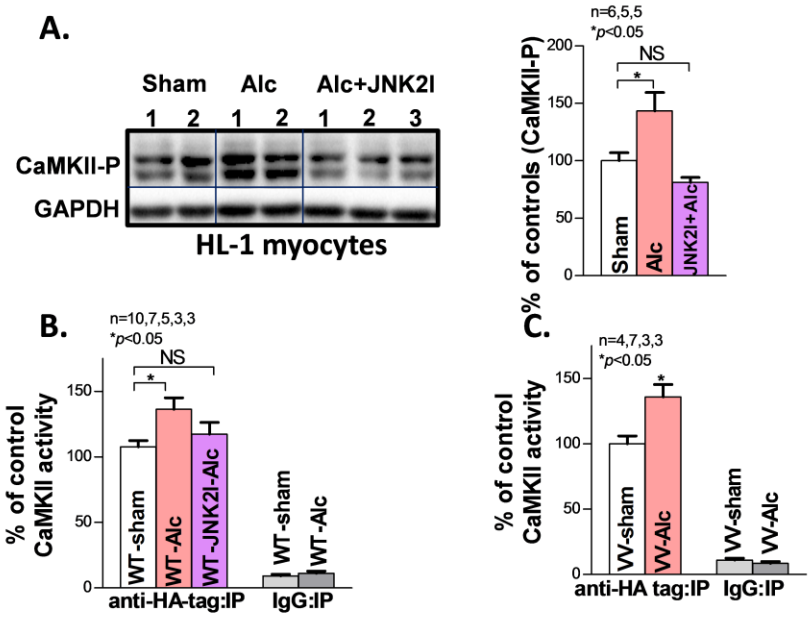

Figure 25. Alcohol-induced CaMKII is dependent on JNK activation but not directly on CaMKII oxidation. **A)** Representative immunoblotting image and summarized data showing increased CaMKII phosphorylation in alcohol-treated HL-1 cells compared with sham controls, while JNK2 inhibition with JNK inhibitor XI (JNK2I) abolished the alcohol-induced CaMKII activation. *I would like to thank Ms.Weiwei Zhao for assistance on immunoblotting.* **B)** HA-tagged wild-type (wt) CaMKII was overexpressed in HEK293 cells before alcohol-treatment with or without pretreatment of JNK2I. CaMKII pulled down from alcohol-treated cell homogenates showed enhanced ADP consumption in ADP-Glo™ assay which suggests increased CaMKII activity, while JNK2 inhibition with JNK2I suppressed alcohol-induced increase in CaMKII activity. For A-B), One-Way ANOVA test with Tukey post hoc test was used to compare the mean of selected groups. **C)** To further understand if alcohol-induced CaMKII activation is mediated by direct CaMKII oxydation, HA-tagged vv-CaMKII (Met280/281Val) was overexpressed in HEK293 cells before alcohol-treatment. Summarized data showing increased alcohol-induced vv-CaMKII activity after alcohol exposure compared to sham controls. Unpaired Student *t*-test was used to compare the mean of the two groups. All data were presented as Mean ± SEM.

6.2.2.3 JNK activation can directly promote CaMKII activation via inducing CaMKII autophosphorylation. Our immunoblotting data and CaMKII activity measurement both indicate that JNK activation promotes CaMKII activation and inhibition of JNK reverses the alcohol treatment-promoted CaMKII activation. To further explore the mechanisms of JNK2-promoted CaMKII activation, the JNK2 and CaMKII interaction was assessed. Incubation of purified active human full-length JNK2 protein (hJNK) with human atrial homogenates has shown that CaMKII phosphorylation was enhance in dose-dependent manner with the addition of active JNK2 protein (Figure 26A). Further, we found that incubating active hJNK2 protein with purified full-length human CaMKII proteins (hCaMKII; without Ca^{2+}/calmodulin present) led to CaMKII phosphorylation in an hJNK2 dose-dependent manner with hJNK2 protein (Figure 26B). To further explore whether JNK2-driven CaMKII activation involves the phosphorylation site of CaMKII autophosphorylation site Thr286, HA-tagged vectors encoding with either wt-CaMKII (wild-type CaMKII), CaMKII carrying loss-of-function mutation of autophosphorylation site T286A (CaMKII-T286A) were constructed from the previous publication (Erickson, 2014; Erickson, He, et al., 2011; Luczak & Anderson, 2014) (courtesy to Dr. Xianlong Gao for constructing the published vectors in the Ai Lab). wt-CaMKII or CaMKII-T286A were respectively overexpressed in HEK293 cells then IP-ed from cell homogenates. Both wt-CaMKII and CaMKII-T286A were both incubated with hJNK2 protein, yet only the wt-CaMKII protein was phosphorylated by the hJNK2 (Figure 22C). The ADP-Glo™ kinase

phosphorylation assay further demonstrated that only wt-CaMKII but not CaMKII-T286A produced a significantly higher level of ADP after incubation with hJNK2 (Figure 26D). This evidence indicates that JNK2 directly leads to CaMKII activation by promoting the phosphorylation of the CaMKII-Thr286.

Figure 26

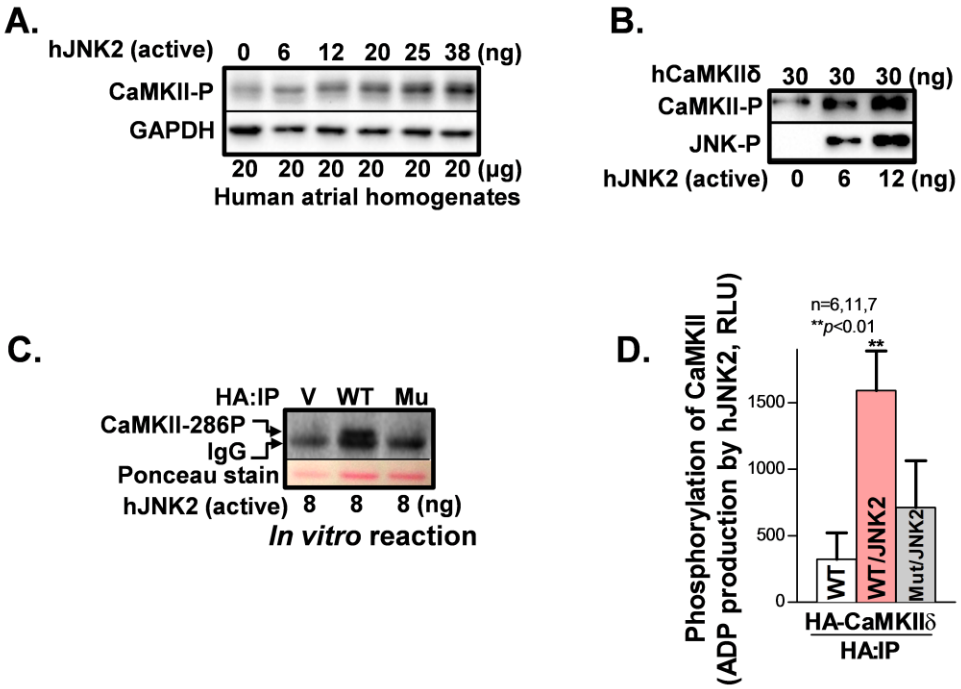

Figure 26. JNK2 directly activates CaMKII. **A)** Immunoblotting images of purified active full-length human JNK2 (hJNK2) dose-dependent increase in phosphorylation of CaMKII in both human atrial tissue homogenates and **(B)** pure recombinant full-length human CaMKIIδ (hCaMKIIδ) proteins. **C)** Immunoblotting images showing increased phosphorylation of CaMKII-P (Thr286) in HA-IPed CaMKII-WT proteins but not HA-IPed CaMKII-T286A mutant (Mu) proteins compared to HA-tagged empty vector (v) controls. Ponseau staining shows equal expression between CaMKII-WT and mutant CaMKII-T286A samples. **D)** Summarized data of increased ADP production from CaMKII phosphorylation by pure active hJNK2 proteins in HA-IPed CaMKII-WT samples but not in the HA-IPed CaMKII-T286A samples compared to CaMKII-WT sham-controls without pure hJNK2 incubation. All data were presented as Mean ± SEM. One-Way ANOVA test with Tukey post hoc test was used to compare the mean of selected groups in this study. *Courtesy of Dr.Xianlong Gao on performing and training Jiajie Yan for the ADP Glo assay. I would like to thank Ms.Weiwei Zhao for assistance on immunoblotting.*

6.3 Discussion

6.3.1 Alcohol Promotes SR Ca^{2+} leak in a JNK-dependent Manner

Previous research has suggested that Ca^{2+} waves occur due to increased RyR opening during the diastolic phase, and Ca^{2+} released from RyR can activate RyRs in the close proximity via inter-RyR CICR (D. A. Eisner, Kashimura, O'Neill, et al., 2009; D. A. Eisner, Kashimura, Venetucci, et al., 2009).

In the previous chapter, I found the intracellular Ca^{2+} decay constant is significantly increased in atria from binge alcohol exposed mice and anisomycin-challenged mice, which suggests the increased diastolic SR Ca^{2+} leak. Using the tetracaine perfusion method, I further found the diastolic SR Ca^{2+} leak was increased in alcohol-treated HL-1 myocytes compared to sham controls. Moreover, the alcohol-induced SR Ca^{2+} leak is JNK-dependent, pre-treatment of JNK2 inhibitor completely abolished this effect. To further confirm that JNK activation without alcohol promotes SR Ca^{2+} leak, HL-1 cells were treated with anisomycin, similar results were found, just as in the alcohol-treated group. These results were also confirmed in freshly isolated mouse and rabbit atrial myocytes. Previous findings from the Ai Lab also demonstrated JNK-dependent SR Ca^{2+} leak in the mouse model of JNK activation (aging model), lending further support for my current results.

Accumulating evidence indicates that increased RyR opening probability contributes to enhanced SR Ca^{2+} leak. Although previous studies yielded conflicting results on the effect of alcohol on RyR opening probability due to different sources

of RyR and different alcohol treatment conditions (Oba, et al., 2000; Oba, et al., 1997; Ye, et al., 2014), Dr. Fill's lab recently provided direct evidence that transient treatment of alcohol (39 mM, 30 min) significantly increased RyR single channel opening probability (Figure 3) similar to anisomycin-treated RyR (manuscript in submission). Also, JNK2 inhibition abolished the alcohol-induced increase in RyR single channel opening, further suggesting that JNK activation mediates the alcohol-induced increase in RyR opening probability. Although previous studies suggest that the direct oxidation of RyR enhances channel opening and promotes the propensity of Ca^{2+} sparks (Bovo, Mazurek, de Tombe, & Zima, 2015), in this part of the study, I focused on the action of JNK on SR Ca^{2+} leak and identified that JNK inhibition alleviated alcohol-induced SR Ca^{2+} leak. These results are not necessarily contradicting the previous finding on oxidation-induced RyR opening, since the cross-talk of enhanced intracellular ROS and JNK activation has been demonstrated in other studies (Jin, et al., 2013) and it is reasonable to hypothesize that JNK-promoted ROS may be contributing to alcohol-enhanced SR Ca^{2+} leak. In the chapter on future studies, I will discuss in detail on how to evaluate the contribution of alcohol-induced ROS elevation in alcohol-induced SR Ca^{2+} leak to further elucidate the detailed mechanism of JNK-enhanced SR Ca^{2+} leak.

One limitation of this session is the potential off-target effect of anisomycin. 0.2 μM anisomycin is used to activate JNK in cells in my thesis. This low concentration of anisomycin (0.2 μM) has been used as a JNK activator by other labs

as well (Hazzalin, Le Panse, Cano, & Mahadevan, 1998; Petrich et al., 2002; Yan, et al., 2013). However, high concentration of anisomycin has been shown to promote the activation of MAPK-p38. For instance, anisomycin treatment (4 μM, 48 hrs) activates MAPK-p38 in glioblastoma cell line (J. Y. Li et al., 2012). 1 μM anisomycin promotes MAPK-p38 activation in perfused mouse heart (Peart et al., 2014).

In my thesis study, a JNK2 null mouse model (JNK2KO) lacking of JNK2 proteins was used to dissect the specific role of JNK2 in Ca^{2+} mishandling. The result showed that knocking out JNK2 precluded anisomycin induced increase in atrial Ca^{2+} wave frequency and intracellular Ca^{2+} decay prolongation (Figure 17A, 17B), suggesting the specific effect of JNK2 in anisomycin-induced atrial Ca^{2+} mishandling.

6.3.2 CaMKII Mediates Alcohol/JNK Promoted SR Ca^{2+} Leak and Atrial Ca^{2+} Waves

CaMKII activation plays a pivotal role in arrhythmogenesis by phosphorylating multiple Ca^{2+} handling proteins in myocytes, including RyR and PLB (Mattiazzi et al., 2015). Hyperphosphorylation of CaMKII and RyR2 as well as diastolic SR Ca^{2+} handling dysfunction were observed in chronic AF (Chelu, et al., 2009; Neef, et al., 2010; Voigt, et al., 2014). Previous research has also documented that hyper-phosphorylation of RyR causes enhanced RyR activity (Camors & Valdivia, 2014; Greenstein, et al., 2014). Studies also have suggested that CaMKII inhibition was able to reverse the diastolic SR Ca^{2+} leak in the failing heart (Sag, et al., 2009; Sossalla, et

al., 2010). To date, it is unknown whether CaMKII contributes to alcohol-prompted atrial arrhythmia.

Here I focused on the role of CaMKII-dependent RyR phosphorylation in alcohol-treated/JNK-activated atrial myocytes. I found that alcohol treatment enhances CaMKII activation in binge alcohol exposed mouse hearts and in HL-1 cells. CaMKII activation plays an essential role in promoting SR Ca^{2+} leak since inhibiting CaMKII with KN93 abolished alcohol/JNK activation induced tetracaine-sensitive SR Ca^{2+} leak (Methods and Materials in Chapter Ten, page 191-192). Moreover, acute CaMKII inhibition via a single dose KN93 injection in binge alcohol exposed mice dramatically decreased arrhythmogenic Ca^{2+} waves in the atria. Currently there are very few available CaMKII inhibitory drugs, and this approach has shown little therapeutic potential to date (Z. Chen et al., 2015). Our data suggest that CaMKII inhibition *in vivo* still lend therapeutic effect in the setting of binge alcohol exposure.

The comments from the committee are well taken. KN93 is a widely used CaMKII inhibitor in various cell types including cardiac myocytes (El-Ani et al., 2014; Shi, Xu, Wei, Ma, & Zhang, 2014; S. Wagner et al., 2006; S. Wagner, et al., 2011). However, a reported nonspecific effect of KN93 is that it can inhibit L-type Ca^{2+} channel (CaV1.3 and CaV1.2) (L. Gao, Blair, & Marshall, 2006). KN92, a structural analogue of KN93, is also shown to inhibit L-type Ca^{2+} channel with similar potency of that observed in KN93, but does not inhibit CaMKII (L. Gao, et al.,

2006). Therefore, KN92 can serve as a control for the specific CaMKII-inhibition effect exerted by KN93.

In my entire thesis, when KN93 was used to inhibit CaMKII, KN92 treatment is always used in parallel to control for the non-specific effect of KN93 (Figure 22A, 22B, 23A, 23B, 28B, 29B). This applies to both *in vitro* and *in vivo* studies. With this design, it is reasonable to interpret that the effect that KN93 exerted on the alcohol or anisomycin-treated cells is due to specific CaMKII.

The next question is: what is the underlying mechanism that alcohol exposure induces CaMKII activation?

6.3.3 The Mechanisms of CaMKII Activation in Alcohol-exposed Tissue/Cells

The canonical mechanism of CaMKII activation relies on the increase of intracellular Ca^{2+} and the presence of calmodulin (Schulman & Greengard, 1978). Recently, it was discovered that CaMKII can be activated by increased intracellular ROS (Luczak & Anderson, 2014). Although there are reports about CaMKII activation in regulating the neuronal function after alcohol exposure (Agoglia, et al., 2015; Salling, et al., 2016), the assessment of CaMKII in the binge alcohol-exposed heart has not been published to date. In the current study, we found enhanced CaMKII activation in binge alcohol-exposed mouse/rabbit heart and HL-1 myocytes.

It is known that alcohol can promote cellular ROS (Ojeda, et al., 2012) (Umoh, Walker, Al-Rubaiee, Jeffress, & Haddad, 2014a), and enhanced ROS has been found to activate CaMKII by directly oxidizing CaMKII (Schulman & Greengard, 1978).

However in this study, I found that the loss-of-function mutation of CaMKII on the ROS-sensing site (Met280/281) did not ablate the alcohol-induced enhancement in CaMKII activity, which suggests that direct CaMKII oxidation by ROS may not be a primary contributor to alcohol-enhanced CaMKII activation. On the contrary, pretreatment of JNK inhibitor before alcohol exposure did suppress CaMKII activation. Similarly, inhibiting JNK in alcohol-treated HL-1 cells suspended CaMKII activation, which further demonstrates that JNK activation plays an essential role in alcohol-induced CaMKII activation.

JNK and ROS can form an augmentation signaling loop in alcohol-exposure models (Jin, et al., 2013). Thus, one possible mechanism is that alcohol-enhanced ROS activates CaMKII in a JNK-dependent manner rather than directly oxidizing CaMKII at Met280/281. Such finding does not exclude the role of ROS in alcohol-triggered activation, and instead further confirms the role of JNK activation in the CaMKII signaling.

6.3.4 The Mechanisms of JNK-enhanced CaMKII Activation

JNK2 is a predominant JNK isoform in the heart (Q. Liang, et al., 2003). JNK activation promotes the occurrence of AF in aged human, rabbit and mouse atria (Benjamin, et al., 1994; Go, et al., 2001; M. W. Rich, 2009; D. Xu, et al., 2012; Yan, et al., 2013). CaMKII is a prominent Ca^{2+} handling protein regulator in the heart, and the arrhythmogenic role of CaMKII activation has been well established (Ai, et al., 2005; Anderson, 2011; Chelu, et al., 2009; Erickson & Anderson, 2008; Maier, et al., 2003;

Neef, et al., 2010; Purohit et al., 2013; Rokita & Anderson, 2012; Sossalla, et al., 2010; Wu, Roden, & Anderson, 1999; R. Zhang et al., 2005; T. Zhang et al., 2003).

The key finding of this work is that we revealed, for the first time, there is a causal link between JNK activation and CaMKII activation in the heart. Moreover, JNK2 inhibition in JNK activated myocytes and alcohol-exposed myocytes both abolished the enhanced CaMKII activation. Most importantly, JNK2 inhibition precluded alcohol-prompted SR Ca^{2+} leak, Ca^{2+} waves and increased propensity for arrhythmia. Therefore, our results demonstrate for the first time that regulating JNK2 could be a potential therapeutic strategy to regulate CaMKII activation hence alleviating the arrhythmogenic Ca^{2+} events in the heart due to CaMKII activation.

The mechanism of JNK-promoted CaMKII activation is a direction of future study. CaMKIIδ is the primary cardiac isoform of CaMKII, and its sequence contains several JNK consensus sequences. As a result, further mutagenesis studies and configuration modeling work will be a future direction of study to gain further insight of the mechanism of CaMKII-JNK binding, anchoring and phosphorylation. This will be discussed in detail in Chapter Eight Future Directions.

6.3.5 Other Factors that may Contribute to SR Ca^{2+} Leak

Besides CaMKII-dependent phosphorylation of RyR, PKA phosphorylates RyR at Ser2809 (Houser, 2014; O'Brien, et al., 2015). However, in the current study our results suggest that PKA-dependent phosphorylation of RyR is comparable between repeated binge alcohol-exposed mice and of sham controls, suggesting that PKA-

dependent phosphorylation may not be a significant contributor of alcohol-induced SR Ca^{2+} leak. It is possible that in our model, BAC has dropped back to sham control level and thus is not able to detect PKA changes by the time of our study, since PKA is a fast-response kinase that is transiently activated during adrenergic response (Greiffenstein, et al., 2007). Future studies will include different time-frames for the study of Ca^{2+} mishandling after binge drinking (such as 6-48 hrs), and the activation of fast-response kinases (e.g., PKA and PKC), kinases that respond in the timeframe of hrs to days (e.g. ERK, MAPK-p38), and potentially long-term remodeling-related proteins (e.g., enzymes and proteins related to extracellular matrix remodeling and fibrosis formation) in binge alcohol-exposure models. Studies involving adrenergic response and PKA will be further discussed in Chapter Eight Future Directions, page 153-154.

Besides RyR, the IP3 receptor is also a prominent Ca^{2+} release channel on the SR in atria (Kupferman, Mitra, Hohenberg, & Wang, 1997), and atrial IP3 expression level is higher than in ventricular myocytes (Domeier et al., 2008; Kockskamper et al., 2008). It is traditionally considered that the IP3 receptor has a lower Ca^{2+} current compared to RyR, making them not a primary contributor in the EC coupling or the diastolic SR Ca^{2+} leak. Yet there are some studies showing the IP3 receptor current has been shown to promote Ca^{2+} waves in multiple type of cells in experimental measurements (Stavermann, et al., 2015; Yuen et al., 2013) and in computational modeling studies (Guisoni, Ferrero, Layana, & Diambra, 2015;

Rudiger, Jung, & Shuai, 2012; Wieder, Fink, & von Wegner, 2015). In addition, there is evidence showing that Ca^{2+} released from IP3 receptor facilitates Ca^{2+} release from RyR thus promotes arrhythmia (X. Li, Zima, Sheikh, Blatter, & Chen, 2005; Zima & Blatter, 2004). Recently, Hohendanner et al. discovered that Ca^{2+} release from IP3 receptors in the nuclear region of the SR contributes to the nuclear signaling in atrial myocytes (Hohendanner et al., 2015; Zima & Blatter, 2004). In my current study, I found alcohol-induced SR Ca^{2+} leak via RyR remodeling, and with CaMKII inhibition, the arrhythmogenic SR Ca leak and Ca^{2+} waves were abolished *in vitro* and *in vivo*. Recent data from the Fill Lab on RyR single channel recording also shows that CaMKII inhibition with KN93 abolished alcohol treatment induced increase in RyR single channel opening probability, further strengthening the findings in my thesis. These results confirmed the pivotal role of CaMKII-dependent RyR remodeling in alcohol-induced arrhythmogenic Ca^{2+} mishandling, however, it does not rule out the participation of IP3 receptor, which could be a future direction of studies.

6.4 Conclusion

My results suggest that alcohol promotes atrial arrhythmia via increasing the frequency of aberrant Ca^{2+} waves. Ca^{2+} waves typically stem from increased diastolic SR Ca^{2+} leak. In this chapter, my discovery suggests that alcohol promotes SR Ca^{2+} leak in a JNK/CaMKII–dependent manner, while inhibiting JNK or CaMKII attenuates the diastolic SR Ca^{2+} leak. Repeated binge alcohol exposure promotes

CaMKII activation in atrial tissue, moreover, alcohol-treatment promotes CaMKII activation in atrial cells and enhances diastolic SR Ca^{2+} leak via promoting CaMKII-dependent RyR phosphorylation. Further exploration of the mechanism underlying alcohol-induced CaMKII activation shows that CaMKII activation is dependent upon JNK activation but not upon direct oxidation of CaMKII on ROS-sensitive amino acid sites (Met 280/281).

CHAPTER SEVEN

BINGE ALCOHOL PROMOTES DIASTOLIC SARCOPLASMIC RETICULUM CALCIUM MISHANDLING VIA ENHANCING SR CALCIUM LOAD

7.1 Abstract

Besides enhanced CaMKII-dependent RyR phosphorylation that promotes SR Ca^{2+} leak and arrhythmogenic Ca^{2+} waves, previous studies also suggest that enhanced SR Ca^{2+} load can further sensitize RyR by triggering the RyR luminal Ca^{2+} sensor. SR Ca^{2+} load is increased in various cardiac pathological conditions that present Ca^{2+} mishandling and arrhythmia. In this section of my thesis, I aim at determining whether enhanced SR Ca^{2+} load is an additional mechanism that underlies the binge alcohol-promoted SR Ca^{2+} leak and Ca^{2+} waves (Figure 27).

SR Ca^{2+} load in alcohol-treated, and JNK-activated (anisomycin-treated) HL-1 myocytes was increased compared to sham controls. The alcohol-induced increase in SR Ca^{2+} load could be reversed with pretreatment of JNK2 inhibitor. These results suggest that alcohol promotes SR Ca^{2+} load in a JNK-dependent mechanism. Meanwhile, CaMKII inhibition did not attenuate alcohol-treatment or JNK activation-induced SR Ca^{2+} overload. SERCA is the primary Ca^{2+} pump that restores the Ca^{2+} back to the SR. Alcohol-treated human SR vesicles displayed increased SERCA

activity in a JNK-dependent manner, as well as JNK2 protein-incubated human SR vesicles. These results suggest that alcohol promotes SERCA activity in JNK-dependent manner.

To further explore whether abolishing SR Ca^{2+} overload-induced RyR Ca^{2+} leak could be a therapeutic strategy, transgenic mice with mutated RyR SR Ca^{2+} luminal sensors (a loss-of-function mutation, RyR-E4872$Q^{+/-}$) were subjected to our repeated binge alcohol protocol. These mutated mice showed decreased Ca^{2+} wave frequency and atrial arrhythmia inducibility compared to WT littermates that were subjected to the same repeated binge alcohol exposure. Moreover, after the JNK activator challenge, E4872$Q^{+/-}$ mice also showed decreased atrial Ca^{2+} waves and atrial arrhythmia inducibility compared to WT littermates, further confirming the role of JNK in SR overload-induced atrial arrhythmia.

Figure 27

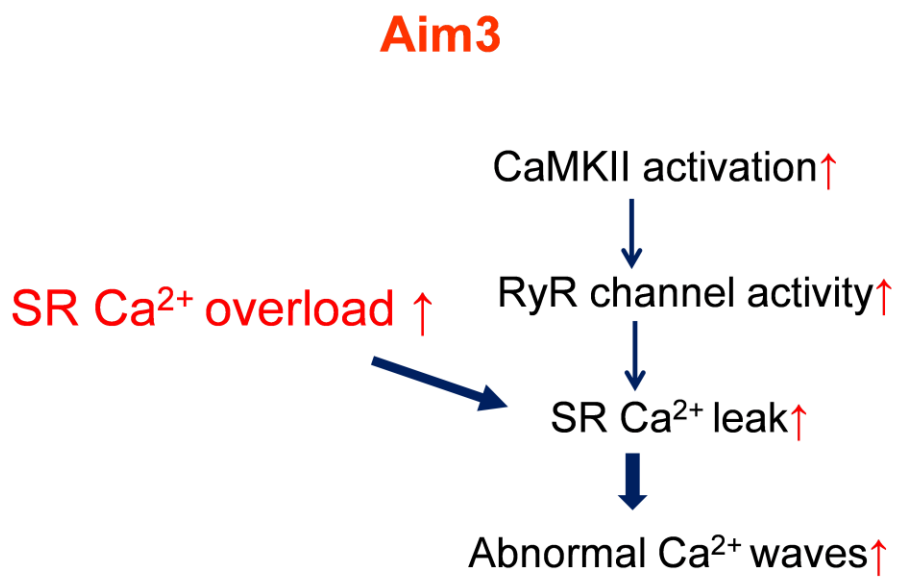

Figure 27. Schematic of the research focus for Aim3. The previous chapter of the thesis indicated that alochol/JNK activation promotes SR Ca^{2+} mishandling via triggering CaMKII activation and CaMKII-dependent RyR phosphorylation. On the other hand, SR Ca^{2+} overload can also promote RyR diastolic leak and SR Ca^{2+} waves via sensitizing RyR. In this part of the thesis, I will further explore whether SR Ca overload contributes to alcohol/JNK activation promoted Ca^{2+} mishandling and atrial arrhythmias.

7.2 Results

7.2.1 Alcohol Prompts SR Ca^{2+} Overload Which Aggravates Ca^{2+} Waves

In the previous section, I showed that binge alcohol contributes to arrhythmogenic Ca^{2+} waves via promoting CaMKII-dependent phosphorylation of RyR. Previous research has established that SR Ca^{2+} overload could also contribute to enhanced RyR opening via sensitizing intra-SR Ca^{2+} load sensor (W. Chen, et al., 2014). To further investigate the potential contribution of SR Ca^{2+} overload in alcohol-induced atrial Ca^{2+} events, SR Ca^{2+} load was measured in alcohol-treated and sham control atrial myocytes using the caffeine surge method (Figure 28 C) in which a fast application of caffeine leads to synchronized RyR opening in the cell hence instantly depletes SR Ca^{2+} load into the cytosol (Materials and Methods, Chapter Ten, page 191-192) (J. W. Bassani, et al., 1994; R. A. Bassani, et al., 1994). The summarized results showed increased SR Ca^{2+} load in alcohol-treated cells compared to sham controls (2.74 ± 0.11 vs. 1.91 ± 0.23 $\Delta F/F_0$ in sham, n = 10, 6, N = 4, $p < 0.01$; Figure 28A), while pretreatment with JNK2 inhibitor reversed the alcohol-induced increase in SR Ca^{2+} load (2.19 ± 0.12 vs. 1.92 ± 0.23 $\Delta F/F_0$ in sham, n = 11, 6, $p < 0.01$; Figure 28A). On the other hand, pretreatment with CaMKII inhibitor KN93 (2.51 ± 0.16 vs. 1.75 ± 0.18 $\Delta F/F_0$ in sham, n = 7, 6, N = 4, $p<0.001$; Figure 28B) or its inactive analog KN92 (2.41 ± 0.14 vs. 1.75 ± 0.18 $\Delta F/F_0$ in sham, n = 7, 6, N = 4, $p < 0.001$; Figure 28B) did not alleviate the alcohol-induced increase in

SR Ca^{2+} load. Such results suggest that JNK activation enhances SR Ca^{2+} load in CaMKII-independent pathways.

Figure 28

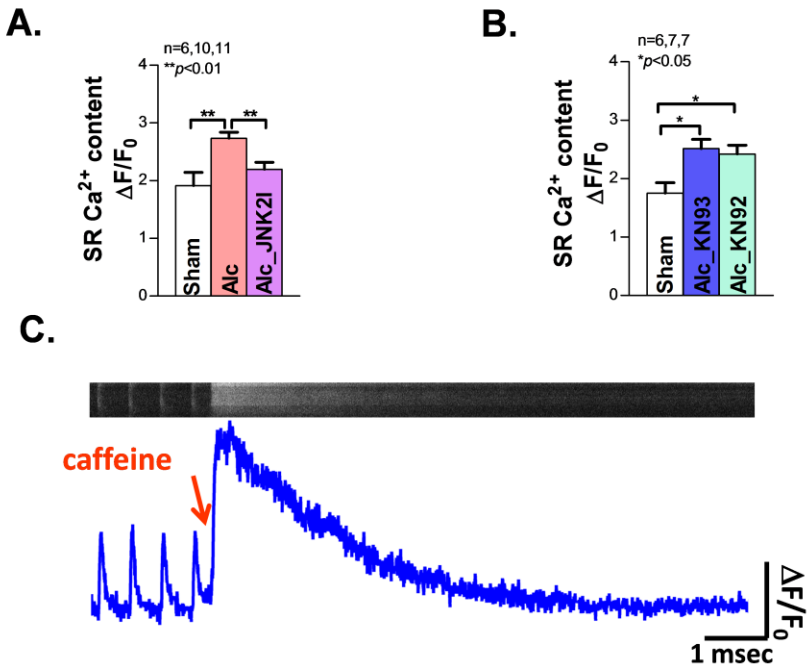

Figure 28. Alcohol exposure increases SR Ca^{2+} load. **A)**. Summarized confocal Ca^{2+} imaging data showing that alcohol treatment increased SR Ca^{2+} load while pretreatment with JNK2 inhibitor JNK inhibitor IX (JNK2I) prevented this alcohol impact. Measurements were carried out in 4 batches of cells (N=4) that totally included 9 sham monlayers, 10 alcohol-treated monolayers, and 11 alcohol-treated monolayers with JNK2I pretreatment (n=9,10,11). **B)**. Summarized data showing that CaMKII inhibition (KN93) or its inactive analogue KN92 failed to prevent the alcohol-driven increase in SR Ca^{2+} load. Measurements were carried out in 4 batches of cells (N=4) that totally included 6 sham monlayers, 7 alcohol-treated monolayers with KN93 pretreatment, and 7 alcohol-treated monolayers with KN92 pretreatment (n=6,7,7). All data were presented as Mean ± SEM. For A-B), One-Way ANOVA test with Tukey post hoc test were used to compare the mean of selected groups. **C)**. An example of confocal recording demonstrating the measurement of SR Ca^{2+} load with the method of instantaneous caffeine surge.

7.2.2 JNK Activation Prompts SR Ca^{2+} Overload which Enhances Ca^{2+} Waves

To gain further evidence on the role of JNK activation in increased SR Ca^{2+} load, confluent HL-1 myocyte monolayers were treated with the JNK activator anisomycin, and the SR Ca^{2+} load was subsequently measured as previously described in Materials and Methods, Chapter Ten, page 190-192. Results showed increased SR Ca^{2+} load in anisomycin-treated cells (3.02 ± 0.14 $\Delta F/F_0$ vs. 2.33 ± 0.14 $\Delta F/F_0$ in sham, n = 8, 6, N = 4, $p < 0.05$; Figure 29A), while JNK2 inhibitor abolished this effect (2.43 ± 0.12 $\Delta F/F_0$ vs. 2.33 ± 0.14 $\Delta F/F_0$ in sham, n= 11, 6, N = 4, p = NS; Figure 29A). Similar to the observations in alcohol-challenged cells, CaMKII inhibition with KN93 did not alleviate the JNK activation induced SR Ca^{2+} load (2.78 ± 0.15 $\Delta F/F_0$ vs. 2.11 ± 0.14 $\Delta F/F_0$ in sham, n = 11, 8, N = 3, $p < 0.05$; Figure 29 B), nor did treatment with KN92 (3.21 ± 0.19 $\Delta F/F_0$ vs. 2.11 ± 0.14 $\Delta F/F_0$ in sham, n = 11, 8, N = 3, $p < 0.001$; Figure 29B). To further explore the isoform-specific effect of JNK in SR Ca^{2+} overload, JNK1dn and JNK2dn protein were respectively overexpressed in HL-1 cells to abolish the JNK1 and JNK2 pathway. The results show that ablating JNK2 (2.39 ± 0.09 $\Delta F/F_0$ vs. 2.22 ± 0.12 $\Delta F/F_0$ in sham JNK2dn, n = 6, 4, N = 4, p = NS; Figure 29C) but not the JNK1 pathway (2.59 ± 0.19 $\Delta F/F_0$ vs. 1.88 ± 0.11 $\Delta F/F_0$ in sham JNK1dn, n = 5, 5, N = 4, $p < 0.01$; Figure 29C) with overexpressing dominant negative JNK proteins abolished the anisomycin-induced increased SR Ca^{2+} load. Moreover, cells overexpressing JNK1dn and JNK2dn showed comparable level SR Ca^{2+} load before challenging with anisomycin (2.59 ± 0.19

$\Delta F/F_0$ vs. 1.88 ± 0.11 $\Delta F/F_0$ vs. 2.22 ± 0.12 $\Delta F/F_0$ in sham JNK2dn, n = 5, 4; *p* = NS), further suggesting that overexpressing these two proteins per se does not alter the SR Ca^{2+} load level. This evidence further supports the role of JNK, specifically JNK2, in increasing SR Ca^{2+} load independent of CaMKII activation.

Figure 29

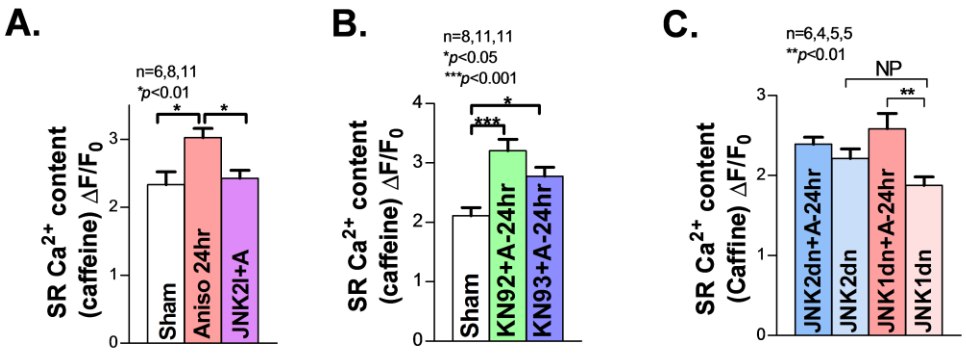

Figure 29. JNK activation increases SR Ca load without alcohol challenge. **A)**. Summarized confocal Ca^{2+} imaging data showing that anisomycin treatment increased SR Ca^{2+} load while pretreatment with JNK2 inhibitor JNK2I-IX (JNK2I) prevented this anisomycin impact. Measurements were carried out in 4 batches of cells (N=4) that totally included 6 sham monlayers, 8 anisomycin-treated monolayers, and 11 anisomycin-treated monolayers with JNK2I pretreatment (n=6,8,11). **B)**. Summarized data showing that CaMKII inhibition (KN93) or its inactive analogue KN92 failed to prevent the JNK activation induced increase in SR Ca^{2+} load. Measurements were carried out in 3 batches of cells (N=3) that totally included 8 sham monlayers, 11 anisomycin-treated monolayers with KN92 pretreatment, and 11 anisomycin-treated monolayers with KN93 pretreatment (n=8,11,11). **C)**. Summarized data suggest that overexpression of inactivated JNK2dn proteins attenuates anisomycin-induced SR overload, while inactivated JNK1dn has no such rescue effects. Measurements were carried out in 4 batches of cells (N=4) that totally included 6 anisomyicin-treated JNK2dn-overexpressing monolayers, 4 sham JNK2dn-overexpressing monolayers, 5 anisomycin-treated JNK1dn-overexpressing monolayers and 5 sham JNK1dn-overexpressing monolayers (n=6,4,5,5). For **A-C)**, One-Way ANOVA test with Tukey post hoc test were used to compare the mean of selected groups in each study.

7.2.3 Alcohol Treatment Promotes SERCA Activity

SERCA is the primary Ca^{2+} pump that contributes to Ca^{2+} reuptake after the systolic Ca^{2+} release from the SR. To understand the mechanism that led to alcohol-JNK promoted SR Ca^{2+} load, SERCA activity was measured in extracted human atrial SR vesicles where intact SERCA protein is concentrated (see Methods and Materials and Methods, Chapter Ten, page 180-182). SERCA activity was measure with an enzyme-linked reaction in which the consumption of NADH was used to calculate the SERCA activity. SERCA activity increased with in vitro alcohol treatment compared with sham preparations (134.2 ± 2.9% vs. 100.0 ± 5.9% in sham, n= 6, 6, $p < 0.001$; Figure 30B). Both sham and alcohol-treated SR vesicles demonstrated strong Ca^{2+}-concentration dependence (Figure 30A). Pretreatment with JNK2 inhibitor before the incubation with alcohol suppressed the alcohol-promoted increase in SERCA activity (134.2 ± 2.9% vs. 110.6 ± 3.0% in Alc+JNK2I, n= 6, 6, $p < 0.001$) and restored the SERCA activity to sham control level (110.6 ± 3.0% vs. 100.0 ± 5.9% in sham, n= 6, 6, p = NS; Figure 30B). Treatment with JNK2 inhibitor alone did not alter SERCA activity in human SR vesicles (103.2 ± 4.6% vs. 100.0 ± 5.9% in sham, n= 6, 6, p = NS; Figure 30B). Moreover, in an experiment to demonstrate that direct JNK activation promotes SERCA activity, activated JNK2 protein was incubated with extracted human atrial SR vesicle *in vitro* before the SERCA activity measurements. The results showed that incubation with purified JNK2 protein increased SERCA activity (144.8 ± 8.8% vs. 100.0 ± 5.9% in sham, n= 8,

8, $p < 0.001$; Figure 30C). CaMKII-dependent phosphorylation of PLB17Thr could lead to the dissociation of PLB and SERCA thus release the inhibition of SERCA. However, pretreatment with CaMKII inhibitor KN93 before the inhibition with active JNK2 protein did not decrease SERCA activity (168.1 ± 4.8% vs. 144.8 ±8.8% in JNK2-incubated, n= 4, 8, p = NS; Figure 30C), which further suggests that JNK directly promoted SERCA activity without CaMKII-dependent mechanisms.

Figure 30

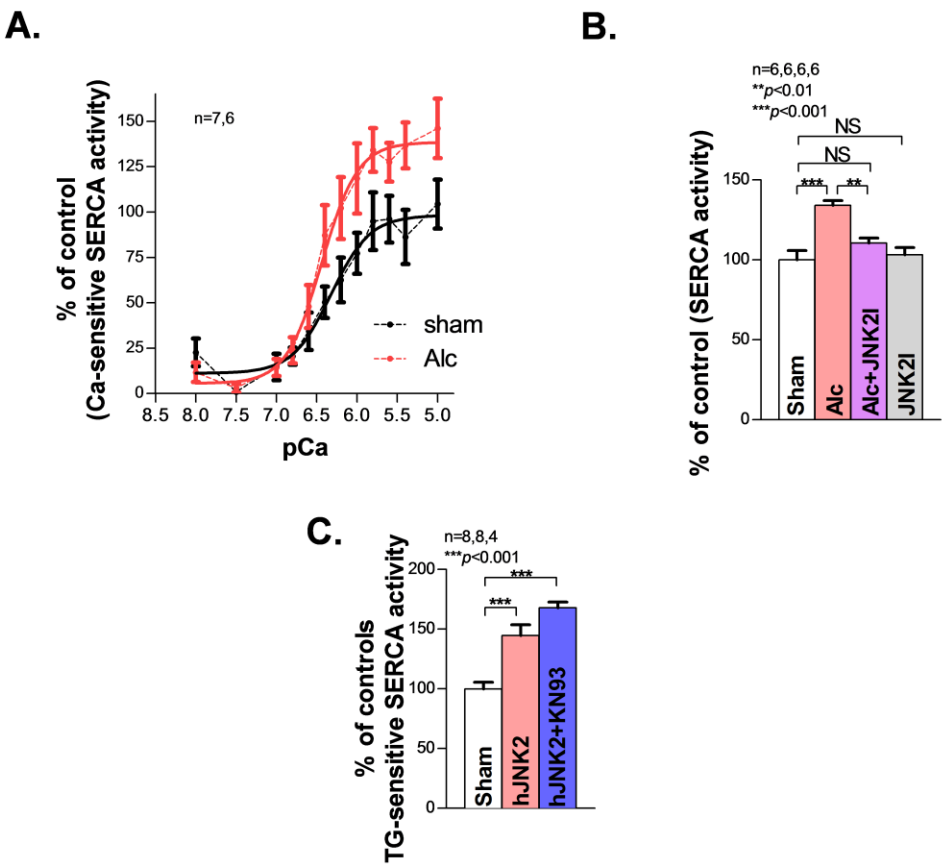

Figure. 30 Alcohol exposure increases SERCA activity in JNK-dependent manner. **A)** Ca^{2+}-dependent SERCA activity is increased in alcohol-incubated human SR vesicle compared to sham controls. **B)** Summarized data showing increased Ca^{2+}-sensitive kinase activity (SERCA activity) in alcohol-treated human SR vesicles compared to sham controls, while JNK2 inhibition with JNK inhibtor IX (JNK2I) abolished the alcohol action. On the other hand, JNK2I alone-treated SR vesicles showed similar level of SERCA activity compared to sham control SR vesicles, suggesting minimal off-target pharmaceutical effects of JNK2I on SERCA activity in extracted human SR vesicles. **C)** Summarized data of SERCA activity assay suggests that incubation with purified active JNK2 protein significantly increased Ca^{2+}-sensitive kinase activity (SERCA activity) in isolated human atrial SR vesicle preparations compared to controls, while treatment with KN93 does not attenuate this JNK2-induced SERCA activity enhancement. All data were presented as Mean ± SEM. One-Way ANOVA test with Tukey post hoc test were used to compare the mean of selected groups. *Courtesy of Ms.Olga N. Raguimova for training Jiajie Yan on the SERCA activity assay.*

7.2.4 The RyR E4872Q$^{+/-}$ Mutation Abolished Alcohol-treatment or JNK Activation-induced Atrial Ca^{2+} Mishandling and Atrial Arrhythmia Inducibility

SR Ca^{2+} overload promotes SR Ca^{2+} leak by sensitizing RyR receptors. To further explore the functional impact of SR Ca^{2+} overload promoted Ca^{2+} waves, mice with loss of function mutation of the SR luminal Ca^{2+} sensor (E4872Q$^{+/-}$) (W. Chen, et al., 2014) and WT littermate controls were subjected to binge alcohol treatment. Subsequent confocal imaging studies showed that Ca^{2+} wave frequency was significantly decreased in E4872Q$^{+/-}$ mice compared to WT littermates after 10Hz burst pacing (0.54 ± 0.15 incidences/mm*sec vs. 3.34 ± 1.61 incidences/mm*sec in EQ-WT, n = 8, 6, $p < 0.05$; Figure 31A), and showed a tendency of decreased Ca^{2+} was during sinus rhythm (0.06 ± 0.04 incidences/mm*sec vs. 1.16 ± 0.78 incidences/mm*sec in EQ-WT, n = 8, 6, p = NS; Figure 31A). At the same time, the intracellular Ca^{2+} decay constant was also shortened compared to WT littermate treated with binge alcohol (31.53 ± 1.05 vs. 37.94 ± 3.08 msec in EQ-WT, n = 8,6, $p < 0.05$; Figure. 31B), further suggesting a decrease in SR Ca^{2+} leak in the challenged heterozygous mice. In addition, burst pacing induced AT/AF was also significantly alleviated in E4872Q$^{+/-}$ mice compared to WT (2.13 ± 1.63 vs. 41.50 ± 25.52 incidences/animal in EQ-WT, n = 8, 6, $p < 0.05$; Figure 31C).

Figure 31

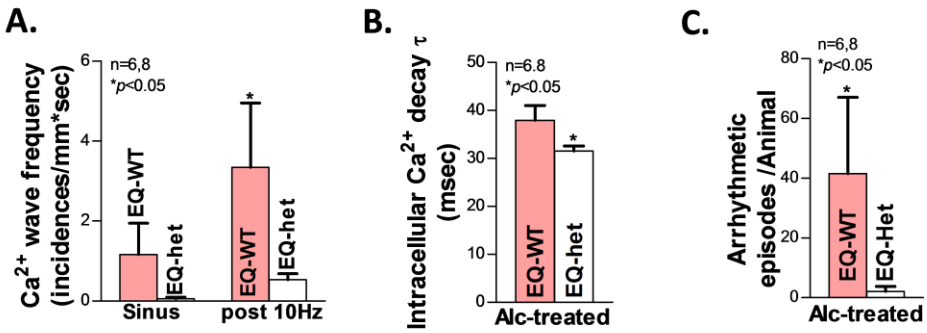

Figure. 31 Ablation of RyR luminal Ca^{2+} sensor attenuate alcohol-induced atrial Ca mishandling and atrial arrhythmogenesis. **A-B)**. RyR luminal Ca^{2+} sensor loss-of-function mutation (RyR E4872Q$^{+/-}$) prevented pacing induced Ca^{2+} waves **(A)**, prolonged Ca^{2+} decay constant τ **(B)**, and inducibility of atrial arrhythmias **(C)** in binge alcohol-exposed Langendorff-perfused mouse atria compared to that of binge alcohol-exposed WT littermate mice. All data were presented as Mean ± SEM. For **A)** Kruskal-Wallis test (One-Way ANOVA on ranks) with post hoc test was used to compare the mean of selected groups; for **B)**, unpaired Student *t*-test was used to compare the mean of the two groups; for **C)**, Mann-Whitney test (non-parametric Student *t*-test for unpaired groups) was used to compare the difference in mean for the two groups.

To specifically evaluate the role of JNK activation in SR Ca^{2+} overload-induced Ca^{2+} waves, E4872Q$^{+/-}$ mice and WT littermate controls were treated with anisomycin, the JNK activator. Confocal imaging studies showed that the E4872Q$^{+/-}$ mice demonstrated decreased frequency of Ca^{2+} waves (0.33 ± 0.05 vs. 3.81 ± 1.25 incidences/mm*sec in EQ-WT, n = 5, 5, $p < 0.01$; Figures 32A) and decreased intracellular decay constant compared to WT littermates subjected to the same anisomycin challenge (32.35 ± 1.67 vs. 37.79 ± 1.41 msec in EQ-WT, n = 5, 5, $p < 0.05$; Figures 32B). Such results further support the role of JNK activation in alcohol-treatment promoted SR Ca^{2+} arrhythmogenic waves, via SR Ca^{2+} overload and sensitization of RyR via the E4872Q$^{+/-}$ luminal Ca^{2+} sensing site.

Figure 32

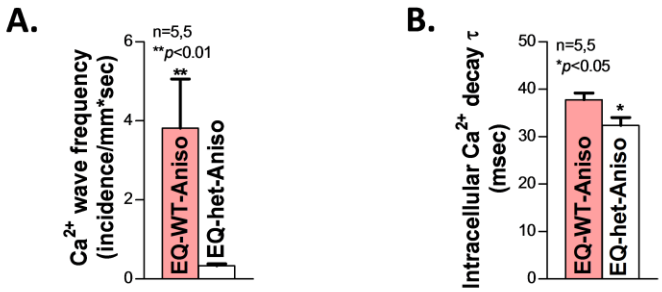

Figure. 32 Ablation of RyR luminal Ca^{2+} sensor attenuate JNK activation-induced atrial Ca^{2+} mishandling and atrial arrhythmogenesis. **A-B)**. RyR luminal Ca^{2+} sensor loss-of-function mutation (RyR E4872Q$^{+/-}$) prevented pacing induced Ca^{2+} waves **(A)**, prolonged Ca^{2+} decay constant τ **(B)**. All data were presented as Mean ± SEM. For **A)**, unpaired Student *t*-test was used to compare the mean of the two groups; for **B)**, Mann-Whitney test (non-parametric Student *t*-test for unpaired groups) was used to compare the difference in mean for the two groups.

7.3 Discussion

Previously, alcohol exposure was shown to increase SR Ca^{2+} load in non-cardiac cells (Souza-Smith, et al., 2015). In this chapter, I discovered that alcohol promotes SR Ca^{2+} load in HL-1 myocytes in a JNK activation-dependent manner. Moreover, previous results in our lab also showed increased SR Ca^{2+} load in freshly-isolated mouse atrial myocytes from a different model with JNK activation (aging-induced JNK activation). JNK2 inhibition with pharmacological inhibitor or overexpressing dominant negative JNK2 protein both abolished the increased SR Ca^{2+} load due to alcohol or anisomycin treatment, further indicating the pivotal role of JNK activation in regulating SR Ca^{2+} load.

7.3.1 The Consequence of Increased SR Ca^{2+} Load

Previous research has shown that increased SR Ca^{2+} load can also promote diastolic SR Ca^{2+} leak via sensitizing the SR luminal Ca^{2+} sensor and promoting the opening of RyR (W. Chen, et al., 2014). For instance, increased SR Ca^{2+} load promotes arrhythmia in multiple pathological conditions such as CPVT and digitalis toxicity. (D. A. Eisner & Lederer, 1980; D. A. Eisner, et al., 1981; Nassal, et al., 2015; Venetucci, et al., 2008; Yeh, et al., 2008). Also, SR Ca^{2+} overload contributes to Ca^{2+} waves *ex vivo* on Langendorff-perfused intact hearts (Mattiazzi, Argenziano, et al., 2015) and *in vitro* on drug-treated (forskolin) ventricular myocytes (Curran, et al., 2010).

In the current study, to further explore the functional consequence of alcohol-induced SR Ca^{2+} overload in arrhythmogenesis, we treated a mouse line that harbors the loss-of-function mutation of RyR SR Ca^{2+} luminal sensor with binge alcohol and found the atrial Ca^{2+} wave frequency significantly decreased compared to the EQ-WT littermates subjected to the same treatment. These results further suggest that alcohol promotes arrhythmogenic Ca^{2+} waves via increasing SR Ca^{2+} load. Also, to determine if JNK activation *per se* can induce Ca^{2+} waves via SR Ca^{2+} overload, E4872Q$^{+/-}$ mice and EQ-WT littermates were treated with the JNK activator anisomycin. Confocal imaging and arrhythmia inducibility results showed E4872Q$^{+/-}$ mice had lower frequency of Ca^{2+} waves, and decreased propensity of burst pacing-induced arrhythmia. These results further suggest that decreasing the luminal SR Ca^{2+} sensing rescues the arrhythmogenic Ca^{2+} waves and atrial arrhythmia when SR Ca^{2+} overload was induced with JNK activation. However, one limitation of this current study is that the SR Ca^{2+} load in alcohol-treated E4872Q$^{+/-}$ mice wasn't directly measured due to the lack available transgenic animals to use. Yet previously, the increased SR Ca^{2+} load was observed in a JNK-activation model of aged mice which lends support to the current study. This part of my work could be accomplished once the experiment animals are available during my further studies.

We have shown in the previous chapter that alcohol treatment increases diastolic SR Ca^{2+} leak, thus it would be expected to observe a decrease in the SR load if the JNK-driven CaMKII-dependent enhancement of RyR2-mediated leak had

occurred alone. On the contrary, the increased SR Ca^{2+} load in alcohol-treated cells implies JNK activation also alters SR Ca^{2+} uptake rate sufficiently to overcome the SR Ca^{2+} loss due to the leak from RyR. SERCA is the primary Ca^{2+} pump that regulates the SR Ca^{2+} homeostasis (Inesi, et al., 1964; Inesi, et al., 1990). In my further investigation of SERCA activity, I found alcohol treatment promotes SERCA activity in extracted human atrial SR vesicles, quantified by the ADP production rate of SERCA in *in vitro* via enzyme-linked ATPase activity assays. Moreover, incubating of human SR vesicles with hJNK2 protein increases SERCA activity, but also pretreatment of human SR vesicles with JNK2 inhibitor abolished the alcohol-promoted increase in SERCA activity. This further suggests that the alcohol-induced increase in SERCA activity is dependent upon JNK activation.

7.3.2 The Role of CaMKII in the Regulation of SR Ca^{2+} Load and SR Diastolic Leak

In the experiment to assess SR Ca^{2+} load, I found that inhibiting CaMKII in alcohol-treated or JNK-activated cells did not attenuate the increase in SR Ca^{2+} load. These phenomena could be due to the fact that although CaMKII activation leads to increased PLB-17 phosphorylation thus alleviates its inhibition of SERCA, yet at the same time, CaMKII activation promotes RyR phosphorylation and the SR Ca^{2+} leak. Thus CaMKII activation itself may not contribute to the increase in of SR Ca^{2+} load. Indeed, previously it was shown that overexpressing CaMKIIδ promotes the RyR phosphorylation and decreases SR Ca^{2+} load in mouse cardiac myocytes (R. Guo &

Ren, 2012; Ling et al., 2009). On the other hand, knocking out PLB in CaMKIIδ-overexpressed mice is shown to restore SR Ca^{2+} load while the cellular Ca^{2+} sparks and Ca^{2+} waves are exacerbated due to the increased SR Ca^{2+} load compared to CaMKIIδ-overexpressing mice alone (T. Zhang et al., 2010), this evidence further demonstrates the role of increased SR Ca^{2+} load in triggering arrhythmogenic Ca^{2+} events. Indirect evidence from our lab shows that CaMKII inhibition by KN93 increases the SR Ca^{2+} uptake rate in cardiac myocytes from aged mice (also a model with significant JNK activation), which further suggests a CaMKII-independent pathway that promotes the SR Ca^{2+} uptake. On the other hand, without CaMKII inhibition, the increased SR Ca^{2+} uptake is masked by the diastolic SR Ca^{2+} leak, further confirming the contribution of CaMKII activation in enhanced SR Ca^{2+} leak.

A limitation of the SERCA activity measurement is that the effect of alcohol on SERCA activity was measured after short term incubation of human SR vesicle with alcohol. This model is different from the model where JNK/CaMKII activation was measured. This difference is due to the limitation of sample choices on human atria, which could be accomplished in future studies when atrial samples from alcohol-intoxication patients are available.

The regulation of SERCA activity is multi-dimensional. Other than the previously described CaMKII-dependent phosphorylation of PLB, other factors such as the phosphorylation of PLB by PKA and the atrial sarcolipin (Shaikh, Sahoo, &

Periasamy, 2016; Stammers, et al., 2015) are examples of factors that could also contribute to the activity of SERCA.

Beyond SERCA activity, intracellular/extracellular Ca^{2+} concentration and the activity of L-type Ca^{2+} channels have also been shown to regulate SR Ca^{2+} load (Correll et al., 2015; Zhao, et al., 2012). Although in the current study, I focused on the action of JNK and alcohol-induced JNK activation on SERCA activity, multiple other factors could contribute to the phenomena being observed. Such studies are considered beyond the scope of the current thesis, but they will be discussed in Chapter Eight Future Studies.

Although SERCA is the primary SR Ca^{2+} pump that maintains Ca^{2+} homeostasis, other factors are also found to regulate SR Ca^{2+} load. For instance, recent studies suggest the increased L-type Ca^{2+} channel current may contribute to enhanced SR Ca^{2+} load. Stokke et al. suggests that inhibiting L-type Ca^{2+} channel decreased total intracellular entry and decrease SR Ca^{2+} load, which further decreases the Ca^{2+} wave propensity in ventricular myocytes (Stokke et al., 2013). On the other hand, Correll et al. shows that in a transgenic mouse model of STIM1, L-type Ca^{2+} current is increased while SR Ca^{2+} load remain unchanged, which could be due to SR Ca^{2+} loss from spontaneous Ca^{2+} release in the form of spontaneous Ca^{2+} transient and Ca^{2+} sparks (Correll, et al., 2015). Such seemingly conflicting results may suggest the multi-dimensional nature of SR Ca^{2+} load regulation. Preliminary data from the Ai Lab shows that L-type Ca^{2+} current in anisomycin-treated mouse

atrial myocyte is not altered compared to sham control (data not shown). Yet whether alcohol treatment has an effect on the L-type Ca^{2+} current, and the contribution of the L-type Ca^{2+} current in the alcohol-exposed cells will be a future direction for the study.

As previously demonstrated, both enhanced SR Ca^{2+} leak and increased SR Ca^{2+} load promote alcohol induced arrhythmogenic Ca^{2+} waves. My studies showed that either inhibiting CaMKII or ablating RyR luminal Ca^{2+} sensitivity both attenuated the occurrence of Ca^{2+} waves and arrhythmia inducibility. These results are indirectly strengthened by previously findings which aimed at modifying the SR Ca^{2+} load or the CaMKII-dependent RyR phosphorylation (Ling, et al., 2009; Maruyama, et al., 2010; T. Zhang, et al., 2010). However, as a limitation, our current research approach does not have the power to resolve whether CaMKII-dependent RyR phosphorylation and SR luminal sensing are independent as such studies require more mutagenesis studies and single channel recording, which could be a valuable future direction of study.

7.4 Conclusion

SR Ca^{2+} overload has been found to promote RyR opening via triggering the RyR luminal Ca^{2+} sensor, which promotes diastolic Ca^{2+} leak. In this chapter, I found increased SR Ca^{2+} load in alcohol-treated atrial myocytes in JNK-dependent and CaMKII-independent manner. Also, anisomycin-treated atrial cells showed JNK activation itself can promote SR Ca^{2+} load. Alcohol-induced JNK activation promotes

SERCA activity which could contribute to the increased SR Ca^{2+} load. Moreover, RyR-E4872Q$^{+/-}$ treated with alcohol showed decreased atrial Ca^{2+} waves and arrhythmogenicity compared with WT littermates subjected to the same treatment, further suggesting that abolishing SR Ca^{2+} overload induced SR Ca^{2+} spontaneous release could serve as a therapeutic strategy.

CHAPTER EIGHT

FUTURE DIRECTIONS

8.1 The Role of Adrenergic Response in Binge Alcohol-induced Arrhythmia

Within 24 hours of alcohol cessation, adrenergic surge is commonly observed (Pohorecky, 1982; Takahashi et al., 2008). For instance, clinical data suggest that within 24 hrs of the last episode of alcohol consumption, elevated heart rate is observed in otherwise healthy patients. Meanwhile, urinary excretion of catecholamine was also present (Denison, et al., 1994). The adrenergic surge lasts a short period, for instance, Greiffenstein et al. have reported that in a repeated binge alcohol mouse model (5 g/kg BW, 1 dose/day for 3 consecutive days, plus 2.5 g/kg BW on the day of terminal study), circulating epinephrine, norepinephrine and stress-responsive hormones glucocorticoids drop to baseline level 2-3 hrs after the last alcohol exposure, on the other hand it took about 4 hrs for BAC to return to baseline level (Greiffenstein, et al., 2007).

PKA is a major downstream signaling pathway of β-adrenergic signaling. The main targets of PKA in Ca^{2+} handling include RyR and PLB. In my current study, PKA-dependent phosphorylation of RyR phosphorylation in not altered by binge

alcohol exposure (Figure 21B), which could be due to the timeframe of my study. Such results lend further evidence to focus the current study on JNK/CaMKII-dependent Ca^{2+} handling alterations as arrhythmogenic substrates remodeling. Functional studies in this work were mainly performed on Langendorff-perfused hearts, a cultured atrial cell line and isolated myocytes. In such experimental systems, it is reasonable to assume that sympathetic and parasympathetic neurons are not playing a major regulating role. In a way, my thesis work is limited by the lack of evaluation of the contribution of adrenergic response in binge alcohol induced atrial arrhythmia. In future studies, it would be valuable to use the telemetry approach on conscious mice to monitor the binge alcohol induced atrial arrhythmia *in vivo*. Also, it would be valuable to probe the role of adrenergic signaling in atrial arrhythmia inducibility at a shorter interval (such as 3-6 hrs after binge alcohol, before the adrenergic signaling wanes) after the last binge episode. For instance, work by Bovo et al. indicates short-term β-adrenergic challenge can increase RyR leak and the occurrence of Ca^{2+} waves (isopreterenol treatment) mediated by ROS-induced RyR oxidation (Bovo, et al., 2015). Moreover, the participation of the α-adrenergic response in the heart is relatively less-studied in alcohol-related studies, yet co-activation of α and β-adrenergic signaling has been observed in multiple conditions (Thomas et al., 2016), which provides a new dimension of studying post-alcohol adrenergic signaling in the heart.

8.2 The Recovery Timeframe of Enhanced Atrial Arrhythmogenicity after Binge Alcohol

Although it is known that HHS appears most frequently within approximately the first 24 hrs of a binge drinking episode, there are currently no clear statistics showing when binge alcohol-promoted arrhythmogenicity starts to wane to a normal level. Our future studies will explore the time frame, recovery of the arrhythmomgenic substrate, and molecular mechanism of the recovery from alcohol-induced atrial arrhythmia.

8.3 Gender Differences in Binge Alcohol-induced Arrhythmia

Alcohol exposure has different impact on male and female subjects, with men having typically higher tolerance compared to women. Also, the drinking pattern of women is different from that of men with more heavy drinking and binge drinking present in men, as previous discussed. Possible mechanisms of the gender difference effect of alcohol involve the role of estrogen in alcohol metabolism in the heart (el-Mas & Abdel-Rahman, 2012, 2014, 2015; Longnecker & Tseng, 1998). In order to exclude the potential effect of different sex hormones in the alcohol-induce atrial arrhythmia, all the animals used in this study were male. Yet binge drinking in women is also an emerging social issue. Recent statistics report 1/8 adult women and 1/5 high school girls engage in binge drinking, and 23,000 women die each year due to excessive drinking in the U.S. ("Vital signs: binge drinking among women and high school girls--United States, 2011," 2013). Thus, the mechanisms of gender

differences in alcohol-induced arrhythmia are of great value for basic/translation research and for benefit of the society.

8.4 Atrial Ca^{2+} Sparks after Alcohol Exposure

As was mentioned in the previous discussion, during the confocal imaging on atrial tissue, the frequency of Ca^{2+} sparks was not used to compare SR Ca^{2+} mishandling between alcohol-exposed hearts compared to controls. Although Ca^{2+} spark frequency has been used by the Wasserstrome Lab (Aistrup, et al., 2006) to quantify the SR Ca^{2+} mishandling, this method is more applicable to cell imaging rather than tissue imaging since the background noise is generally higher in the latter case. However, Ca^{2+} spark imaging is worthy of exploration considering it reflects the spatial pattern of the local SR Ca^{2+} release events and the distribution of the leaking RyR clusters. This work could be done in circumstances where the dye loading conditions or microscopy are further improved.

8.5 IP3 Receptor in Alcohol-treated Tissue/Cells

In my current study, I focused on the alcohol-induced SR Ca^{2+} leak via RyR modeling, and with CaMKII inhibition, the arrhythmogenic SR Ca^{2+} leak and Ca^{2+} waves were abolished *in vitro* and *in vivo*. Yet it does not rule out the role of other Ca^{2+} release channels in alcohol-induced atrial arrhythmia.

For instance, besides RyR, atrial myocytes also expresses the IP3 receptor as Ca^{2+} release channel on the SR (Domeier, et al., 2008; Kockskamper, et al., 2008; Kupferman, et al., 1997). Previous studies suggest that the IP3 receptor current

facilitate Ca^{2+} waves in multiple type of cells (Guisoni, et al., 2015; Rudiger, et al., 2012; Stavermann, et al., 2015; Wieder, et al., 2015; Yuen, et al., 2013). Also, the IP3 Ca^{2+} current has been found to promote arrhythmia (X. Li, et al., 2005; Zima & Blatter, 2004) and nuclear signaling due to its close proximity to the nuclei (Hohendanner, et al., 2015; Zima & Blatter, 2004). The role of IP3 receptor in arrhythmogenicity and in intracellular Ca^{2+} signaling, which could be future direction of study.

8.6 Other Ion Channels that may be Involved in Alcohol-induced Atrial Arrhythmia

In the current study, I found enhanced diastolic SR Ca^{2+} leak and Ca^{2+} waves in alcohol treated mouse atria and HL-1 cells, and CaMKII inhibition reversed the alcohol-induced alteration. Yet it is a possibility that the increased diastolic cytosolic Ca^{2+} level plays a role in triggering the spontaneous Ca^{2+} release events and enhanced CaMKII activation. It is previously reported that hyper-activity of L-type Ca^{2+} current can promote the increased diastolic cytosolic Ca^{2+} concentration (Lu, Wang, Hu, Fang, & Mei, 2016; Zhao, et al., 2012), while decreased L-type Ca^{2+} current contributes to the decrease in intracellular Ca^{2+} concentration (Judenherc-Haouzi et al., 2016), although Dr. Eisner's review provided a different conclusion (Trafford, Diaz, & Eisner, 2001). Preliminary data from the Ai Lab shows that L-type Ca^{2+} current in anisomycin-treated mouse atrial myocyte is not altered compared to sham control. To date, whether alcohol-treatment has effect on the cardiac L-type

Ca^{2+} current, and the contribution of the L-type Ca^{2+} current in the alcohol-exposed cardiac cells haven't been studied thus it can be a future direction for the study.

Also, in the triggered mechanism, Ca^{2+} waves increased intracellular Ca^{2+} concentration which can result in the activation of the NCX (Lehnart, et al., 2008; Sugai, et al., 2009). NCX allows 3 Na^{+} into the cytosol in exchange of 1 Ca^{2+} out of the cell which further depolarize the plasma membrane and causes DADs (Bers, 2000, 2014; Maruyama, et al., 2010; Rubart & Zipes, 2005). Thus, NCX is a pivotal link between the intracellular Ca^{2+} elevation and triggered activity. Previously, I found no significant differences in NCX-mediated Ca extrusion (time constant of NCX, τ_{NCX}) in JNK-activated HL-1 cells compared to sham control cells, and the Ai Lab found no significant increase in NCX protein level in HL-1 cells compared to control. On the contrary, our very preliminary data suggest increased NCX protein level in alcohol-treated HL-1 cells. However, this finding may suggest but does not indicate increased NCX current in alcohol-treated HL-1 cells considering the various factors that regulate the function of NCX including but not limited to the signaling pathways involving neurohormonal regulation (G. Chen, Yang, Alber, Shusterman, & Salama, 2011), redox signaling (T. Liu & O'Rourke, 2013) and the colocalization of NCX with other channels (Doleschal et al., 2015). To date, the alcohol-induced alteration in the NCX current remains unknown. Thus it would be valuable to evaluate the expression level of NCX and function of NCX, and the pathways that regulate the NCX expression and function in alcohol-exposed atrial myocytes for future studies.

Besides L-type Ca^{2+} channel and NCX, there are other ion channels (such as K^+ channels) and pumps (such as Na^+/K^+ ATPase) that could be affected by binge alcohol treatment. However, previous studies suggest that ion channel remodeling tends to occur long before the AF onset, and is not always correlated with the occurrence of sustained AF in patients and animal models (Kanagaratnam, et al., 2008; van der Velden, et al., 2000). In my thesis study, I found SR Ca^{2+} mishandling due to enhanced SR Ca^{2+} leak promotes the initiation of atrial arrhythmia in repeated binge alcohol-exposed mice. These results indicate the pivotal role of SR Ca^{2+} mishandling yet do not exclude the potential role of other ion channels that could be valuable directions of future research.

8.7 Factors that may Contribute to SR Ca^{2+} Overload in Alcohol-treated Cells

Multiple factors could lead to SR Ca^{2+} overload. In my thesis study, I found alcohol treatment promotes SERCA activity in JNK-dependent yet CaMKII-independent manner. A limitation of the current study is that alcohol-induced SERCA activity alteration was measured by incubating human SR with alcohol for a short period of time, due to the limited choice of human patient samples. It is a plan for the future to evaluate the SERCA activity from samples of binge drinking/chronicle drinking patients.

The regulation of SERCA activity is multi-dimensional. Other than the previously described CaMKII-dependent phosphorylation of PLB, other factors such

as the phosphorylation of PLB by PKA, the atrial sarcolipin (Shaikh, et al., 2016) could also contribute to the activity of SERCA.

Other factors besides the altered SERCA activity could lead to SR Ca^{2+} overload. For instance, exposure to hypokalaemia induces Ca^{2+} overload and Ca^{2+} waves in ventricular myocytes by reducing Na^{+}/K^{+} ATPase function (Aronsen et al., 2015), similar to the findings in digoxin toxicity induced SR Ca^{2+} overload.

The contribution of L-type Ca^{2+} channel function to SR Ca^{2+} overload is still controversial. Although Li et al. reported that enhanced L-type Ca^{2+} current can promote SR Ca^{2+} overload (H. Li, et al., 2013). In Dr. Eisner's review (D. A. Eisner, Kashimura, Venetucci, et al., 2009), it is suggested that the changes in L-type Ca^{2+} channel alone is not enough to affect SR Ca^{2+} load (Trafford, et al., 2001), since although enhanced L-type Ca^{2+} channel can load cell with more Ca^{2+}, it also triggers more release from SR, thus the net effects are an unaltered SR Ca^{2+} load (D. A. Eisner & Trafford, 2000). Further study from the same group showing that RyR opening probability alone cannot induce Ca^{2+} waves as well in conditions that the SR load is small (for instance, with low concentration of caffeine present) (Venetucci, et al., 2007). Thus SR Ca^{2+} load is a regulated by multiple factors including at least L-type Ca^{2+} channel and RyR opening probability.

Enhanced intracellular ROS can also promote SR Ca^{2+} overload. For instance, treating ventricular myocytes with H_2O_2 (200 μM) led to CaMKII activation and SR Ca^{2+} overload, which further enhanced SR Ca^{2+} leak; the SR Ca^{2+} overload is largely

due to the increased L-type Ca^{2+} channel activity (H. Li, et al., 2013). In my thesis work, I focused on the role of SERCA activity in alcohol-induced SR Ca^{2+} overload. The evaluation of the role of ROS and L-type Ca^{2+} current channel could be a future direction of study.

8.8 Intracellular Ca^{2+} Buffer in Alcohol-treated Atrial Tissue/Cells

As was previously discussed, intracellular Ca^{2+} buffers also play an important role in determining the propagation of Ca^{2+} waves. Previous research suggests that increasing intracellular buffer decreases the frequency and kinetics of Ca^{2+} waves in multiple cells types (M. Chen, et al., 2010; Rintoul & Baimbridge, 2003; Stavermann, et al., 2015; Sugai, et al., 2009). In cardiac myocytes, myofilaments act as one of the prominent Ca^{2+} buffer systems and bind about 50% of SR Ca^{2+} release in each cardiac cycle) (Shannon, et al., 2000). When myofilament Ca^{2+} sensitivity is increased, the contractile machinery activated at lower intracellular Ca^{2+} concentration (Huke & Knollmann, 2010), thus for the same Ca^{2+} release from SR, the amount of free Ca^{2+} increases. Increased myofilament Ca^{2+} sensitivity has been shown to promote arrhythmogenicity (Fentzke et al., 1999; Hernandez et al., 2005; Huke & Knollmann, 2010; Knollmann et al., 2001; Knollmann, et al., 2003; Puglisi, et al., 1999; Sugai, et al., 2009; J. White, Lee, Shah, & Orchard, 1993; Wolff, et al., 1996). Yet controversial results abound (Miura, et al., 2015; Sheehan, et al., 2009). In the current study, I focused on the contribution of alcohol-induced SR Ca^{2+} release properties rather than the Ca^{2+} buffer property of the cell. Ca^{2+} buffers such as

myofilament Ca^{2+} sensitivity or other intracellular Ca^{2+} buffer systems could be future direction for this study.

8.9 The Differential Contribution of SR Ca^{2+} Load or CaMKII-dependent RyR Phosphorylation in Alcohol-promoted SR Ca^{2+} Leak

As was shown in the results, both enhanced SR Ca^{2+} leak and increased SR Ca^{2+} load promote alcohol induced arrhythmogenic Ca^{2+} waves. My studies showed that either inhibiting CaMKII thus alleviating the SR Ca^{2+} leak or blocking RyR luminal Ca^{2+} sensitivity attenuated the occurrence of Ca^{2+} waves and arrhythmia inducibility. To further differentiate the role of SR Ca^{2+} overload and CaMKII-dependent RyR phosphorylation in RyR channel sensitivity, single channel recording methods could be used incrementally elevating the Ca^{2+} concentration on the side of recording chamber that mimics the SR lumen. Also, single channel recording on mutated RyR without CaMKII-dependent phosphorylation site and SR preparations with pharmacologically inhibited CaMKII could provide further information on the mapping the RyR behavior under different phosphorylation status/SR load conditions. To gain further knowledge on whether ROS plays an important role in tuning RyR behavior under different phosphorylation/SR load condition, single RyR channel recording with ROS scavenger such as NAC could yield further information.

8.10 Gap Junction Remodeling in Alcohol Exposure

In my thesis research, I focused on Ca^{2+} mishandling in binge alcohol as cause of atrial arrhythmia rather than the potential reentrant mechanisms. In my preliminary optical mapping studies, my preliminary data suggest the mis-matched pattern of atrial action potential propagation and Ca^{2+} transient propagation in alcohol-exposed hearts which further indicates the existence of ectopic Ca^{2+} activities. Also, my preliminary data suggest unaltered action potential conduction velocity in binge alcohol exposed mice. Moreover, the Ai Lab found that Cx43 expression level is not significantly altered in alcohol-treated HL-1 cells or binge alcohol exposed mouse atria. However, such findings do not exclude gap junction protein remodeling in binge alcohol exposed hearts, especially considering that Cx43 and other connexin can be post-translationally modified, such as phosphorylation. Thus, the expression level, post-translational modifications and the channel function of gap junction proteins are a future direction of study.

8.11 Fibrosis and Inflammation Cytokines and in Alcohol-exposure Model

Long term alcohol exposure has been shown to increase fibrosis formation in humans (Sokolova, 2016) and in various animal models (W. Liu, et al., 2011; Steiner, et al., 2015; Vasdev, et al., 1975), although there are still controversial findings (Matyas, et al., 2016). Fibrosis formation plays an important role in the maintaining of arrhythmia by forming non-conductive zones that block the action potential propagation and promote reentry. In a mouse model of chronic alcohol feeding (24

weeks), upregulation of collagenα I-V was found in both mRNA and protein level in the heart (Steiner, et al., 2015). The reason that fibrosis is usually observed in long term alcohol exposure is that cardiac interstitial formation relies on the activation of inflammatory cytokines, which further activates the cardiac fibroblast.

The interplay between inflammatory cytokines such as TNF-α, TGF-β and NFκB has been observed in fibrosis formation in the heart and in other organs after alcohol challenge (Araujo Junior et al., 2016; Z. Ren, Wang, et al., 2016). And cardiac interstitial formation relies on the activation of inflammatory cytokines, which further activates the cardiac fibroblast; and ECM remodeling typically happens in the time scale of weeks after the tissue insult (Czubryt, 2012). It would be an interesting future study to closely monitor the interaction of inflammatory cytokines and the multiple cell types involved in fibrosis formation in the setting of alcohol exposure. Previously, it was discovered that suppressing inflammatory cytokines such as TNF-α and IL-6 in an acute treatment setting alleviates the atrial arrhythmia inducibility (Moradi et al., 2016). Thus, it would be valuable to evaluate the different cytokine levels in binge alcohol exposed hearts and examine the roles of inflammatory cytokines in Ca^{2+} handling, ion channel function and reentrant substrate remodeling.

8.12 The Mechanisms of Alcohol-induced JNK Activation and JNK Alternative Splicing

Increased cellular ROS has been proved to promote JNK activation, and alcohol exposure has been shown to increase intracellular ROS (R. H. Zhang, et al., 2013). The current study focuses on alcohol-activated JNK and its downstream functional consequences, especially in arrhythmogenesis and aims at exploring the possibility of using JNK as a therapeutic target. Thus the molecular mechanisms of binge alcohol exposure-induced JNK activation were not included in the current study. It is possible that the activation of JNK is ROS-dependent, or through the canonical pathways of the MAPK cascade or potentially other mechanisms. It is of great interest to elucidate the detailed mechanisms of alcohol-induced JNK activation and the essential motifs/amino acid sites that are vital for alcohol-induced JNK activation if it is different from the canonical amino acid sites on JNK that the upstream MKK4 and MKK7 phosphorylate. Mutagenesis, proteomics and bioinformatics approaches, as well as molecular biology methods will be beneficial to study this topic.

Treating SR vesicle *in vitro* with anisomycin or alcohol both showed enhanced JNK activation and the functional consequences, such as increased RyR single channel opening probability and increased SERCA activity. The mechanism is currently an ongoing study in the Ai Lab with the main goal of identifying the contribution of ROS in the anisomycin or alcohol-promoted JNK activation in vitro.

As previously discussed, JNK is a multi-isoform protein including JNK1, JNK2 and JNK3, while JNK1 and JNK2 are the primary isoforms in cardiac tissue (Bogoyevitch & Kobe, 2006; Q. Liang, et al., 2003). Alternative splicing in JNK exists for each isoform and about 10 isoforms are produced by JNK alternative splicing from the three genes encoding JNK1, JNK2 and JNK3 (Gupta, et al., 1996). The JNK isoforms resulted from alternative splicing usually carry variations on the C-terminus which may facilitate the JNK localization in different cellular domains (Casanova, et al., 2000; Yang, et al., 2007) , while the sequence (Thr-Pro-Tyr) recognized by upstream kinase (MKK4 and MKK&) remain conservative (Barr & Bogoyevitch, 2001). To date, the specific roles of the JNK isoforms produced by alternative splicing are currently unknown (Barr & Bogoyevitch, 2001). Although this thesis did not focus on the potential alterations in alcohol exposure induced alternative splicing, such information is of great importance for targeting JNK in a specific and effective manner as a potential treatment strategy.

8.13 Mechanisms of JNK-mediated CaMKII Activation

Besides the canonical pathway, in which CaMKII is activated dependent upon increased Ca^{2+} concentration and calmodulin, we discovered in this work that JNK can directly promote CaMKII phosphorylation by promoting the phosphorylation of CaMKII at the auto-phosphorylation site Thr286. Yet the detailed mechanisms of how JNK induces CaMKII activation are unknown. For instance, determination of the amino acid sites on CaMKII (primarily CaMKIIδ in the heart) that JNK binds to and

phosphorylates is the next topic to study. Preliminary investigations suggest that there are amino acids on CaMKIIδ that fit the consensus sequence of JNK, yet whether these amino acid sites are on accessible to JNK, and whether JNK binds to CaMKII via an anchoring protein are future directions for this project.

CHAPTER NINE

CLINICAL IMPLICATIONS

9.1 Binge Alcohol-induced Atrial Arrhythmias

As previously described, binge drinking can induce cardiac arrhythmias in young and otherwise healthy patients with no clinical evidence of increased risk for cardiac diseases (Beets, et al., 2009; Mitka, 2009; Tonelo, et al., 2013; Trejbal & Mitro, 2008). AF is the most frequently diagnosed rhythm disturbance among HHS (Ettinger, 1984; Ettinger, et al., 1978; Mandyam, et al., 2012; Tonelo, et al., 2013). Emerging evidence indicates that AF brings threatening health risks to the patient by increasing the risk for stroke by 5 fold (Krahn, et al., 1995; Wolf, Dawber, Thomas, & Kannel, 1978), and tripling the risk for HF (Krahn, et al., 1995) and by increasing the all-cause mortality 40-90% (Benjamin, et al., 2009; Benjamin, et al., 1998; Krahn, et al., 1995). AF accounts for more than 350000 U.S. hospital admission annually (Wattigney, et al., 2003) and costs the U.S. health care system approximately $26 billion each year (Calkins, et al., 2012), thus causing significant financial burden to our society. Also, current treatment strategies for AF, including anti-coagulation therapy, ablation

therapy and surgical ablation are not effective in long term (Touze & Ciocanu, 2014) (Furberg, et al., 1994) (Skelly, et al., 2015) (Aryana, et al., 2012).

Despite an extensive prevention effort nationwide, binge alcohol consumption still affects large percentage of the population and continues to rise. Moreover, binge drinkers tend to repeat this behavior within a short period of time, for instance, once per week ("Vital signs: binge drinking prevalence, frequency, and intensity among adults-U.S., 2010," 2012). Indeed, about 30% of binge drinkers report >10 binge episodes while 15% report 5-9 binge episodes within a 30-day period (Esser, et al., 2014). This repeated binge drinking pattern not only increases the chance of alcohol exposure that could significantly facilitate the formation of arrhythmogenic substrates, it could even promote the development of persistent AF via 'AF begets AF' mechanisms (Lo et al., 2016; Wijffels, Kirchhof, Dorland, & Allessie, 1995).

In this study, I used a newly developed mouse model of repeated binge alcohol exposure, and confirmed that during the 24 hrs after the last binge alcohol episode when BAC returned to normal, atrial arrhythmia inducibility was still dramatically increased. This phenomena is similar to the findings in human patients, where the highest frequency of binge alcohol-induced arrhythmia tends to happen when the patient already recovers from alcohol intoxication.

9.2 Binge Alcohol-induced JNK Activation and JNK Inhibitor as a Potential Therapeutic Target

I also discovered repeated binge alcohol induced atrial arrhythmia depends on JNK activation. It is well established that alcohol exposure leads to the activation of JNK in multiple tissues and organs including the liver, the pancreas, and the heart (Aroor, et al., 2010; C. H. Lang, et al., 2014; Lee, et al., 2002; S. Y. Li, et al., 2009; Masamune, et al., 2002; McCarroll, et al., 2003; Meriin, et al., 1999; Nishitani & Matsumoto, 2006), especially in organs that are involved in alcohol metabolism (Dinu, et al., 2005; Liew, et al., 2013; Rodrigo & Rivera, 2002).

JNK activation has been shown in various human cardiac diseases including myocardial infarction (T. Li et al., 2016), and HF of both ischemic and non-ischemic etiology (Haq, et al., 2001) (Cook, Sugden, & Clerk, 1999). Studies from a wide spectrum of animal models of cardiac diseases further strengthen the role of JNK activation in cardiac pathology. For instance, JNK activation is enhanced in a mouse model of non-ischemic HF induced with transverse aortic constriction; inhibition of JNK activation reversed the decreased EF (S. Cao et al., 2013). Enhanced JNK activation in cardiac myocytes by overexpressing active mutant of MKK7 induces characteristic profile of hypertrophy, including increased cell size and macromere reorganization (Wang et al., 1998). Moreover, in a mouse model of epilepsy-associated cardiac comorbidity, JNK activation co-existed with cardiac dysfunction (F. Chen et al., 2013). On the other hand, blocking JNK signaling in a model of cardiac overload by

overexpressing dominant negative JNK has been shown to abolish the pressure-overload induced cardiac hypertrophy (Choukroun et al., 1999). JNK activation has also been shown to promote arrhythmia. Additionally, JNK activation is reported in reperfusion phase of the ischemic reperfusion injury during which high propensity of arrhythmia was demonstrated (Bogoyevitch, et al., 1996; Siow, et al., 2000). JNK phosphorylation was significantly increased in a mouse model of Rheb1 (a small GTPase) in which malignant arrhythmia, HF and premature death were observed (Y. Cao, et al., 2013).

Multiple JNK inhibitors have been used to suppress JNK pathways. SP600125 has been shown to inhibit JNK pathway efficiently and is commonly used as JNK inhibitor (W. Guo et al., 2016; Han et al., 2001; J. H. Kim, Chae, Choi, Sik Kim, & Yoon, 2014; Yan, et al., 2013). However, SP600125 inhibits JNK1, JNK2 and JNK3 with IC50 of 40 nM, 40 nM and 90 nM in cell-free assays, suggesting it does not have JNK isoform-specific action (Bennett et al., 2001). CEP-1347 has been used as a JNK inhibitor (Kujime, Hashimoto, Gon, Shimizu, & Horie, 2000). However, the specificity of this drug is questionable since findings suggest that it may inhibit JNK upstream regulators in the meantime (Maroney et al., 1998). Also, Wagner et al. have shown in a pancreatitis animal model that the *in vivo* efficiency of CEP-1347 in JNK inhibition may need further investigation (A. C. Wagner, Mazzucchelli, Miller, Camoratto, & Goke, 2000). In my thesis study, I used a newly developed JNK inhibitor IX (JNK2I, EMD), which is shown to be highly specific that selects against other MAPKs

including MAPK-p38, ERK and JNK1 (Angell et al., 2007). The development and application of JNK inhibitors is a currently very active field of research with the goal of finding the JNK inhibitor with high isoform-specific action, organ-specificity and drug efficiency (Koch, Gehringer, & Laufer, 2015).

JNK inhibition has been used as a treatment strategy in clinical trial in non-cardiac diseases. In preclinical animal models, JNK inhibition has also been tested for tested in treating cerebral ischemia (Hirt et al., 2004), hepatic damage (Lehnert et al., 2008) and Alzheimer's disease (Ploia et al., 2011). In clinical trials, CNI-1943, an inhibitor of both JNK and MAPK-p38, has been tested in a small short-term clinical study (12 patients) for treating Chrone's disease and has shown beneficial results (Hommes et al., 2002). Another JNK inhibitor CC-930 was recently advanced to Phase II clinical trial to examine the pharmacokinetics and biological activity in patients with idiopathic pulmonary fibrosis ("Trial Identifier NCT0120394,"). Another multi-center double blind clinical trial using a single dose injection of AM-111, a JNK-inhibiting peptide to treat idiopathic sudden sensorineural hearing loss showed persistent improvements in severe-to-profound hearing loss patients but not in mild-to moderate cases (Suckfuell et al., 2014). However, to the best of my knowledge, there are no clinical studies using JNK inhibition to treat cardiovascular diseases. Since there are JNK inhibitors that are being used in clinical trial for non-cardiac diseases, it would be interesting to test JNK inhibitors in the treatment alcohol induced atrial arrhythmias.

Although it is known that JNK and ROS form augmentation signaling loop in alcohol-exposed tissue, and that suppressing one component theoretically limit the function of the other component (Cederbaum, et al., 2009; Jin, et al., 2013; Knockaert, et al., 2011). Clinical trials targeting ROS in cardiovascular diseases are abundant, I chose to focus this study on JNK because the ROS suppression studies yielded mixed results (Goszcz et al., 2015). For instance, it has been shown that anti-oxidant vitamins reduced arteriosclerosis progress in patients with endothelial dysfunction (Behrendt et al., 2006). However, long term anti-oxidant vitamin C (500 mg/day) supplementation does not reduce the incidence of major cardiovascular diseases including myocardial infarction, total stroke, cardiovascular death, and CHF (Sesso et al., 2008). Similarly, a large scale randomized clinical study of 8112 patients showed that taking antioxidant vitamin blend did not exert benefits on ischemic cardiovascular disease incidence compared to placebo group (Hercberg et al., 2004). There are also reports showing that taking antioxidant vitamin blend (500mg vitamin C and 400 IU vitamin E twice per day) accelerates coronary artery disease progression (Levy et al., 2004; Waters et al., 2002). NAC, the ROS scavenger used in my thesis, has appealing antioxidant activity, yet its oral bioavailability is generally considered too low to bring an effect in vivo (Rushworth & Megson, 2014). Yet previous reports suggest that long term oral administration of NAC and L-arginine brings endothelial function improvement in diabetes patients (Martina et al., 2008). The controversial results may due to the differences in bioavailability due

to the difference in doses and routes used in each study; also, it could be due to the fact that ROS mediates various signaling pathways including but not limited to angiotensin (Gopi, Subramanian, Manivasagam, & Vellaichamy, 2015) and adrenergic receptors (Odnoshivkina et al., 2015), thus non-selectively limiting ROS could affect many pathways.

9.3 CaMKII Inhibition as a Potential Therapeutic Target for SR Ca^{2+} Mishandling

In this thesis, I found that alcohol-promoted JNK activation causes Ca^{2+} mishandling via CaMKII activation. The activation of CaMKII has been found in many cardiac diseases including HF (van Oort, Brown, & Westenbrink, 2014). Suppressing the activation of CaMKII with CaMKII inhibitor KN93 or CaMKII inhibiting peptide AIP both decreases alcohol/JNK induced arrhythmogenic Ca^{2+} events and atrial arrhythmia.

Inhibiting CaMKII could be a therapeutic strategy to treat Ca^{2+} mishandling prompted arrhythmogenic cardiac diseases. Yet obstacles still exist. For instance, the CaMKII inhibitor used in this study, KN-93, is highly selective yet not optimized for its potency (Y. Gao et al., 2013; Pellicena & Schulman, 2014). Peptide AIP also demonstrates highly specificity of CaMKII inhibition, yet the delivery of a peptide in vivo present considerable challenge. In experimental models, AIP is usually expressed in cell systems via adenoviral constructs or direct cell injection (Payne et al., 1988; Pellicena & Schulman, 2014). Currently, clinical trials exploring the

therapeutic effects of CaMKII inhibition are relatively rare and the results are not optimal (Hund & Mohler, 2015). In my thesis study, I found single dose CaMKII inhibitor KN93 (I.P.) was able to attenuate the frequency of atrial Ca^{2+} waves and burst pacing induced atrial arrhythmia, suggesting that CaMKII inhibition could be a therapeutic strategy for treating alcohol induced atrial arrhythmia, especially when the molecular optimization for the compound and the drug delivery methods are improved.

In summary, my findings in this thesis work strongly indicate that JNK-promoted CaMKII activation plays a pivotal role in the arrhythmogenic Ca^{2+} mishandling in repeated binge alcohol-prompted arrhythmia. Inhibition of JNK and potentially suppression of CaMKII could be a therapeutic strategy for binge alcohol induced atrial arrhythmias.

9.4 Pleiotropic Effect of JNK and CaMKII Inhibition

The key finding of my thesis is that alcohol-prompted JNK activation can further promote SR Ca^{2+} mishandling by activation CaMKII, in addition, activated JNK can promote SR Ca^{2+} load which further causes SR Ca^{2+} mishandling. Thus, JNK plays a pivotal role in regulating intracellular Ca^{2+} handling, thus enhancing arrhythmogenicity. Inhibition of JNK and CaMKII has the potential to become therapeutic approach. However, pleotropic effects of JNK and CaMKII are a vital aspect when considering JNK and CaMKII inhibition for treatment strategies.

CaMKII effect on SR Ca^{2+} handling protein: My thesis findings have shown that alcohol-prompted JNK activation can promote CaMKII activation, which causes CaMKII-dependent RyR phosphorylation at Ser2815 that further enhances SR diastolic Ca^{2+} leak. Moreover, this effect can be abolished with CaMKII inhibitor KN93 treatment. CaMKII inhibition or knocking out has been shown to decrease SR Ca^{2+} leak in multiple studies (Ai, et al., 2005; J. Cheng et al., 2012; Curran, et al., 2010). However, besides RyR, CaMKII can regulate multiple proteins involved in SR Ca^{2+} handling. For instance, CaMKII is known to phosphorylate PLB17 and promotes its dissociation with SERCA thus enhances SERCA activity (DeSantiago, Maier, & Bers, 2004; Mattiazzi, Mundina-Weilenmann, Vittone, & Said, 2004; Tsui, Inagaki, & Schulman, 2005). Thus, the inhibition of CaMKII could potentially bring off-target effect of lowering SERCA activity and decrease SR Ca^{2+} load (H. K. Kim et al., 2015). The decreased SERCA activity could on one hand, decrease the risk of triggered arrhythmia (Picht et al., 2007), yet on the other hand, it poses an increased risk of hindering cardiac contractile function (Haghighi, Bidwell, & Kranias, 2014).

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CaMKII effect on L-type Ca^{2+} current: CaMKII activation is implicated in the facilitation of L-type Ca^{2+} current. It is previously shown that enhanced CaMKII activation increases L-type-Ca^{2+} amplitude and slowing of the channel inactivation (Anderson, Braun, Schulman, & Premack, 1994; R. P. Xiao, Cheng, Lederer, Suzuki, &

Lakatta, 1994; Yuan & Bers, 1994). Also, knocking out CaMKII has been shown to preclude the challenge-induced increase in L-type-Ca^{2+} current; such challenge include *in vivo* cardiac pressure overload (J. Cheng, et al., 2012), and *in vitro* isopreterenol treatment (L. Xu et al., 2010). Thus, blocking CaMKII may give rise to decreased L-type-Ca^{2+} current, decreased RyR Ca^{2+} release due to decreased CICR (Bryant et al., 2015), hence hindered cardiac contraction that was observed in models of L-type-Ca^{2+} channel inhibition (S. J. Liu, 2007; T. Liu et al., 2016; Santos et al., 2011).

CaMKII effect on Na^+ currents: CaMKII is known to phosphorylate Na^+ channel, which leads to intermediate inactivation of Na^+ channel and prolongation of the Na^+ channel inactivation, while enhancing the late persistent inward Na^+ current (S. Wagner, et al., 2006). CaMKII-dependent Na^+ channel activation can result in increased intracellular Na^+ concentration, prolonged QRS complex and increased propensity for ventricular arrhythmia (S. Wagner, et al., 2006). Recently, CaMKII has also been found to promote arrhythmogenic events including EAD, DAD and altered automaticity via enhancing late Na^+ channel activity (Belardinelli, Giles, Rajamani, Karagueuzian, & Shryock, 2015; Shryock, Song, Rajamani, Antzelevitch, & Belardinelli, 2013). Similarly, altered Na^+ channel gating was found when CaMKII was activated in cardiac myocytes by increasing ROS with H_2O_2 treatment (Foteinou, Greenstein, & Winslow, 2015). On the other hand, enhanced late Na^+ current can

promote CaMKII activation by increasing intracellular Ca^{2+} via NCX activation, which further leads to arrhythmogenic SR Ca^{2+} leak (Fischer et al., 2015).

In this thesis, I found JNK2 plays major role in binge alcohol-prompted atrial arrhythmia. Isoform specificity is of vital importance when inhibiting JNK is considered a therapeutic strategy since JNK has 3 isoforms, while JNK1 and JNK2 are the major isoforms in the heart (Q. Liang, et al., 2003). Dr.Ai's lab has recently found that enhanced JNK2 activation promotes arrhythmogenic remodeling in aged rabbits (Yan, et al., 2013).

Pleiotropic JNK function due to multiple isoforms: in It has also been shown that JNK is important in cardiac development, especially ventricular morphology, due to its crosstalk with Wnt signaling (Cohen, Tian, & Morrisey, 2008; Zhou et al., 2007). Multiple studies have also suggested that JNK offers cardioprotection and myocytes survival in stress conditions including hypoxia and oxidative stress (Dougherty et al., 2002; Shao et al., 2006). Interestingly, it has been shown that JNK1 but not JNK2 suppresses apoptosis pathway after ischemic/reoxygenation injury (Hreniuk et al., 2001). Also, previous finding has shown that JNK1 activation may convey protection effect during acute but not prolonged cardiac ischemia injury (Wei et al., 2011). In addition, JNK1 has been shown to preserve the contractility of the heart and protect the heart against hypertrophy during mechanical overload (Tachibana et al., 2006). These data suggest that JNK1 activation could offer protection in acute stress conditions, while JNK2 activation could cause arrhythmogenic remodeling.

Thus, in order to choose an effective pharmacological inhibitor to target the binge alcohol-prompted JNK/CaMKII activation, it is of vital importance to take into consideration of the various proteins that are subjected to JNK/CaMKII regulation, and to differentiate the specific isoforms of the kinase of interest.

CHAPTER TEN

MATERIALS AND METHODS

10.1 Human Sample and SR Vesicle Extraction

Human right atrial tissue was obtained from a human donor heart that was not used for heart transplantation but had no history of major cardiovascular diseases. The human donor heart was provided by Illinois Gift of Hope Organ & Tissue Donor Network (GOH). Table1 shows de-identified general data information from the donor. The study was approved by the Human Study Committees of Loyola University Chicago and Illinois GOH.

To extract SR vesicles, flash frozen right atrial tissue was dissected into small pieces and homogenized in Solution 1 (0.9% NaCl, 10mM Tris-Maleate). To remove the tissue remnants, the tissue homogenates was centrifuged at 4000 g for 20 min and the supernatant was collected. The supernatant was further centrifuged at 20,000g for 20min to collect the supernatant again to remove remnant mitochondria. Then the newly collected supernatant was centrifuged at 70,000g for 40min to collect the pellet. The pellet was resuspended in Solution II (Solution I supplemented with DTT and protease inhibitor), flash frozen and stored in liquid nitrogen.

I graciously thank Ms.Alma Nani at Dr. Fill's lab for instructing and helping me isolating the SR vesicles.

Table 1

Gender	Age	EF	BMI	CHF	Cardiac valve diseases	CAD	HTN	DM	Alcohol consumption
M	64	65%	32	-	-	-	+	+[1]	-

1. Patient hemoglobin A1c is 4.8%, within the normal range of 4.0-5.6% , which suggests that DM is under control.

10.2 Animal Preparations

All animal studies followed the Guide for the Care and Use of Laboratory Animals (NIH Publication, 8th Edition, 2011) and were approved by the Institutional Animal Care and Use Committees of Loyola University Chicago and University of Alabama at Birmingham.

Young (6 months; n=6) New Zealand White male rabbits were infused with alcohol (2 g/kg BW, 12.67% v/v, I.V.; every other day for a total of four injections) and sham control rabbits (n=5) were used for the atrial arrhythmia induction and biochemical assays. To further study the role of JNK activation in atrial arrhythmogenesis, JNK activator anisomycin was also used to challenge young rabbit (20 mg/kg BW, I.V., EMD; every other day for a total of four injections). To further assess the role of JNK activation in atrial arrhythmogenesis, a Tg mouse line that overexpresses inducible cardiac-specific isoform of constitutively activated MKK7 (MKK7D), was used in the atrial arrhythmia inducibility studies (a generous gift from Dr. Yibin Wang's lab at UCLA (Andersson et al., 2009)). Tamoxifen injection (1 mg/kg BW, I.P., 4 consecutive days) was used to induce the MKK7D expression (Andersson, et al., 2009).

Wild-type C57/Bj (Jackson laboratory, ME) and Tg mice (8-12 weeks of age) were treated with alcohol (2 g/kg BW, 12.67% v/v, I.P.) every other day for four doses. A Tg mouse line with over-expression of cardiac-specific dominant-negative JNK1 and JNK2 protein (JNK1/2dn) (Q. Liang, et al., 2003) was used to assess the

contribution of JNK protein in binge alcohol-induced Ca^{2+} handling alterations and AF genesis. Another transgenic mouse model overexpressing a mutated SR luminal Ca^{2+} sensor on RyR ($E4872Q^{+/-}$) (W. Chen, et al., 2014) was used to explore the involvement of alcohol-induced SR overload in RyR dysfunction and arrhythmogenesis. To further explore the potential rescue effect of CaMKII inhibition in binge-alcohol exposed mice, alcohol-injected mice were treated with CaMKII inhibitor KN93 (7.2 mg/kg BW, I.P.) or its inactive analogue KN92 (7.2 mg/kg BW, I.P.) 12-16 hrs after the last dose of alcohol injection and 3 hrs prior to terminal studies.

For this entire thesis, the sham control animal controls are animals of the same age, gender (male) and injected with equal volume of saline as of the drug at the same treatment schedule of the drug treatment.

10.3 Rabbit atrial Arrhythmia Induction *in vivo*

Atrial arrhythmia inducibility was examined in binge alcohol challenged rabbits compared to sham control ones. Rabbits were sedated with ketamine (45 mg/kg, I.M.) and followed by intubation and mechanical inhalation of 2-4% isofluorane delivered via 100% oxygen to maintain surgical plane during open-chest AT/AF induction procedure. The same *in vivo* AF induction procedure via burst pacing was performed in alcohol-challenged and sham rabbits. A bipolar pacing electrode was sutured on the right atrium (RA) while four unipolar electrodes were sutured at four sites (2mm apart) on the left atrium (LA) posterior free wall for

electrical signal recording through a multi-channel data acquisition system based on the method by Berul et al. with modifications (Berul, Aronovitz, Wang, & Mendelsohn, 1996). Diastolic threshold (TH) was determined at baseline pacing of 200 msec as the lowest current capable of pacing with 1:1 atrial capture. Atrial effective refractory period (AERP) was determined at baseline pacing of 200 msec. AERP was measured by giving a train of 10 basic stimuli (S1, 2x diastolic threshold, 2 msec pulse duration) at a cycle length (CL) of 200 msec, followed by a premature stimulus (S2). The S2 was delivered at 2x diastolic threshold in 10 msec decrements and followed in 5 msec decrements until a response was not evoked. The AERP was defined as the longest S1–S2 interval which failed to produce a propagated response, and the procedure was repeated twice for the AERP measurement. Burst pacing was delivered at a pacing CL of 100 msec and then reduced to 50 msec in 10 msec decrements. For each recording, burst pacing last for 2 sec at 3xTH. The same pacing protocol was then repeated at 6xTH strength. Finally, 30 sec rapid pacing (CL = ± 5 msec of AERP) at 6xTH was attempted. The induced rhythm was defined as AT/AF when the atrial bipolar electrogram showed fast (>8 Hz) and regular or irregular cycles that lasted for at least 1 sec. If the arrhythmia lasted for more than 30 sec, it would be terminated by electrical shock. The duration of AT/AF was analyzed as the mean value of all episodes in each rabbit. AF inducibility was calculated as number of AF incidence per pacing attempt per animal.

I graciously thank Drs. Cheryl Killingsworth and Qiang Zhang's help for the in vivo atrial arrhythmia inducibility studies in rabbits.

10.4 Mouse Preparation and BAC Measurement

Mouse terminal studies were performed within 24 hrs after the last dose of alcohol injection. To harvest the mouse heart for confocal Ca^{2+} imaging studies, the mice were injected with heparin (50 unit/g BW, I.P.) 15 minutes prior to the sacrifice to avoid coagulation. Ketamine hydrochloride (100 mg/kg BW, I.P.) and xylazine (5 mg/kg BW, I.P.) was used to induce anesthesia, and surgical plane was maintained with isofluorane (0.5-1.5% delivered with pure oxygen) (Pachon, Scharf, Vatner, & Vatner, 2015). The hearts were harvested and immersed in cold cardioplegic solution (NaCl 120.4 mM, Na_2HPO_4 0.6 mM, $NaHCO_3$ 4.6 mM, KCl 14.7 mM, KH_2PO_4 0.6 mM, $MgSO_4$ 1.2 mM, Hepes 10 mM, glucose 5.5 mM, pH 7.4). To harvest cardiac tissue for biochemical studies, blood was rinsed off with Ca^{2+}-free Tyrodes' solution before flash freezing the tissue in liquid nitrogen. Blood samples were collected at the time of sacrifice from the chest immediately after the heart was harvested, and temporarily put into 1.5 mL eppendorff centrifuge tubes pre-coated with heparin. Serum was separated from the blood cells by centrifugation (1000 g, 10 min) and collected carefully without disturbing the pellets. The serum was then stored in -80°C freezer before usage. BAC was measured using an alcohol concentration assay kit (Abcam ab65343). In brief, stand curve for was prepared using step-diluted pure alcohol (10 nM- 0.1 μM) as published by Abcam online,

http://www.abcam.com/ethanol-assay-kit-ab65343-protocols.html. The sample was diluted 1:20 with saline before measurement, and 10μL sample was incubated with 40 μL reaction mixture (47.8 μL Ethanol Assay Buffer, 0.2 μL Probe, 2 μL Ethanol Enzyme Mix for each 50 μL reaction mixture) for 30 min at 37°C in dark. Fluorometric assay was performed at Ex/Em 535/587 nm to detect fluorescent signals (PHERAstar fluorescent measurement setting).

I graciously thank Dr. Xianlong Gao for his performing and training me in the BAC measurements.

10.5 *ex vivo* Confocal Ca^{2+} Imaging and Atrial Arrhythmia Inducibility in Intact Mouse Atria

Freshly-harvested mouse hearts were immediately submerged and cannulated in cold cardiac plegic solution. Then the hearts were perfused on Langendorff setting to rinse off the blood completely using Ca^{2+}-free Tyrode's solution (NaCl 140 mM, KCl 4 mM, $MgCl_2$ 1 mM, HEPES 4 mM, $CaCl_2$ 1.8 mM, pH 7.4) before dye loading (D. Lang, Sulkin, Lou, & Efimov, 2011). 5μM Ca^{2+}-sensitive dye Rhod2-AM (5 mM stock solution dissolved in DMSO, 1:1000 diluted with Ca^{2+}-free Tyrode's solution; AAT Bioquest) was used to stain the heart at room temperature for 1 hour. After dye loading, the mouse heart was connected to the perfusion chamber mounted on the confocal microscope (Nikon Eclipse TE2000-U, 40x objective, NA 1.3) and perfused with oxygenated 37°C Normal Tyrode's solution containing 1.8 mM Ca^{2+} (NaCl 140 mM, KCl 4 mM, $MgCl_2$ 1 mM, HEPES 4 mM, $CaCl_2$

1.8 mM, pH 7.4). Electro-mechanical uncouplers of combined BDM (5 mM) and blebbistatin (3 μM) were used control the movement of the cardiac muscle and stabilized the heart for confocal imaging (B. Chen, et al., 2012; W. Chen, et al., 2014; J. Zhang, et al., 2014b; J. Zhang, et al., 2015). Rhod2-AM was excited with the 561 nm line of a diode laser and fluorescence was detected at wavelength 570-620 nm. Line scan was performed on both left and right atria at a rate of 2 msec/line. Diastolic pacing threshold was determined as the lowest current capable of pacing with 1:1 atrial capture at a pacing cycle length of 250 msec. Scanning was performed at intrinsic sinus rhythm, during and after the burst pacing of (4, 8, 10 and 20 Hz) at 2x diastolic threshold. Ca^{2+} wave frequency, intracellular Ca^{2+} decay constant τ and Ca^{2+} transient rise time (time to peak) was calculated using a custom-developed algorithm in Matlab, and compared between binge alcohol-treated and sham mice of different genotypes.

After confocal Ca^{2+} imaging, a bipolar recording electrode was positioned on the left atrium while a bipolar pacing electrode on the right atrium. A series of burst pacing protocols were then performed at pacing frequency of 8Hz, 10Hz, 20Hz and 50Hz for 30 sec, at pacing strength of 3xTH respectively. Each run of burst pacing stimulation was repeated for 10 times. Then the pacing strength was raised to 6xTH and 10xTH to repeat the same pacing protocol (Figure 9C). Local tissue electrogram was detected with bipolar electrode, amplified and recorded with the AD instrument recording system. The pacing-induced rhythm was defined as atrial arrhythmias

when the atrial bipolar electrogram showed at least three fast (> 8 Hz) of regular or irregular atrial beats.

10.6 Mouse Atrial Arrhythmia Induction *in vivo*

To further understand the role of JNK activation in atrial arrhythmogenesis, JNK activator anisomycin (20 mg/kg BW I.P.; EMD) challenged mice and mice overexpressing inducible active MKK7 (MKK7D) (a generous gift from Dr. Yibin Wang at UCLA) were used in *in vivo* atrial arrhythmia inducibility studies. Mice were sedated with ketamine hydrochloride (100 mg/kg BW, I.P.) and xylazine (5 mg/kg BW, I.P.) and maintained in a surgical plane of anesthesia with 0.5-1.5% isoflurane inhalation delivered through 100% oxygen. *In vivo* AF induction was conducted in sedated mice using a 1.1 F (Millar) octapolar catheter inserted into the right atrium. Pacing current was generated from Grass Stimulator and delivered through the intracardiac catheter. Cardiac electrogram was recorded with intracardiac cathether, and surface ECG was monitored simultaneously with limb leads. Diastolic pacing threshold was determined as the lowest current capable of pacing with 1:1 atrial capture at a pacing CL of 120% of the intrinsic heart rhythm. Short burst pacing of 1 sec at pacing CL of 30 msec and 20 msec were performed with 10 repeats respectively, followed by long burst pacing of 30 sec at pacing CL of 30 msec and 20 msec with 3 repeats. Pacing strength of 3xTH was used for the entire series of burst pacing stimulations, then the series of pacing stimulations was repeated at pacing strength of 6 x TH and 10 x TH (Figure 9C). The pacing-induced rhythm was defined

as atrial arrhythmias when the atrial bipolar electrogram showed at least three fast (>20Hz) and regular or irregular atrial beats with less than 1:1 AV conduction. AF inducibility was calculated as the number of AF incidences per pacing attempt per animal. After the AF induction procedure, mouse hearts were carefully harvested and flash-frozen in liquid nitrogen for biochemical studies.

10.7 Rabbit Atrial Myocytes Isolation

Rabbits were sedated with ketamine (45 mg/kg, I.M.) and followed by intubation and mechanical inhalation of 2-4% isoflurane delivered via 100% oxygen to maintain surgical plane. The heart was immediately harvested and rinsed in cold Ca^{2+}-free Tyrodes' solution. The heart was cannulated and perfused on Langendorff setting with oxygenated normal Tyrode's solution to further rinse off blood and pre-digested with 37°C collagenase (0.75 mg/mL with 0.05% albumin) until the ventricles become flaccid (15-25 min) (Ai & Pogwizd, 2005). Then the atria were carefully dissected from the ventricle, cut into 0.5cm*0.5cm pieces and further digested in stir-flask with the same collagenase (3-5 mL) in a 37°C water bath. For every 5 min of digestion, the enzyme was separated from the tissue pieces with a tissue strainer (100 μm), and neutralized with same volume of 37°C bovine serum. New collagenase was added into the atrial tissue to continue the digestion cycle until the tissue pieces cannot be separated from the enzyme with the tissue strainer. Isolated myocytes from neutralized collagenase was collected via centrifugation (500 g, 5 min), and gently resuspended with Ca^{2+}-free Tyrodes' solution. Ca^{2+}

concentration was slowly brought up to 18 mM by carefully dripping in normal Tyrode's solution into the suspended myocytes. For anisomycin-treatment group, cells were treated with anisomycin (0.2 μM) for 5 hrs before imaging studies (Hazzalin, et al., 1998; Petrich, et al., 2002; Yan, et al., 2013). JNK2I (JNK inhibitor IX; EMD) pretreatment was applied 3 hrs before the anisomycin treatment for the JNK2I-anisomycin treated groups. Cells were plated in imaging chambers 1 hr before the imaging study to achieve stable attachment. The imaging condition for freshly isolated myocytes was the same as for HL-1 cell imaging studies.

10.8 HL-1 Confocal Imaging

A well-characterized cultured atrial myocyte line (HL-1, from Dr. William Claycomb, Louisiana State University) was used for my thesis studies. The cells were cultured to maintain the differentiated phenotype of morphologic, genetic, electrophysiological and pharmacologic characteristics of adult atrial myocytes (Yan et al., 2015). HL-1 myocytes were seeded on fibronectin-coated glass coverslips for confocal imaging studies. On day 4 after seeding, the cells were treated with 50mM alcohol for 24 hrs prior to confocal imaging studies. To understand the role of JNK, a specific JNK2 inhibitor (170 nM; JNK inhibitor IX, EMD) was applied 24 hrs before alcohol treatment. To explore the contribution of CaMKII activation in alcohol-induced Ca^{2+} dysfunction, CaMKII inhibitor KN93 (0.2 μM; EMD) and its inactive analogue KN92 (0.2 μM; EMD) were used to pretreat cells 24 hrs before the alcohol treatment. To further dissect the role of JNK activation in SR Ca^{2+} mishandling, JNK

activator anisomycin (0.2 μM) (Hazzalin, et al., 1998; Petrich, et al., 2002; Yan, et al., 2013) was used to treat HL-1 cells and rabbit atrial myocytes were treated for 24 hrs before imaging studies.

Before confocal imaging, cells from each group were loaded with Fluo-4 AM (4.6 μM, in normal Hanks Salt solution; Invitrogen) at 34.5°C for 15 min. The monolayer was then put into a perfusion chamber mounted on the microscope (Nikon Eclipse TE2000-U, 40x objective, NA 1.3), and continuously superfused with 34.5°C normal Hanks Salt solution (NaCl 136 mM, $NaHCO_3$ 4.16 mM, Hepes 5.04 mM, KCl 5.36 mM, KH_2PO4 0.44 mM, NaH_2PO4 0.4 mM, MgSO4 0.81 mM, $CaCl_2$ 1.26 mM, glucose 5.05 mM, pH 7.4), and paced at 2Hz with a micro-electrode. Fluo-4 AM was excited with an Argon laser (488 nm), and the emission light was collected at wavelength > 515 nm. Ca^{2+} transient signals were presented as the fluorescent signals normalized to the background level of fluorescence ($\Delta F/F_0$), where F_0 is the resting fluorescence under steady-state conditions at the beginning of the recording. To measure the diastolic SR Ca^{2+} leak, the monolayer was superfused with Na^+-free Ca^{2+}-free Tyrode's solution (140 mM LiCl, 4 mM KCl, 1 mM MgCl2, 4 mM HEPES,10 mM EGTA, pH 7.4) (Shannon, et al., 2002) for 15 min followed by perfusion with tetracaine (1 mM, dissolved in Na^+-free and Ca^{2+}-free Tyrode's solution) (Shannon, et al., 2003). Tetracaine blocks diastolic SR Ca^{2+} leak through RyR channels, thus decreasing diastolic intracellular Ca^{2+} fluorescence. To measure the SR Ca^{2+} load, a fast caffeine surge was supplied into the perfusion chamber during the line scanning

while the cells were superfused with normal Hanks' salt solution. The signals were normalized to F_0, while the normalized height of the caffeine-induced Ca^{2+} release was used as SR Ca^{2+} content (Figure.28C).

10.9 Immunoblotting and Kinase Activity Assays

The cardiac tissue from humans, rabbits and mice were homogenized in RIPA buffer (Tris 25 mM, NaCl 150 mM, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, pH 8) with protease inhibitors (aprotinin 10 μg/mL, leupeptin 10 μg/mL, Pefabloc 1 mM) for immunoblotting. The expression and phosphorylation of multiple proteins of interest, including JNK, CaMKII, RyR and PLB, were probed with specific antibodies (Ai, Jiang, Ke, Solaro, & Pogwizd, 2011; Yan, et al., 2013).

To further measure the activity of JNK1 and JNK2 in binge alcohol-exposed mouse cardiac tissue compared to sham control, mouse cardiac tissue was homogenized in the tissue lysis buffer (Tris·HCl 40 mM, NaCl 150 mM, β-glycerophosphate 5 mM, NaF 10 mM, Na_3VO_4 0.2 mM and 1% protease inhibitor cocktail, pH 7.4). Protein was further extracted with tissue lysis buffer containing 1% Triton X-100 for 30 min. JNK1 or JNK2 protein was IP-ed from cardiac tissue homogenates by incubating with anti-JNK1 or anti-JNK2 specific antibody and protein-A/protein-G coated algarose beads (Millipore EMD16-663). JNK1 and JNK2 protein from binge-alcohol and sham mice were used to incubate with its specific substrate c-Jun (Abcam) in kinase reaction buffer (Tris·HCl 40 mM, MgCl2 10 mM, β-glycerophosphate 5 mM, Na_3VO_4 0.2 mM, pH 7.4) at the presence of 20 μM ATP for

45min. The ADP produced from the kinase reaction was measured using an ADP-Glo™ kinase assay kit (Promega V6930). The luminescent signal intensity was used to quantify the JNK activity in sham and alcohol-treated WT mouse hearts.

To determine the direct phosphorylation action of JNK2 on CaMKII at the site of Thr286, HA-tagged wt-CaMKII and mutant CaMKII-T286A vectors were constructed as previously described as described by Erickson et al. (Erickson, Patel, et al., 2011). HEK293 cells were transfected with HA-tagged wt-CaMKII and CaMKII-T286A (mu) vectors as well as an empty vector (v) to transiently express HA-tagged proteins using lipofectamine transfection reagents (Invitrogen). Next, pure active JNK2 protein (hJNK2) was incubated with HA-antibody immunoprecipitated HA-wt-CaMKII or HA-CaMKIIT286A mutant proteins. Then, JNK2 mediated phosphorylation of CaMKII was detected using an immunoblotting assay with a phosphorylation site-specific (Thr286) antibody of CaMKII-P. In addition, direct action of JNK2 on CaMKII phosphorylation was confirmed by measuring the produced luminescent ADP signals during the phosphorylation reaction using an ADP-Glo™ assay. For all assays involving IP, protein pull down with IgG was used to serve as background controls.

I graciously thank Ms.Weiwei Zhao for her assistance in the immunoblotting studies, and Dr. Xianlong Gao for performing and training me in the JNK activity measurements.

10.10 SERCA Activity Assay

To probe whether alcohol induces alterations of SERCA activity, SR vesicles was prepared from right atrial tissue from non-failing human donor hearts. The SR vesicle was treated with 50mM alcohol and incubated with supplemented purified activated JNK2 protein and ATP at 37 °C for 30 min. The rate of ADP production was measured indirectly using an enzyme-coupled reaction in which ADP production correlated with NADH consumption (Abrol et al., 2014; Warren, Toon, Birdsall, Lee, & Metcalfe, 1974). NADH concentration was determined by UV absorption at 340nm wavelength every 30 sec for 30 min of reaction time. SERCA activity was calculated from the rate of NADH consumption. ADP production rate in each sample was measured with and without the presence of thapsigargin which inhibits SERCA function (Witayavanitkul, Woranush, Bupha-Intr, & Wattanapermpool, 2013). SERCA activity was analyzed as the difference between the ATP consumption rate with and without thapsigargin treatment.

I graciously thank Ms.Olga N. Raguimova for training and helping me in the SERCA activity studies.

10.11 CaMKII Activity Assay

To confirm that alcohol promotes CaMKII activity, wt-CaMKII was overexpressed in HEK293 cells using an adenoviral vector before alcohol treatment (50 mM, 24 hrs) before harvest in cold PBS solution. JNK2 inhibitor (JNK2I, 170 nM) was used to pretreat cells before alcohol exposure to further explore whether

alcohol-induced CaMKII activation was JNK-dependent. To further assess whether alcohol alters CaMKII activity by directly oxidizing ROS-sensitive amino acid sites, point mutagenesis was performed in wt-CaMKII to substitute the ROS-sensing 280/281Met with Val (vv-CaMKII). vv-CaMKII was also overexpressed in HEK293 cells before alcohol treatment. Both wt-CaMKII and vv-CaMKII overexpressed in HEK293 cells carry HA tag. Overexpressed wt and vv-CaMKII was pulled-down with anti-HA tag antibody from the cell homogenates. wt-CaMKII and vv-CaMKII were IP-ed via incubating the cell homogenates with anti-HA antibodies (Abcam ab9110) and protein A/G-coated magnetic beads (Millipore EMD16-663). For all assays involving IP, protein pull down with IgG was used to serve as background control (IgG pull down).

IP-ed wt-CaMKII and vv-CaMKII were incubated with a reaction mixture containing: 1) 17.5μL kinase assay buffer (25 mM MOPS, 12.5 mM beta-glycerol-phosphate, 25 mM $MgCl_2$, 5 mM EGTA, 2 mM EDTA,0.25 mM DTT; pH 7.2); 2) 10 μL 10 mM ATP; 3) 5 μL 6 mM CaCl2; 4) 15 μL autocamtide2 (a CaMKII substrate with high specificity, Santa Cruz sc-3029, 1 mg/mL) according to Abcam protocol (http://www.abcam.com/recombinant-human-camkii-delta-protein-ab84552.html). A reaction with all the kinase reaction components but without adding protein A/G-coated magnetic beads was used as a negative control (buffer control). The reaction system was gently agitated during incubation (30 min) to keep the beads in suspension. At the end of the reaction, the reaction mixture

(supernatant) was collected for subsequent ADP measurement. ADP production was probed using ADP Glo™ assay kit (Promega) as a measurement for CaMKII activity. Equal volume of reaction mixture (35 µL) and ADP Glo reagent (35 µL) was mixed and incubated for 40 min in dark at room temperature. Then 2 x volumes (70 µL) of Kinase Detection Reagent was added into the mixture and incubated further for 30 min at room temperature. The final reaction was loaded into 96-well plate in dark and detected for luminescence (PhERAStar, LumPlus setting).

10.12 Recipe for Solutions

SR vesicle extraction Solution 1 (NaCl 0.9%, Tris-Maleate 10 mM)

RIPA buffer (Tris 25 mM, NaCl 150 mM, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, pH 8) with protease inhibitors (aprotinin 10 µg/mL, leupeptin 10 µg/mL, Pefabloc 1 mM)

Cardioplegic solution (NaCl 120.4 mM, Na_2HPO_4 0.6 mM, $NaHCO_3$ 4.6 mM, KCl 14.7 mM, KH_2PO_4 0.6 mM, $MgSO_4$ 1.2 mM, Hepes 10 mM, glucose 5.5 mM, pH 7.4)

Normal Hanks Salt solution (NaCl 136 mM, $NaHCO_3$ 4.16 mM, Hepes 5.04 mM, KCl 5.36 mM, KH_2PO4 0.44 mM, NaH_2PO4 0.4 mM, MgSO4 0.81 mM, $CaCl_2$ 1.26 mM, glucose 5.05 mM, pH 7.4)

Cardioplegic solution(NaCl 120.4 mM, Na_2HPO_4 0.6 mM, $NaHCO_3$ 4.6 mM, KCl 14.7 mM, KH_2PO_4 0.6 mM, $MgSO_4$ 1.2 mM, Hepes 10 mM, glucose 5.5 mM, pH 7.4)

Normal Tyrode's solution (NaCl 140 mM, KCl 4 mM, $MgCl_2$ 1 mM, HEPES 4 mM, $CaCl_2$ 1.8 mM, pH 7.4)

Ca^{2+}-free Tyrode's solution (NaCl 140 mM, KCl 4 mM, $MgCl_2$ 1 mM, HEPES 4 mM, $CaCl_2$ 1.8 mM, pH 7.4)

Na^{+}-free Ca^{2+}-free Tyrode's solution (LiCl 140 mM, KCl 4 mM, $MgCl_2$ 1 mM, HEPES 4 mM, EGTA 10 mM, pH 7.4)

Tissue lysis buffer (Tris·HCl 40 mM, NaCl 150 mM, β-glycerophosphate 5mM, NaF 10 mM, Na_3VO_4 0.2 mM and 1% protease inhibitor cocktail, pH 7.4)

JNK Kinase reaction buffer (Tris·HCl 40 mM, $MgCl_2$ 10 mM, β-glycerophosphate 5 mM, Na_3VO_4 0.2 mM, pH 7.4)

CaMKII Kinase assay buffer (MOPS 25 mM, beta-glycerol-phosphate 12.5 mM, $MgCl_2$ 25 mM, EGTA 5 mM, EDTA 2 mM, DTT 0.25 mM; pH 7.2)

10.13 Drug Treatments *in vivo* and *in vitro*

Treatment for HL-1 cells and isolated rabbit atrial myocytes:

Alcohol: 50 mM, 24 hrs, in sealed containers

Anisomycin: 0.2 μM, 24 hrs; EMD

JNK2I (JNK inhibitor IX; EMD): 170 nM, added 24 hrs prior to addition of anisomycin or alcohol in HL-1 cells, added 3 hrs prior to addition of anisomycin in rabbit atrial myocytes, anisomycin incubation continued after the adding of JNK2I; EMD

KN93: 0.2 μM, 24 hrs; EMD

KN92: 0.2 μM, 24 hrs; EMD

Caffeine: 10 mM; Sigma

Tetracaine: 1 mM; Sigma

Protocol for drug treatment for rabbit:

Rabbit model of repeated binge alcohol exposure: Young (6 months) New Zealand White male rabbits were infused with alcohol (2 g/kg BW, 12.67% v/v, I.V.; every other day for a total of four injections)

Rabbit model of JNK activation: Young New Zealand White male rabbits (6 months) were challenged with anisomycin (20 mg/kg BW, I.V., EMD; every other day for totally four injections)

Protocol for drug treatment for mice:

Mouse model of repeated binge alcohol exposure: Wild-type C57/Bj and Tg mice including JNK1/2dn mice, RyR-E4872Q$^{+/-}$ mice were treated with alcohol (2 g/kg BW, 12.67% v/v, I.P.) every other day for four doses. All mice used were 8-12 wks of age.

Repeated binge alcohol exposed-mice treated with KN93 or KN92: repeated binge alcohol exposed mice were treated with CaMKII inhibitor KN93 (7.2 mg/kg BW, I.P.) or its inactive analogue KN92 (7.2 mg/kg BW, I.P.) 12-16 hrs after the last dose of alcohol injection and 3 hrs prior to terminal studies.

MKK7D mice: MKK7D mice were injected with tamoxifen (1 mg/kg BW, I.P., 4 consecutive days) to induce the MKK7D overexpression (Andersson, et al., 2009).

10.14 Statistical Analysis

All data are presented as Mean ± SEM. In this entire thesis, One-way ANOVA with post-hoc Tukey test was used to compare the differences between the means of multiple groups; the difference between the means of two groups were analyzed using Student *t*-test. For data of non-parametric nature (including Ca^{2+} wave frequency and atrial arrhythmia inducibility), group comparison for the differences of mean are analyzed using the methods of: 1) Kruskal-Wallis test (One-way ANOVA on ranks) with post-hoc test when there are more than 2 groups to compare. 2) Mann-Whitney test (non-parametric Student t-test for unpaired groups) when there are two groups to compare. A $p < 0.05$ was considered to be significant for this entire study.

BIBLIOGRAPHY

Abrol, N., Smolin, N., Armanious, G., Ceholski, D. K., Trieber, C. A., Young, H. S., & Robia, S. L. (2014). Phospholamban C-terminal residues are critical determinants of the structure and function of the calcium ATPase regulatory complex. *J Biol Chem, 289*(37), 25855-25866

Agoglia, A. E., Holstein, S. E., Reid, G., & Hodge, C. W. (2015). CaMKIIalpha-GluA1 Activity Underlies Vulnerability to Adolescent Binge Alcohol Drinking. *Alcohol Clin Exp Res, 39*(9), 1680-1690

Ai, X. (2015). SR calcium handling dysfunction, stress-response signaling pathways, and atrial fibrillation. *Front Physiol, 6*, 46

Ai, X., Curran, J. W., Shannon, T. R., Bers, D. M., & Pogwizd, S. M. (2005). Ca2+/calmodulin-dependent protein kinase modulates cardiac ryanodine receptor phosphorylation and sarcoplasmic reticulum Ca2+ leak in heart failure. *Circ Res, 97*(12), 1314-1322

Ai, X., Jiang, A., Ke, Y., Solaro, R. J., & Pogwizd, S. M. (2011). Enhanced activation of p21-activated kinase 1 in heart failure contributes to dephosphorylation of connexin 43. *Cardiovascular research, 92*(1), 106-114

Ai, X., & Pogwizd, S. M. (2005). Connexin 43 downregulation and dephosphorylation in nonischemic heart failure is associated with enhanced colocalized protein phosphatase type 2A. *Circulation research, 96*(1), 54-63

Aistrup, G. L., Kelly, J. E., Piano, M. R., & Wasserstrom, J. A. (2006). Biphasic changes in cardiac excitation-contraction coupling early in chronic alcohol exposure. *American journal of physiology. Heart and circulatory physiology, 291*(3), H1047-1057

Aleong, R. G., Sauer, W. H., Davis, G., & Bristow, M. R. (2014). New-onset atrial fibrillation predicts heart failure progression. *Am J Med, 127*(10), 963-971

Almehmadi, F., Joncas, S. X., Nevis, I., Zahrani, M., Bokhari, M., Stirrat, J., . . . White, J. A. (2014). Prevalence of myocardial fibrosis patterns in patients with systolic dysfunction: prognostic significance for the prediction of sudden cardiac

arrest or appropriate implantable cardiac defibrillator therapy. *Circulation. Cardiovascular imaging, 7*(4), 593-600

Altura, M. B., Zhang, A., Cheng, T. P., & Altura, B. T. (1996). Exposure of piglet coronary arterial muscle cells to low alcohol results in elevation of intracellular free Ca2+: relevance to fetal alcohol syndrome. *Eur J Pharmacol, 314*(3), R9-11

Anadon, M. J., Almendral, J., Gonzalez, P., Zaballos, M., Delcan, J. L., & De Guevara, J. L. (1996). Alcohol concentration determines the type of atrial arrhythmia induced in a porcine model of acute alcoholic intoxication. *Pacing and clinical electrophysiology : PACE, 19*(11 Pt 2), 1962-1967

Anderson, M. E. (2011). Pathways for CaMKII activation in disease. *Heart Rhythm, 8*(9), 1501-1503

Anderson, M. E. (2015). Oxidant stress promotes disease by activating CaMKII. *J Mol Cell Cardiol, 89*(Pt B), 160-167

Anderson, M. E., Braun, A. P., Schulman, H., & Premack, B. A. (1994). Multifunctional Ca2+/calmodulin-dependent protein kinase mediates Ca(2+)-induced enhancement of the L-type Ca2+ current in rabbit ventricular myocytes. *Circ Res, 75*(5), 854-861

Andersson, K. B., Birkeland, J. A., Finsen, A. V., Louch, W. E., Sjaastad, I., Wang, Y., . . . Christensen, G. (2009). Moderate heart dysfunction in mice with inducible cardiomyocyte-specific excision of the Serca2 gene. *J Mol Cell Cardiol, 47*(2), 180-187

Angell, R. M., Atkinson, F. L., Brown, M. J., Chuang, T. T., Christopher, J. A., Cichy-Knight, M., . . . Wang, R. (2007). N-(3-Cyano-4,5,6,7-tetrahydro-1-benzothien-2-yl)amides as potent, selective, inhibitors of JNK2 and JNK3. *Bioorg Med Chem Lett, 17*(5), 1296-1301

Araujo Junior, R. F., Garcia, V. B., Leitao, R. F., Brito, G. A., Miguel Ede, C., Guedes, P. M., & de Araujo, A. A. (2016). Carvedilol Improves Inflammatory Response, Oxidative Stress and Fibrosis in the Alcohol-Induced Liver Injury in Rats by Regulating Kuppfer Cells and Hepatic Stellate Cells. *PLoS One, 11*(2), e0148868

Aronsen, J. M., Skogestad, J., Lewalle, A., Louch, W. E., Hougen, K., Stokke, M. K., . . . Sjaastad, I. (2015). Hypokalaemia induces Ca(2+) overload and Ca(2+) waves in ventricular myocytes by reducing Na(+),K(+)-ATPase alpha2 activity. *J Physiol, 593*(6), 1509-1521

Aroor, A. R., James, T. T., Jackson, D. E., & Shukla, S. D. (2010). Differential changes in MAP kinases, histone modifications, and liver injury in rats acutely treated with ethanol. *Alcoholism, clinical and experimental research, 34*(9), 1543-1551

Aroor, A. R., & Shukla, S. D. (2004). MAP kinase signaling in diverse effects of ethanol. *Life sciences, 74*(19), 2339-2364

Aryana, A., Saad, E. B., & d'Avila, A. (2012). Left atrial appendage occlusion and ligation devices: what is available, how to implement them, and how to manage and avoid complications. *Curr Treat Options Cardiovasc Med, 14*(5), 503-519

Bai, Y., Jones, P. P., Guo, J., Zhong, X., Clark, R. B., Zhou, Q., . . . Chen, S. R. (2013). Phospholamban knockout breaks arrhythmogenic Ca(2)(+) waves and suppresses catecholaminergic polymorphic ventricular tachycardia in mice. *Circ Res, 113*(5), 517-526

Barr, R. K., & Bogoyevitch, M. A. (2001). The c-Jun N-terminal protein kinase family of mitogen-activated protein kinases (JNK MAPKs). *Int J Biochem Cell Biol, 33*(11), 1047-1063

Bassani, J. W., Bassani, R. A., & Bers, D. M. (1994). Relaxation in rabbit and rat cardiac cells: species-dependent differences in cellular mechanisms. *J Physiol, 476*(2), 279-293

Bassani, R. A., Bassani, J. W., & Bers, D. M. (1994). Relaxation in ferret ventricular myocytes: unusual interplay among calcium transport systems. *J Physiol, 476*(2), 295-308

Beavers, D. L., Wang, W., Ather, S., Voigt, N., Garbino, A., Dixit, S. S., . . . Wehrens, X. H. (2013). Mutation E169K in junctophilin-2 causes atrial fibrillation due to impaired RyR2 stabilization. *J Am Coll Cardiol, 62*(21), 2010-2019

Beets, M. W., Flay, B. R., Vuchinich, S., Li, K. K., Acock, A., & Snyder, F. J. (2009). Longitudinal patterns of binge drinking among first year college students with a history of tobacco use. *Drug and alcohol dependence, 103*(1-2), 1-8

Behrendt, D., Beltrame, J., Hikiti, H., Wainstein, M., Kinlay, S., Selwyn, A. P., . . . Fang, J. C. (2006). Impact of coronary endothelial function on the progression of cardiac transplant-associated arteriosclerosis: effect of anti-oxidant vitamins C and E. *J Heart Lung Transplant, 25*(4), 426-433

Belardinelli, L., Giles, W. R., Rajamani, S., Karagueuzian, H. S., & Shryock, J. C. (2015). Cardiac late Na(+) current: proarrhythmic effects, roles in long QT

syndromes, and pathological relationship to CaMKII and oxidative stress. *Heart Rhythm, 12*(2), 440-448

Benjamin, E. J., Chen, P. S., Bild, D. E., Mascette, A. M., Albert, C. M., Alonso, A., . . . Wyse, D. G. (2009). Prevention of atrial fibrillation: report from a national heart, lung, and blood institute workshop. *Circulation, 119*(4), 606-618

Benjamin, E. J., Levy, D., Vaziri, S. M., D'Agostino, R. B., Belanger, A. J., & Wolf, P. A. (1994). Independent risk factors for atrial fibrillation in a population-based cohort. The Framingham Heart Study. *JAMA, 271*(11), 840-844

Benjamin, E. J., Wolf, P. A., D'Agostino, R. B., Silbershatz, H., Kannel, W. B., & Levy, D. (1998). Impact of atrial fibrillation on the risk of death: the Framingham Heart Study. *Circulation, 98*(10), 946-952

Bennett, B. L., Sasaki, D. T., Murray, B. W., O'Leary, E. C., Sakata, S. T., Xu, W., . . . Anderson, D. W. (2001). SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc Natl Acad Sci U S A, 98*(24), 13681-13686

Bers, D. M. (2000). Calcium fluxes involved in control of cardiac myocyte contraction. *Circulation research, 87*(4), 275-281

Bers, D. M. (2002). Cardiac excitation-contraction coupling. *Nature, 415*(6868), 198-205

Bers, D. M. (2014). Cardiac sarcoplasmic reticulum calcium leak: basis and roles in cardiac dysfunction. *Annual review of physiology, 76*, 107-127

Berul, C. I., Aronovitz, M. J., Wang, P. J., & Mendelsohn, M. E. (1996). In vivo cardiac electrophysiology studies in the mouse. *Circulation, 94*(10), 2641-2648

Bogoyevitch, M. A., Gillespie-Brown, J., Ketterman, A. J., Fuller, S. J., Ben-Levy, R., Ashworth, A., . . . Sugden, P. H. (1996). Stimulation of the stress-activated mitogen-activated protein kinase subfamilies in perfused heart. p38/RK mitogen-activated protein kinases and c-Jun N-terminal kinases are activated by ischemia/reperfusion. *Circ Res, 79*(2), 162-173

Bogoyevitch, M. A., & Kobe, B. (2006). Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases. *Microbiol Mol Biol Rev, 70*(4), 1061-1095

Bompotis, G. C., Deftereos, S., Angelidis, C., Choidis, E., Panagopoulou, V., Kaoukis, A., . . . Giannopoulos, G. (2015). Altered Calcium Handling in Reperfusion Injury. *Medicinal chemistry*

Bovo, E., Mazurek, S. R., de Tombe, P. P., & Zima, A. V. (2015). Increased Energy Demand during Adrenergic Receptor Stimulation Contributes to Ca(2+) Wave Generation. *Biophys J, 109*(8), 1583-1591

Boyden, P. A., Dun, W., & Stuyvers, B. D. (2015). What is a Ca(2+) wave? Is it like an Electrical Wave? *Arrhythm Electrophysiol Rev, 4*(1), 35-39

Brown, N. R., Noble, M. E., Endicott, J. A., & Johnson, L. N. (1999). The structural basis for specificity of substrate and recruitment peptides for cyclin-dependent kinases. *Nat Cell Biol, 1*(7), 438-443

Bryant, S. M., Kong, C. H., Watson, J., Cannell, M. B., James, A. F., & Orchard, C. H. (2015). Altered distribution of ICa impairs Ca release at the t-tubules of ventricular myocytes from failing hearts. *J Mol Cell Cardiol, 86*, 23-31

Burashnikov, A., & Antzelevitch, C. (2003). Reinduction of atrial fibrillation immediately after termination of the arrhythmia is mediated by late phase 3 early afterdepolarization-induced triggered activity. *Circulation, 107*(18), 2355-2360

Cabrales-Romero Mdel, P., Marquez-Rosado, L., Fattel-Fazenda, S., Trejo-Solis, C., Arce-Popoca, E., Aleman-Lazarini, L., & Villa-Trevino, S. (2006). S-adenosyl-methionine decreases ethanol-induced apoptosis in primary hepatocyte cultures by a c-Jun N-terminal kinase activity-independent mechanism. *World journal of gastroenterology, 12*(12), 1895-1904

Calkins, H., Kuck, K. H., Cappato, R., Brugada, J., Camm, A. J., Chen, S. A., . . . Wilber, D. (2012). 2012 HRS/EHRA/ECAS expert consensus statement on catheter and surgical ablation of atrial fibrillation: recommendations for patient selection, procedural techniques, patient management and follow-up, definitions, endpoints, and research trial design: a report of the Heart Rhythm Society (HRS) Task Force on Catheter and Surgical Ablation of Atrial Fibrillation. Developed in partnership with the European Heart Rhythm Association (EHRA), a registered branch of the European Society of Cardiology (ESC) and the European Cardiac Arrhythmia Society (ECAS); and in collaboration with the American College of Cardiology (ACC), American Heart Association (AHA), the Asia Pacific Heart Rhythm Society (APHRS), and the Society of Thoracic Surgeons (STS). Endorsed by the governing bodies of the American College of Cardiology Foundation, the American Heart Association, the European Cardiac Arrhythmia Society, the European Heart Rhythm Association, the Society of Thoracic Surgeons, the Asia Pacific Heart Rhythm Society, and the Heart Rhythm Society. *Heart Rhythm, 9*(4), 632-696 e621

Camors, E., & Valdivia, H. H. (2014). CaMKII regulation of cardiac ryanodine receptors and inositol triphosphate receptors. *Front Pharmacol, 5*, 101

Cao, S., Zeng, Z., Wang, X., Bin, J., Xu, D., & Liao, Y. (2013). Pravastatin slows the progression of heart failure by inhibiting the c-Jun N-terminal kinase-mediated intrinsic apoptotic signaling pathway. *Mol Med Rep, 8*(4), 1163-1168

Cao, Y., Tao, L., Shen, S., Xiao, J., Wu, H., Li, B., . . . Li, X. (2013). Cardiac ablation of Rheb1 induces impaired heart growth, endoplasmic reticulum-associated apoptosis and heart failure in infant mice. *Int J Mol Sci, 14*(12), 24380-24398

Cardin, S., Li, D., Thorin-Trescases, N., Leung, T. K., Thorin, E., & Nattel, S. (2003). Evolution of the atrial fibrillation substrate in experimental congestive heart failure: angiotensin-dependent and -independent pathways. *Cardiovasc Res, 60*(2), 315-325

Carson, P. E., Johnson, G. R., Dunkman, W. B., Fletcher, R. D., Farrell, L., & Cohn, J. N. (1993). The influence of atrial fibrillation on prognosis in mild to moderate heart failure. The V-HeFT Studies. The V-HeFT VA Cooperative Studies Group. *Circulation, 87*(6 Suppl), VI102-110

Casanova, E., Callejo, A. I., Calvo, P., & Chinchetru, M. A. (2000). Analysis of splicing of four mouse JNK/SAPKalpha variants. *Neuroreport, 11*(2), 305-309

Cederbaum, A. I., Lu, Y., & Wu, D. (2009). Role of oxidative stress in alcohol-induced liver injury. *Arch Toxicol, 83*(6), 519-548

Chelu, M. G., Sarma, S., Sood, S., Wang, S., van Oort, R. J., Skapura, D. G., . . . Wehrens, X. H. (2009). Calmodulin kinase II-mediated sarcoplasmic reticulum Ca2+ leak promotes atrial fibrillation in mice. *J Clin Invest, 119*(7), 1940-1951

Chen, B., Guo, A., Gao, Z., Wei, S., Xie, Y. P., Chen, S. R., . . . Song, L. S. (2012). In situ confocal imaging in intact heart reveals stress-induced Ca(2+) release variability in a murine catecholaminergic polymorphic ventricular tachycardia model of type 2 ryanodine receptor(R4496C+/-) mutation. *Circ Arrhythm Electrophysiol, 5*(4), 841-849

Chen, F., Cao, Y. G., Qi, H. P., Li, L., Huang, W., Wang, Y., & Sun, H. L. (2013). Involvement of cardiomyocyte apoptosis in myocardial injury of hereditary epileptic rats. *Can J Physiol Pharmacol, 91*(10), 804-811

Chen, G., Yang, X., Alber, S., Shusterman, V., & Salama, G. (2011). Regional genomic regulation of cardiac sodium-calcium exchanger by oestrogen. *J Physiol, 589*(Pt 5), 1061-1080

Chen, M., Wang, Y., & Qu, A. (2010). PGC-1 alpha accelerates cytosolic Ca2+ clearance without disturbing Ca2+ homeostasis in cardiac myocytes. *Biochem Biophys Res Commun, 396*(4), 894-900

Chen, P. S., Ogawa, M., Maruyama, M., Chua, S. K., Chang, P. C., Rubart-von der Lohe, M., . . . Lin, S. F. (2012). Imaging arrhythmogenic calcium signaling in intact hearts. *Pediatr Cardiol, 33*(6), 968-974

Chen, W., Aistrup, G., Wasserstrom, J. A., & Shiferaw, Y. (2011). A mathematical model of spontaneous calcium release in cardiac myocytes. *Am J Physiol Heart Circ Physiol, 300*(5), H1794-1805

Chen, W., Wang, R., Chen, B., Zhong, X., Kong, H., Bai, Y., . . . Chen, S. R. (2014). The ryanodine receptor store-sensing gate controls Ca2+ waves and Ca2+-triggered arrhythmias. *Nature medicine, 20*(2), 184-192

Chen, X., Guo, L., Kang, J., Huo, Y., Wang, S., & Tan, W. (2014). Calcium waves initiating from the anomalous subdiffusive calcium sparks. *J R Soc Interface, 11*(91), 20130934

Chen, Z., Sohal, M., Voigt, T., Sammut, E., Tobon-Gomez, C., Child, N., . . . Rinaldi, C. A. (2015). Myocardial tissue characterization by cardiac magnetic resonance imaging using T1 mapping predicts ventricular arrhythmia in ischemic and non-ischemic cardiomyopathy patients with implantable cardioverter-defibrillators. *Heart rhythm : the official journal of the Heart Rhythm Society, 12*(4), 792-801

Chenet, L., McKee, M., Leon, D., Shkolnikov, V., & Vassin, S. (1998). Alcohol and cardiovascular mortality in Moscow; new evidence of a causal association. *J Epidemiol Community Health, 52*(12), 772-774

Cheng, H., Lederer, M. R., Lederer, W. J., & Cannell, M. B. (1996). Calcium sparks and [Ca2+]i waves in cardiac myocytes. *The American journal of physiology, 270*(1 Pt 1), C148-159

Cheng, H. G., & Anthony, J. C. (2016). Does our legal minimum drinking age modulate risk of first heavy drinking episode soon after drinking onset? Epidemiological evidence for the United States, 2006-2014. *PeerJ, 4*, e2153

Cheng, J., Xu, L., Lai, D., Guilbert, A., Lim, H. J., Keskanokwong, T., & Wang, Y. (2012). CaMKII inhibition in heart failure, beneficial, harmful, or both. *Am J Physiol Heart Circ Physiol, 302*(7), H1454-1465

Choi, B. R., & Salama, G. (2000). Simultaneous maps of optical action potentials and calcium transients in guinea-pig hearts: mechanisms underlying concordant alternans. *J Physiol, 529 Pt 1*, 171-188

Choukroun, G., Hajjar, R., Fry, S., del Monte, F., Haq, S., Guerrero, J. L., . . . Force, T. (1999). Regulation of cardiac hypertrophy in vivo by the stress-activated protein kinases/c-Jun NH(2)-terminal kinases. *J Clin Invest, 104*(4), 391-398

Christ, T., Boknik, P., Wohrl, S., Wettwer, E., Graf, E. M., Bosch, R. F., . . . Dobrev, D. (2004). L-type Ca2+ current downregulation in chronic human atrial fibrillation is associated with increased activity of protein phosphatases. *Circulation, 110*(17), 2651-2657

Clark, D. M., Plumb, V. J., Epstein, A. E., & Kay, G. N. (1997). Hemodynamic effects of an irregular sequence of ventricular cycle lengths during atrial fibrillation. *Journal of the American College of Cardiology, 30*(4), 1039-1045

Clark, J. C. (1988). Sudden death in the chronic alcoholic. *Forensic Sci Int, 36*(1-2), 105-111

Claycomb, W. C., Lanson, N. A., Jr., Stallworth, B. S., Egeland, D. B., Delcarpio, J. B., Bahinski, A., & Izzo, N. J., Jr. (1998). HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proceedings of the National Academy of Sciences of the United States of America, 95*(6), 2979-2984

Cohen, E. D., Tian, Y., & Morrisey, E. E. (2008). Wnt signaling: an essential regulator of cardiovascular differentiation, morphogenesis and progenitor self-renewal. *Development, 135*(5), 789-798

Conen, D., Tedrow, U. B., Cook, N. R., Moorthy, M. V., Buring, J. E., & Albert, C. M. (2008). Alcohol consumption and risk of incident atrial fibrillation in women. *JAMA, 300*(21), 2489-2496

Cook, S. A., Sugden, P. H., & Clerk, A. (1999). Activation of c-Jun N-terminal kinases and p38-mitogen-activated protein kinases in human heart failure secondary to ischaemic heart disease. *Journal of molecular and cellular cardiology, 31*(8), 1429-1434

Corbin, W. R., Zalewski, S., Leeman, R. F., Toll, B. A., Fucito, L. M., & O'Malley, S. S. (2014). In with the old and out with the new? A comparison of the old and new binge drinking standards. *Alcoholism, clinical and experimental research, 38*(10), 2657-2663

Correll, R. N., Goonasekera, S. A., van Berlo, J. H., Burr, A. R., Accornero, F., Zhang, H., . . . Molkentin, J. D. (2015). STIM1 elevation in the heart results in aberrant Ca(2)(+) handling and cardiomyopathy. *J Mol Cell Cardiol, 87*, 38-47

Crawford, J. R., Haudek, S. B., Cieslik, K. A., Trial, J., & Entman, M. L. (2012). Origin of developmental precursors dictates the pathophysiologic role of cardiac fibroblasts. *J Cardiovasc Transl Res, 5*(6), 749-759

Curran, J., Brown, K. H., Santiago, D. J., Pogwizd, S., Bers, D. M., & Shannon, T. R. (2010). Spontaneous Ca waves in ventricular myocytes from failing hearts depend on Ca(2+)-calmodulin-dependent protein kinase II. *Journal of molecular and cellular cardiology, 49*(1), 25-32

Czubryt, M. P. (2012). Common threads in cardiac fibrosis, infarct scar formation, and wound healing. *Fibrogenesis Tissue Repair, 5*(1), 19

Davis, R. J. (2000). Signal transduction by the JNK group of MAP kinases. *Cell, 103*(2), 239-252

Denison, H., Jern, S., Jagenburg, R., Wendestam, C., & Wallerstedt, S. (1994). Influence of increased adrenergic activity and magnesium depletion on cardiac rhythm in alcohol withdrawal. *British heart journal, 72*(6), 554-560

DeSantiago, J., Maier, L. S., & Bers, D. M. (2004). Phospholamban is required for CaMKII-dependent recovery of Ca transients and SR Ca reuptake during acidosis in cardiac myocytes. *J Mol Cell Cardiol, 36*(1), 67-74

Dinu, D., Nechifor, M. T., & Movileanu, L. (2005). Ethanol-induced alterations of the antioxidant defense system in rat kidney. *J Biochem Mol Toxicol, 19*(6), 386-395

Dobrev, D., Friedrich, A., Voigt, N., Jost, N., Wettwer, E., Christ, T., . . . Ravens, U. (2005). The G protein-gated potassium current I(K,ACh) is constitutively active in patients with chronic atrial fibrillation. *Circulation, 112*(24), 3697-3706

Doleschal, B., Primessnig, U., Wolkart, G., Wolf, S., Schernthaner, M., Lichtenegger, M., . . . Groschner, K. (2015). TRPC3 contributes to regulation of cardiac contractility and arrhythmogenesis by dynamic interaction with NCX1. *Cardiovasc Res, 106*(1), 163-173

Domeier, T. L., Zima, A. V., Maxwell, J. T., Huke, S., Mignery, G. A., & Blatter, L. A. (2008). IP3 receptor-dependent Ca2+ release modulates excitation-contraction coupling in rabbit ventricular myocytes. *Am J Physiol Heart Circ Physiol, 294*(2), H596-604

Dougherty, C. J., Kubasiak, L. A., Prentice, H., Andreka, P., Bishopric, N. H., & Webster, K. A. (2002). Activation of c-Jun N-terminal kinase promotes survival of cardiac myocytes after oxidative stress. *Biochem J, 362*(Pt 3), 561-571

Dries, D. L., Exner, D. V., Gersh, B. J., Domanski, M. J., Waclawiw, M. A., & Stevenson, L. W. (1998). Atrial fibrillation is associated with an increased risk for mortality and heart failure progression in patients with asymptomatic and symptomatic left ventricular systolic dysfunction: a retrospective analysis of the SOLVD trials. Studies of Left Ventricular Dysfunction. *Journal of the American College of Cardiology, 32*(3), 695-703

Duan, J., McFadden, G. E., Borgerding, A. J., Norby, F. L., Ren, B. H., Ye, G., . . . Ren, J. (2002). Overexpression of alcohol dehydrogenase exacerbates ethanol-induced contractile defect in cardiac myocytes. *American journal of physiology. Heart and circulatory physiology, 282*(4), H1216-1222

Easton, A. C., Lucchesi, W., Mizuno, K., Fernandes, C., Schumann, G., Giese, K. P., & Muller, C. P. (2013). alphaCaMKII autophosphorylation controls the establishment of alcohol-induced conditioned place preference in mice. *Behav Brain Res, 252*, 72-76

Eisner, D., Bode, E., Venetucci, L., & Trafford, A. (2013). Calcium flux balance in the heart. *Journal of molecular and cellular cardiology, 58*, 110-117

Eisner, D. A., Kashimura, T., O'Neill, S. C., Venetucci, L. A., & Trafford, A. W. (2009). What role does modulation of the ryanodine receptor play in cardiac inotropy and arrhythmogenesis? *J Mol Cell Cardiol, 46*(4), 474-481

Eisner, D. A., Kashimura, T., Venetucci, L. A., & Trafford, A. W. (2009). From the ryanodine receptor to cardiac arrhythmias. *Circ J, 73*(9), 1561-1567

Eisner, D. A., & Lederer, W. J. (1980). The relationship between sodium pump activity and twitch tension in cardiac Purkinje fibres. *J Physiol, 303*, 475-494

Eisner, D. A., Lederer, W. J., & Vaughan-Jones, R. D. (1981). The dependence of sodium pumping and tension on intracellular sodium activity in voltage-clamped sheep Purkinje fibres. *J Physiol, 317*, 163-187

Eisner, D. A., & Trafford, A. W. (2000). No Role for the Ryanodine Receptor in Regulating Cardiac Contraction? *News Physiol Sci, 15*, 275-279

El-Ani, D., Philipchik, I., Stav, H., Levi, M., Zerbib, J., & Shainberg, A. (2014). Tumor necrosis factor alpha protects heart cultures against hypoxic damage via activation of PKA and phospholamban to prevent calcium overload. *Can J Physiol Pharmacol, 92*(11), 917-925

el-Mas, M. M., & Abdel-Rahman, A. A. (2012). Exacerbation of myocardial dysfunction and autonomic imbalance contributes to the estrogen-dependent chronic hypotensive effect of ethanol in female rats. *Eur J Pharmacol, 679*(1-3), 95-100

El-Mas, M. M., & Abdel-Rahman, A. A. (2014). Nongenomic effects of estrogen mediate the dose-related myocardial oxidative stress and dysfunction caused by acute ethanol in female rats. *Am J Physiol Endocrinol Metab, 306*(7), E740-747

El-Mas, M. M., & Abdel-Rahman, A. A. (2015). Estrogen modulation of the ethanol-evoked myocardial oxidative stress and dysfunction via DAPK3/Akt/ERK activation in male rats. *Toxicol Appl Pharmacol, 287*(3), 284-292

Engel, T. R., & Luck, J. C. (1983). Effect of whiskey on atrial vulnerability and "holiday heart". *Journal of the American College of Cardiology, 1*(3), 816-818

Erickson, J. R. (2014). Mechanisms of CaMKII Activation in the Heart. *Frontiers in pharmacology, 5*, 59

Erickson, J. R., & Anderson, M. E. (2008). CaMKII and its role in cardiac arrhythmia. *J Cardiovasc Electrophysiol, 19*(12), 1332-1336

Erickson, J. R., He, B. J., Grumbach, I. M., & Anderson, M. E. (2011). CaMKII in the cardiovascular system: sensing redox states. *Physiol Rev, 91*(3), 889-915

Erickson, J. R., Patel, R., Ferguson, A., Bossuyt, J., & Bers, D. M. (2011). Fluorescence resonance energy transfer-based sensor Camui provides new insight into mechanisms of calcium/calmodulin-dependent protein kinase II activation in intact cardiomyocytes. *Circulation research, 109*(7), 729-738

Esser, M. B., Hedden, S. L., Kanny, D., Brewer, R. D., Gfroerer, J. C., & Naimi, T. S. (2014). Prevalence of alcohol dependence among US adult drinkers, 2009-2011. *Preventing chronic disease, 11*, E206

Ettinger, P. O. (1984). Holiday heart arrhythmias. *International journal of cardiology, 5*(4), 540-542

Ettinger, P. O., Wu, C. F., De La Cruz, C., Jr., Weisse, A. B., Ahmed, S. S., & Regan, T. J. (1978). Arrhythmias and the "Holiday Heart": alcohol-associated cardiac rhythm disorders. *American heart journal, 95*(5), 555-562

Faccidomo, S., Reid, G. T., Agoglia, A. E., Ademola, S. A., & Hodge, C. W. (2016). CaMKII inhibition in the prefrontal cortex specifically increases the positive

reinforcing effects of sweetened alcohol in C57BL/6J mice. *Behav Brain Res, 298*(Pt B), 286-290

Farman, G. P., Tachampa, K., Mateja, R., Cazorla, O., Lacampagne, A., & de Tombe, P. P. (2008). Blebbistatin: use as inhibitor of muscle contraction. *Pflugers Archiv : European journal of physiology, 455*(6), 995-1005

Fedorov, V. V., Lozinsky, I. T., Sosunov, E. A., Anyukhovsky, E. P., Rosen, M. R., Balke, C. W., & Efimov, I. R. (2007). Application of blebbistatin as an excitation-contraction uncoupler for electrophysiologic study of rat and rabbit hearts. *Heart Rhythm, 4*(5), 619-626

Fentzke, R. C., Buck, S. H., Patel, J. R., Lin, H., Wolska, B. M., Stojanovic, M. O., . . . Leiden, J. M. (1999). Impaired cardiomyocyte relaxation and diastolic function in transgenic mice expressing slow skeletal troponin I in the heart. *J Physiol, 517 (Pt 1)*, 143-157

Ferrier, G. R. (1977). Digitalis arrhythmias: role of oscillatory afterpotentials. *Prog Cardiovasc Dis, 19*(6), 459-474

Fischer, T. H., Eiringhaus, J., Dybkova, N., Forster, A., Herting, J., Kleinwachter, A., . . . Sossalla, S. (2014). Ca(2+) /calmodulin-dependent protein kinase II equally induces sarcoplasmic reticulum Ca(2+) leak in human ischaemic and dilated cardiomyopathy. *European journal of heart failure, 16*(12), 1292-1300

Fischer, T. H., Herting, J., Mason, F. E., Hartmann, N., Watanabe, S., Nikolaev, V. O., . . . Sossalla, S. (2015). Late INa increases diastolic SR-Ca2+-leak in atrial myocardium by activating PKA and CaMKII. *Cardiovasc Res, 107*(1), 184-196

Foteinou, P. T., Greenstein, J. L., & Winslow, R. L. (2015). Mechanistic Investigation of the Arrhythmogenic Role of Oxidized CaMKII in the Heart. *Biophys J, 109*(4), 838-849

Franzini-Armstrong, C., Protasi, F., & Ramesh, V. (1999). Shape, size, and distribution of Ca(2+) release units and couplons in skeletal and cardiac muscles. *Biophys J, 77*(3), 1528-1539

Furberg, C. D., Psaty, B. M., Manolio, T. A., Gardin, J. M., Smith, V. E., & Rautaharju, P. M. (1994). Prevalence of atrial fibrillation in elderly subjects (the Cardiovascular Health Study). *Am J Cardiol, 74*(3), 236-241

Gao, L., Blair, L. A., & Marshall, J. (2006). CaMKII-independent effects of KN93 and its inactive analog KN92: reversible inhibition of L-type calcium channels. *Biochem Biophys Res Commun, 345*(4), 1606-1610

Gao, Y., Davies, S. P., Augustin, M., Woodward, A., Patel, U. A., Kovelman, R., & Harvey, K. J. (2013). A broad activity screen in support of a chemogenomic map for kinase signalling research and drug discovery. *Biochem J, 451*(2), 313-328

Gao, Y., Li, P., Ma, L. X., Du, K. X., Wang, X. H., Tang, M. J., . . . Hu, H. (2012). Effects of acute administration of ethanol on experimental arrhythmia. *Chin J Physiol, 55*(5), 307-313

Ge, W., Guo, R., & Ren, J. (2011). AMP-dependent kinase and autophagic flux are involved in aldehyde dehydrogenase-2-induced protection against cardiac toxicity of ethanol. *Free Radic Biol Med, 51*(9), 1736-1748

Go, A. S., Hylek, E. M., Phillips, K. A., Chang, Y., Henault, L. E., Selby, J. V., & Singer, D. E. (2001). Prevalence of diagnosed atrial fibrillation in adults: national implications for rhythm management and stroke prevention: the AnTicoagulation and Risk Factors in Atrial Fibrillation (ATRIA) Study. *JAMA, 285*(18), 2370-2375

Gonano, L. A., Sepulveda, M., Rico, Y., Kaetzel, M., Valverde, C. A., Dedman, J., . . . Vila Petroff, M. (2011). Calcium-calmodulin kinase II mediates digitalis-induced arrhythmias. *Circulation. Arrhythmia and electrophysiology, 4*(6), 947-957

Gonthier, B., Jeunet, A., & Barret, L. (1991). Electron spin resonance study of free radicals produced from ethanol and acetaldehyde after exposure to a Fenton system or to brain and liver microsomes. *Alcohol, 8*(5), 369-375

Gopi, V., Subramanian, V., Manivasagam, S., & Vellaichamy, E. (2015). Angiotensin II down-regulates natriuretic peptide receptor-A expression and guanylyl cyclase activity in H9c2 (2-1) cardiac myoblast cells: Role of ROS and NF-kappaB. *Mol Cell Biochem, 409*(1-2), 67-79

Goszcz, K., Deakin, S. J., Duthie, G. G., Stewart, D., Leslie, S. J., & Megson, I. L. (2015). Antioxidants in Cardiovascular Therapy: Panacea or False Hope? *Front Cardiovasc Med, 2*, 29

Greenstein, J. L., Foteinou, P. T., Hashambhoy-Ramsay, Y. L., & Winslow, R. L. (2014). Modeling CaMKII-mediated regulation of L-type Ca(2+) channels and ryanodine receptors in the heart. *Front Pharmacol, 5*, 60

Greiffenstein, P., Mathis, K. W., Stouwe, C. V., & Molina, P. E. (2007). Alcohol binge before trauma/hemorrhage impairs integrity of host defense mechanisms during recovery. *Alcohol Clin Exp Res, 31*(4), 704-715

Gu, L., Fink, A. M., Chowdhury, S. A., Geenen, D. L., & Piano, M. R. (2013). Cardiovascular responses and differential changes in mitogen-activated

protein kinases following repeated episodes of binge drinking. *Alcohol and alcoholism, 48*(2), 131-137

Guisoni, N., Ferrero, P., Layana, C., & Diambra, L. (2015). Abortive and propagating intracellular calcium waves: analysis from a hybrid model. *PLoS One, 10*(1), e0115187

Guo, R., & Ren, J. (2010). Alcohol dehydrogenase accentuates ethanol-induced myocardial dysfunction and mitochondrial damage in mice: role of mitochondrial death pathway. *PloS one, 5*(1), e8757

Guo, R., & Ren, J. (2012). Deficiency in AMPK attenuates ethanol-induced cardiac contractile dysfunction through inhibition of autophagosome formation. *Cardiovasc Res, 94*(3), 480-491

Guo, R., Xu, X., Babcock, S. A., Zhang, Y., & Ren, J. (2015). Aldehyde dedydrogenase-2 plays a beneficial role in ameliorating chronic alcohol-induced hepatic steatosis and inflammation through regulation of autophagy. *J Hepatol, 62*(3), 647-656

Guo, T., Gillespie, D., & Fill, M. (2012). Ryanodine receptor current amplitude controls Ca2+ sparks in cardiac muscle. *Circulation research, 111*(1), 28-36

Guo, W., Cao, S., Yan, B., Zhang, G., Li, J., Zhao, Y., & Zhang, S. (2016). Myocardial protective effects of a c-Jun N-terminal kinase inhibitor in rats with brain death. *J Cell Mol Med, 20*(7), 1214-1218

Guo, X., Yuan, S., Liu, Z., & Fang, Q. (2014). Oxidation- and CaMKII-Mediated Sarcoplasmic Reticulum Ca(2+) Leak Triggers Atrial Fibrillation in Aging. *Journal of cardiovascular electrophysiology, 25*(6), 645-652

Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., Derijard, B., & Davis, R. J. (1996). Selective interaction of JNK protein kinase isoforms with transcription factors. *EMBO J, 15*(11), 2760-2770

Guzzo-Merello, G., Dominguez, F., Gonzalez-Lopez, E., Cobo-Marcos, M., Gomez-Bueno, M., Fernandez-Lozano, I., . . . Garcia-Pavia, P. (2015). Malignant ventricular arrhythmias in alcoholic cardiomyopathy. *Int J Cardiol, 199*, 99-105

Haghighi, K., Bidwell, P., & Kranias, E. G. (2014). Phospholamban interactome in cardiac contractility and survival: A new vision of an old friend. *J Mol Cell Cardiol, 77*, 160-167

Haghighi, K., Pritchard, T. J., Liu, G. S., Singh, V. P., Bidwell, P., Lam, C. K., . . . Kranias, E. G. (2015). Human G109E-inhibitor-1 impairs cardiac function and promotes arrhythmias. *J Mol Cell Cardiol, 89*(Pt B), 349-359

Han, Z., Boyle, D. L., Chang, L., Bennett, B., Karin, M., Yang, L., . . . Firestein, G. S. (2001). c-Jun N-terminal kinase is required for metalloproteinase expression and joint destruction in inflammatory arthritis. *J Clin Invest, 108*(1), 73-81

Hansson, A., Madsen-Hardig, B., & Olsson, S. B. (2004). Arrhythmia-provoking factors and symptoms at the onset of paroxysmal atrial fibrillation: a study based on interviews with 100 patients seeking hospital assistance. *BMC cardiovascular disorders, 4*, 13

Haq, S., Choukroun, G., Lim, H., Tymitz, K. M., del Monte, F., Gwathmey, J., . . . Molkentin, J. D. (2001). Differential activation of signal transduction pathways in human hearts with hypertrophy versus advanced heart failure. *Circulation, 103*(5), 670-677

Hazzalin, C. A., Le Panse, R., Cano, E., & Mahadevan, L. C. (1998). Anisomycin selectively desensitizes signalling components involved in stress kinase activation and fos and jun induction. *Mol Cell Biol, 18*(4), 1844-1854

Heijman, J., Voigt, N., Nattel, S., & Dobrev, D. (2014). Cellular and molecular electrophysiology of atrial fibrillation initiation, maintenance, and progression. *Circulation research, 114*(9), 1483-1499

Heijman, J., Voigt, N., Wehrens, X. H., & Dobrev, D. (2014). Calcium dysregulation in atrial fibrillation: the role of CaMKII. *Frontiers in pharmacology, 5*, 30

Hell, J. W. (2014). CaMKII: claiming center stage in postsynaptic function and organization. *Neuron, 81*(2), 249-265

Hercberg, S., Galan, P., Preziosi, P., Bertrais, S., Mennen, L., Malvy, D., . . . Briancon, S. (2004). The SU.VI.MAX Study: a randomized, placebo-controlled trial of the health effects of antioxidant vitamins and minerals. *Arch Intern Med, 164*(21), 2335-2342

Hernandez, O. M., Szczesna-Cordary, D., Knollmann, B. C., Miller, T., Bell, M., Zhao, J., . . . Potter, J. D. (2005). F110I and R278C troponin T mutations that cause familial hypertrophic cardiomyopathy affect muscle contraction in transgenic mice and reconstituted human cardiac fibers. *J Biol Chem, 280*(44), 37183-37194

Hirt, L., Badaut, J., Thevenet, J., Granziera, C., Regli, L., Maurer, F., . . . Bogousslavsky, J. (2004). D-JNKI1, a cell-penetrating c-Jun-N-terminal kinase inhibitor,

protects against cell death in severe cerebral ischemia. *Stroke, 35*(7), 1738-1743

Hohendanner, F., Walther, S., Maxwell, J. T., Kettlewell, S., Awad, S., Smith, G. L., . . . Blatter, L. A. (2015). Inositol-1,4,5-trisphosphate induced Ca2+ release and excitation-contraction coupling in atrial myocytes from normal and failing hearts. *J Physiol, 593*(6), 1459-1477

Hommes, D., van den Blink, B., Plasse, T., Bartelsman, J., Xu, C., Macpherson, B., . . . Van Deventer, S. (2002). Inhibition of stress-activated MAP kinases induces clinical improvement in moderate to severe Crohn's disease. *Gastroenterology, 122*(1), 7-14

Houser, S. R. (2014). Role of RyR2 phosphorylation in heart failure and arrhythmias: protein kinase A-mediated hyperphosphorylation of the ryanodine receptor at serine 2808 does not alter cardiac contractility or cause heart failure and arrhythmias. *Circ Res, 114*(8), 1320-1327; discussion 1327

Hreniuk, D., Garay, M., Gaarde, W., Monia, B. P., McKay, R. A., & Cioffi, C. L. (2001). Inhibition of c-Jun N-terminal kinase 1, but not c-Jun N-terminal kinase 2, suppresses apoptosis induced by ischemia/reoxygenation in rat cardiac myocytes. *Mol Pharmacol, 59*(4), 867-874

Huke, S., & Knollmann, B. C. (2010). Increased myofilament Ca2+-sensitivity and arrhythmia susceptibility. *J Mol Cell Cardiol, 48*(5), 824-833

Hund, T. J., & Mohler, P. J. (2015). Role of CaMKII in cardiac arrhythmias. *Trends Cardiovasc Med, 25*(5), 392-397

Hunyady, L., & Catt, K. J. (2006). Pleiotropic AT1 receptor signaling pathways mediating physiological and pathogenic actions of angiotensin II. *Mol Endocrinol, 20*(5), 953-970

Inesi, G., Ebashi, S., & Watanabe, S. (1964). Preparation of Vesicular Relaxing Factor from Bovine Heart Tissue. *Am J Physiol, 207*, 1339-1344

Inesi, G., Sumbilla, C., & Kirtley, M. E. (1990). Relationships of molecular structure and function in Ca2(+)-transport ATPase. *Physiol Rev, 70*(3), 749-760

Jin, M., Ande, A., Kumar, A., & Kumar, S. (2013). Regulation of cytochrome P450 2e1 expression by ethanol: role of oxidative stress-mediated pkc/jnk/sp1 pathway. *Cell Death Dis, 4*, e554

Jones, S. A., & Lancaster, M. K. (2015). Progressive age-associated activation of JNK associates with conduction disruption in the aged atrium. *Mech Ageing Dev, 146-148*, 72-80

Judenherc-Haouzi, A., Zhang, X. Q., Sonobe, T., Song, J., Rannals, M. D., Wang, J., . . . Haouzi, P. (2016). Methylene blue counteracts H2S toxicity-induced cardiac depression by restoring L-type Ca channel activity. *Am J Physiol Regul Integr Comp Physiol, 310*(11), R1030-1044

Kamata, H., Honda, S., Maeda, S., Chang, L., Hirata, H., & Karin, M. (2005). Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell, 120*(5), 649-661

Kanagaratnam, P., Kojodjojo, P., & Peters, N. S. (2008). Electrophysiological abnormalities occur prior to the development of clinical episodes of atrial fibrillation: observations from human epicardial mapping. *Pacing Clin Electrophysiol, 31*(4), 443-453

Kandadi, M. R., Hu, N., & Ren, J. (2013). ULK1 plays a critical role in AMPK-mediated myocardial autophagy and contractile dysfunction following acute alcohol challenge. *Curr Pharm Des, 19*(27), 4874-4887

Kannel, W. B., Abbott, R. D., Savage, D. D., & McNamara, P. M. (1983). Coronary heart disease and atrial fibrillation: the Framingham Study. *Am Heart J, 106*(2), 389-396

Kass, R. S., Lederer, W. J., Tsien, R. W., & Weingart, R. (1978). Role of calcium ions in transient inward currents and aftercontractions induced by strophanthidin in cardiac Purkinje fibres. *J Physiol, 281*, 187-208

Kass, R. S., Tsien, R. W., & Weingart, R. (1978). Ionic basis of transient inward current induced by strophanthidin in cardiac Purkinje fibres. *J Physiol, 281*, 209-226

Khan, Y., & Pandy, V. (2016). Methanolic Extract of Morinda citrifolia L. (Noni) Unripe Fruit Attenuates Ethanol-Induced Conditioned Place Preferences in Mice. *Front Pharmacol, 7*, 352

Kim, H. K., Youm, J. B., Jeong, S. H., Lee, S. R., Song, I. S., Ko, T. H., . . . Han, J. (2015). Echinochrome A regulates phosphorylation of phospholamban Ser16 and Thr17 suppressing cardiac SERCA2A Ca(2)(+) reuptake. *Pflugers Arch, 467*(10), 2151-2163

Kim, J. H., Chae, M., Choi, A. R., Sik Kim, H., & Yoon, S. (2014). SP600125 overcomes antimitotic drug-resistance in cancer cells by increasing apoptosis with independence of P-gp inhibition. *Eur J Pharmacol, 723*, 141-147

Kimberly, W. T., Zheng, J. B., Town, T., Flavell, R. A., & Selkoe, D. J. (2005). Physiological regulation of the beta-amyloid precursor protein signaling domain by c-Jun N-terminal kinase JNK3 during neuronal differentiation. *J Neurosci, 25*(23), 5533-5543

Kneller, J., Zou, R., Vigmond, E. J., Wang, Z., Leon, L. J., & Nattel, S. (2002). Cholinergic atrial fibrillation in a computer model of a two-dimensional sheet of canine atrial cells with realistic ionic properties. *Circ Res, 90*(9), E73-87

Knockaert, L., Fromenty, B., & Robin, M. A. (2011). Mechanisms of mitochondrial targeting of cytochrome P450 2E1: physiopathological role in liver injury and obesity. *The FEBS journal, 278*(22), 4252-4260

Knollmann, B. C., Blatt, S. A., Horton, K., de Freitas, F., Miller, T., Bell, M., . . . Potter, J. D. (2001). Inotropic stimulation induces cardiac dysfunction in transgenic mice expressing a troponin T (I79N) mutation linked to familial hypertrophic cardiomyopathy. *J Biol Chem, 276*(13), 10039-10048

Knollmann, B. C., Kirchhof, P., Sirenko, S. G., Degen, H., Greene, A. E., Schober, T., . . . Morad, M. (2003). Familial hypertrophic cardiomyopathy-linked mutant troponin T causes stress-induced ventricular tachycardia and Ca2+-dependent action potential remodeling. *Circ Res, 92*(4), 428-436

Koch, P., Gehringer, M., & Laufer, S. A. (2015). Inhibitors of c-Jun N-terminal kinases: an update. *J Med Chem, 58*(1), 72-95

Kockskamper, J., Zima, A. V., Roderick, H. L., Pieske, B., Blatter, L. A., & Bootman, M. D. (2008). Emerging roles of inositol 1,4,5-trisphosphate signaling in cardiac myocytes. *J Mol Cell Cardiol, 45*(2), 128-147

Koskinen, P., Kupari, M., Leinonen, H., & Luomanmaki, K. (1987). Alcohol and new onset atrial fibrillation: a case-control study of a current series. *British heart journal, 57*(5), 468-473

Krahn, A. D., Manfreda, J., Tate, R. B., Mathewson, F. A., & Cuddy, T. E. (1995). The natural history of atrial fibrillation: incidence, risk factors, and prognosis in the Manitoba Follow-Up Study. *Am J Med, 98*(5), 476-484

Krishnamoorthy, S., Lip, G. Y., & Lane, D. A. (2009). Alcohol and illicit drug use as precipitants of atrial fibrillation in young adults: a case series and literature review. *The American journal of medicine, 122*(9), 851-856 e853

Kujime, K., Hashimoto, S., Gon, Y., Shimizu, K., & Horie, T. (2000). p38 mitogen-activated protein kinase and c-jun-NH2-terminal kinase regulate RANTES production by influenza virus-infected human bronchial epithelial cells. *J Immunol, 164*(6), 3222-3228

Kukielka, E., Dicker, E., & Cederbaum, A. I. (1994). Increased production of reactive oxygen species by rat liver mitochondria after chronic ethanol treatment. *Arch Biochem Biophys, 309*(2), 377-386

Kupferman, R., Mitra, P. P., Hohenberg, P. C., & Wang, S. S. (1997). Analytical calculation of intracellular calcium wave characteristics. *Biophys J, 72*(6), 2430-2444

Lang, C. H., Derdak, Z., & Wands, J. R. (2014). Strain-dependent differences for suppression of insulin-stimulated glucose uptake in skeletal and cardiac muscle by ethanol. *Alcoholism, clinical and experimental research, 38*(4), 897-910

Lang, D., Sulkin, M., Lou, Q., & Efimov, I. R. (2011). Optical mapping of action potentials and calcium transients in the mouse heart. *Journal of visualized experiments : JoVE*(55)

Larsson, S. C., Drca, N., & Wolk, A. (2014). Alcohol consumption and risk of atrial fibrillation: a prospective study and dose-response meta-analysis. *J Am Coll Cardiol, 64*(3), 281-289

Lederer, W. J., & Tsien, R. W. (1976). Transient inward current underlying arrhythmogenic effects of cardiotonic steroids in Purkinje fibres. *J Physiol, 263*(2), 73-100

Lee, Y. J., Aroor, A. R., & Shukla, S. D. (2002). Temporal activation of p42/44 mitogen-activated protein kinase and c-Jun N-terminal kinase by acetaldehyde in rat hepatocytes and its loss after chronic ethanol exposure. *The Journal of pharmacology and experimental therapeutics, 301*(3), 908-914

Lehnart, S. E., Mongillo, M., Bellinger, A., Lindegger, N., Chen, B. X., Hsueh, W., . . . Marks, A. R. (2008). Leaky Ca2+ release channel/ryanodine receptor 2 causes seizures and sudden cardiac death in mice. *J Clin Invest, 118*(6), 2230-2245

Lehnert, M., Relja, B., Sun-Young Lee, V., Schwestka, B., Henrich, D., Czerny, C., . . . Marzi, I. (2008). A peptide inhibitor of C-jun N-terminal kinase modulates hepatic damage and the inflammatory response after hemorrhagic shock and resuscitation. *Shock, 30*(2), 159-165

Lerman, B. B., Belardinelli, L., West, G. A., Berne, R. M., & DiMarco, J. P. (1986). Adenosine-sensitive ventricular tachycardia: evidence suggesting cyclic AMP-mediated triggered activity. *Circulation, 74*(2), 270-280

Levy, A. P., Friedenberg, P., Lotan, R., Ouyang, P., Tripputi, M., Higginson, L., . . . Howard, B. V. (2004). The effect of vitamin therapy on the progression of coronary artery atherosclerosis varies by haptoglobin type in postmenopausal women. *Diabetes Care, 27*(4), 925-930

Li, H., Jia, Z., Li, A., Jenkins, G., Yang, X., Hu, J., & Guo, W. (2013). Resveratrol repressed viability of U251 cells by miR-21 inhibiting of NF-kappaB pathway. *Mol Cell Biochem, 382*(1-2), 137-143

Li, J. Y., Huang, J. Y., Li, M., Zhang, H., Xing, B., Chen, G., . . . Hu, W. X. (2012). Anisomycin induces glioma cell death via down-regulation of PP2A catalytic subunit in vitro. *Acta Pharmacol Sin, 33*(7), 935-940

Li, N., Chiang, D. Y., Wang, S., Wang, Q., Sun, L., Voigt, N., . . . Wehrens, X. H. (2014). Ryanodine receptor-mediated calcium leak drives progressive development of an atrial fibrillation substrate in a transgenic mouse model. *Circulation, 129*(12), 1276-1285

Li, S. Y., Gilbert, S. A., Li, Q., & Ren, J. (2009). Aldehyde dehydrogenase-2 (ALDH2) ameliorates chronic alcohol ingestion-induced myocardial insulin resistance and endoplasmic reticulum stress. *Journal of molecular and cellular cardiology, 47*(2), 247-255

Li, T., Wei, X., Evans, C. F., Sanchez, P. G., Li, S., Wu, Z. J., & Griffith, B. P. (2016). Left Ventricular Unloading After Acute Myocardial Infarction Reduces MMP/JNK Associated Apoptosis and Promotes FAK Cell-Survival Signaling. *Ann Thorac Surg*

Li, X., Zima, A. V., Sheikh, F., Blatter, L. A., & Chen, J. (2005). Endothelin-1-induced arrhythmogenic Ca2+ signaling is abolished in atrial myocytes of inositol-1,4,5-trisphosphate(IP3)-receptor type 2-deficient mice. *Circ Res, 96*(12), 1274-1281

Liang, Q., Bueno, O. F., Wilkins, B. J., Kuan, C. Y., Xia, Y., & Molkentin, J. D. (2003). c-Jun N-terminal kinases (JNK) antagonize cardiac growth through cross-talk with calcineurin-NFAT signaling. *EMBO J, 22*(19), 5079-5089

Liang, Y., Mente, A., Yusuf, S., Gao, P., Sleight, P., Zhu, J., . . . Teo, K. K. (2012). Alcohol consumption and the risk of incident atrial fibrillation among people with cardiovascular disease. *CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne, 184*(16), E857-866

Liew, R., Khairunnisa, K., Gu, Y., Tee, N., Yin, N. O., Naylynn, T. M., & Moe, K. T. (2013). Role of tumor necrosis factor-alpha in the pathogenesis of atrial fibrosis and development of an arrhythmogenic substrate. *Circulation journal : official journal of the Japanese Circulation Society, 77*(5), 1171-1179

Lindegger, N., Hagen, B. M., Marks, A. R., Lederer, W. J., & Kass, R. S. (2009). Diastolic transient inward current in long QT syndrome type 3 is caused by Ca2+ overload and inhibited by ranolazine. *Journal of molecular and cellular cardiology, 47*(2), 326-334

Ling, H., Zhang, T., Pereira, L., Means, C. K., Cheng, H., Gu, Y., . . . Brown, J. H. (2009). Requirement for Ca2+/calmodulin-dependent kinase II in the transition from pressure overload-induced cardiac hypertrophy to heart failure in mice. *The Journal of clinical investigation, 119*(5), 1230-1240

Lip, G. Y. (2013). Stroke and bleeding risk assessment in atrial fibrillation: when, how, and why? *Eur Heart J, 34*(14), 1041-1049

Liu, B., Ho, H. T., Brunello, L., Unudurthi, S. D., Lou, Q., Belevych, A. E., . . . Gyorke, S. (2015). Ablation of HRC alleviates cardiac arrhythmia and improves abnormal Ca handling in CASQ2 knockout mice prone to CPVT. *Cardiovasc Res, 108*(2), 299-311

Liu, S. J. (2007). Inhibition of L-type Ca2+ channel current and negative inotropy induced by arachidonic acid in adult rat ventricular myocytes. *Am J Physiol Cell Physiol, 293*(5), C1594-1604

Liu, T., Chu, X., Wang, H., Zhang, X., Zhang, Y., Guo, H., . . . Zhang, J. (2016). Crocin, a carotenoid component of Crocus cativus, exerts inhibitory effects on L-type Ca(2+) current, Ca(2+) transient, and contractility in rat ventricular myocytes. *Can J Physiol Pharmacol, 94*(3), 302-308

Liu, T., & O'Rourke, B. (2013). Regulation of the Na+/Ca2+ exchanger by pyridine nucleotide redox potential in ventricular myocytes. *J Biol Chem, 288*(44), 31984-31992

Liu, W., Li, J., Tian, W., Xu, T., & Zhang, Z. (2011). Chronic alcohol consumption induces cardiac remodeling in mice from Th1 or Th2 background. *Exp Mol Pathol, 91*(3), 761-767

Lo, L. W., Chang, H. Y., Scherlag, B. J., Lin, Y. J., Chou, Y. H., Lin, W. L., . . . Po, S. S. (2016). Temporary Suppression of Cardiac Ganglionated Plexi Leads to Long-Term Suppression of Atrial Fibrillation: Evidence of Early Autonomic Intervention to Break the Vicious Cycle of "AF Begets AF". *J Am Heart Assoc, 5*(7)

Longnecker, M. P., & Tseng, M. (1998). Alcohol, hormones, and postmenopausal women. *Alcohol Health Res World, 22*(3), 185-189

Lowenstein, S. R., Gabow, P. A., Cramer, J., Oliva, P. B., & Ratner, K. (1983). The role of alcohol in new-onset atrial fibrillation. *Archives of internal medicine, 143*(10), 1882-1885

Lu, J. M., Wang, C. Y., Hu, C., Fang, Y. J., & Mei, Y. A. (2016). GDF-15 enhances intracellular Ca2+ by increasing Cav1.3 expression in rat cerebellar granule neurons. *Biochem J, 473*(13), 1895-1904

Luczak, E. D., & Anderson, M. E. (2014). CaMKII oxidative activation and the pathogenesis of cardiac disease. *J Mol Cell Cardiol, 73*, 112-116

Lukyanenko, V., Subramanian, S., Gyorke, I., Wiesner, T. F., & Gyorke, S. (1999). The role of luminal Ca2+ in the generation of Ca2+ waves in rat ventricular myocytes. *J Physiol, 518*(Pt 1), 173-186

Luo, X. J., Liu, B., Dai, Z., Li, T. B., Li, N. S., Zhang, X. J., . . . Peng, J. (2013). Expression of apoptosis-associated microRNAs in ethanol-induced acute gastric mucosal injury via JNK pathway. *Alcohol, 47*(6), 481-493

Luo, Y., Hargraves, R. H., Belle, A., Bai, O., Qi, X., Ward, K. R., . . . Najarian, K. (2013). A hierarchical method for removal of baseline drift from biomedical signals: application in ECG analysis. *ScientificWorldJournal, 2013*, 896056

Ma, H., Yu, L., Byra, E. A., Hu, N., Kitagawa, K., Nakayama, K. I., . . . Ren, J. (2010). Aldehyde dehydrogenase 2 knockout accentuates ethanol-induced cardiac depression: role of protein phosphatases. *J Mol Cell Cardiol, 49*(2), 322-329

Magnani, J. W., Rienstra, M., Lin, H., Sinner, M. F., Lubitz, S. A., McManus, D. D., . . . Benjamin, E. J. (2011). Atrial fibrillation: current knowledge and future directions in epidemiology and genomics. *Circulation, 124*(18), 1982-1993

Maier, L. S., Zhang, T., Chen, L., DeSantiago, J., Brown, J. H., & Bers, D. M. (2003). Transgenic CaMKIIdeltaC overexpression uniquely alters cardiac myocyte Ca2+ handling: reduced SR Ca2+ load and activated SR Ca2+ release. *Circulation research, 92*(8), 904-911

Makela, P., Paljarvi, T., & Poikolainen, K. (2005). Heavy and nonheavy drinking occasions, all-cause and cardiovascular mortality and hospitalizations: a follow-up study in a population with a low consumption level. *J Stud Alcohol, 66*(6), 722-728

Mandyam, M. C., Vedantham, V., Scheinman, M. M., Tseng, Z. H., Badhwar, N., Lee, B. K., . . . Marcus, G. M. (2012). Alcohol and vagal tone as triggers for paroxysmal atrial fibrillation. *The American journal of cardiology, 110*(3), 364-368

Mansouri, A., Demeilliers, C., Amsellem, S., Pessayre, D., & Fromenty, B. (2001). Acute ethanol administration oxidatively damages and depletes mitochondrial dna in mouse liver, brain, heart, and skeletal muscles: protective effects of antioxidants. *J Pharmacol Exp Ther, 298*(2), 737-743

Maroney, A. C., Glicksman, M. A., Basma, A. N., Walton, K. M., Knight, E., Jr., Murphy, C. A., . . . Dionne, C. A. (1998). Motoneuron apoptosis is blocked by CEP-1347 (KT 7515), a novel inhibitor of the JNK signaling pathway. *J Neurosci, 18*(1), 104-111

Martina, V., Masha, A., Gigliardi, V. R., Brocato, L., Manzato, E., Berchio, A., . . . Iannone, A. (2008). Long-term N-acetylcysteine and L-arginine administration reduces endothelial activation and systolic blood pressure in hypertensive patients with type 2 diabetes. *Diabetes Care, 31*(5), 940-944

Maruyama, M., Joung, B., Tang, L., Shinohara, T., On, Y. K., Han, S., . . . Chen, P. S. (2010). Diastolic intracellular calcium-membrane voltage coupling gain and postshock arrhythmias: role of purkinje fibers and triggered activity. *Circ Res, 106*(2), 399-408

Marx, S. O., Reiken, S., Hisamatsu, Y., Jayaraman, T., Burkhoff, D., Rosemblit, N., & Marks, A. R. (2000). PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. *Cell, 101*(4), 365-376

Maryniak, A., Walczak, F., Bodalski, R., Szumowski, L., Derejko, P., Urbanek, P., . . . Szufladowicz, E. (2006). Atrial fibrillation onset circumstances and their relation to patients' quality of life. *Kardiologia polska, 64*(10), 1102-1108; discussion 1109

Masamune, A., Kikuta, K., Satoh, M., Satoh, A., & Shimosegawa, T. (2002). Alcohol activates activator protein-1 and mitogen-activated protein kinases in rat pancreatic stellate cells. *The Journal of pharmacology and experimental therapeutics, 302*(1), 36-42

Mattiazzi, A., Argenziano, M., Aguilar-Sanchez, Y., Mazzocchi, G., & Escobar, A. L. (2015). Ca2+ Sparks and Ca2+ waves are the subcellular events underlying Ca2+ overload during ischemia and reperfusion in perfused intact hearts. *J Mol Cell Cardiol, 79*, 69-78

Mattiazzi, A., Bassani, R. A., Escobar, A. L., Palomeque, J., Valverde, C. A., Vila Petroff, M., & Bers, D. M. (2015). Chasing cardiac physiology and pathology down the CaMKII cascade. *Am J Physiol Heart Circ Physiol, 308*(10), H1177-1191

Mattiazzi, A., Mundina-Weilenmann, C., Vittone, L., & Said, M. (2004). Phosphorylation of phospholamban in ischemia-reperfusion injury: functional role of Thr17 residue. *Mol Cell Biochem, 263*(1-2), 131-136

Matyas, C., Varga, Z. V., Mukhopadhyay, P., Paloczi, J., Lajtos, T., Erdelyi, K., . . . Pacher, P. (2016). Chronic plus binge ethanol feeding induces myocardial oxidative stress, mitochondrial and cardiovascular dysfunction, and steatosis. *Am J Physiol Heart Circ Physiol, 310*(11), H1658-1670

McCarroll, J. A., Phillips, P. A., Park, S., Doherty, E., Pirola, R. C., Wilson, J. S., & Apte, M. V. (2003). Pancreatic stellate cell activation by ethanol and acetaldehyde: is it mediated by the mitogen-activated protein kinase signaling pathway? *Pancreas, 27*(2), 150-160

McMillen, B. A., Hillis, S. M., & Brown, J. M. (2009). College students' responses to a 5/4 drinking question and maximum blood alcohol concentration calculated from a timeline followback questionnaire. *J Stud Alcohol Drugs, 70*(4), 601-605

Meriin, A. B., Yaglom, J. A., Gabai, V. L., Zon, L., Ganiatsas, S., Mosser, D. D., & Sherman, M. Y. (1999). Protein-damaging stresses activate c-Jun N-terminal kinase via inhibition of its dephosphorylation: a novel pathway controlled by HSP72. *Molecular and cellular biology, 19*(4), 2547-2555

Mitka, M. (2009). College binge drinking still on the rise. *JAMA, 302*(8), 836-837

Miura, M., Taguchi, Y., Nagano, T., Sasaki, M., Handoh, T., & Shindoh, C. (2015). Effect of myofilament Ca(2+) sensitivity on Ca(2+) wave propagation in rat ventricular muscle. *J Mol Cell Cardiol, 84*, 162-169

Moradi, S., Nikoui, V., Imran Khan, M., Amiri, S., Jazaeri, F., & Bakhtiarian, A. (2016). Involvement of Inflammatory Cytokines in Antiarrhythmic Effects of Clofibrate in Ouabain-Induced Arrhythmia in Isolated Rat Atria. *Adv Pharmacol Sci, 2016*, 9128018

Morio, Y., Tsuji, M., Inagaki, M., Nakagawa, M., Asaka, Y., Oyamada, H., . . . Oguchi, K. (2013). Ethanol-induced apoptosis in human liver adenocarcinoma cells (SK-Hep1): Fas- and mitochondria-mediated pathways and interaction with MAPK signaling system. *Toxicology in vitro : an international journal published in association with BIBRA, 27*(6), 1820-1829

Mukamal, K. J., Tolstrup, J. S., Friberg, J., Jensen, G., & Gronbaek, M. (2005). Alcohol consumption and risk of atrial fibrillation in men and women: the Copenhagen City Heart Study. *Circulation, 112*(12), 1736-1742

Murphy, M. P. (2009). How mitochondria produce reactive oxygen species. *Biochem J, 417*(1), 1-13

Murray, R. P., Connett, J. E., Tyas, S. L., Bond, R., Ekuma, O., Silversides, C. K., & Barnes, G. E. (2002). Alcohol volume, drinking pattern, and cardiovascular disease morbidity and mortality: is there a U-shaped function? *Am J Epidemiol, 155*(3), 242-248

Nassal, M. M., Wan, X., Laurita, K. R., & Cutler, M. J. (2015). Atrial SERCA2a Overexpression Has No Affect on Cardiac Alternans but Promotes Arrhythmogenic SR Ca2+ Triggers. *PloS one, 10*(9), e0137359

. National Institute on Alcohol Abuse and Alcoholism Moderate & binge drinking. (2012). Rockville, MD: NIAAA.

Nattel, S., Burstein, B., & Dobrev, D. (2008). Atrial remodeling and atrial fibrillation: mechanisms and implications. *Circ Arrhythm Electrophysiol, 1*(1), 62-73

Nattel, S., Maguy, A., Le Bouter, S., & Yeh, Y. H. (2007). Arrhythmogenic ion-channel remodeling in the heart: heart failure, myocardial infarction, and atrial fibrillation. *Physiol Rev, 87*(2), 425-456

Neef, S., Dybkova, N., Sossalla, S., Ort, K. R., Fluschnik, N., Neumann, K., . . . Maier, L. S. (2010). CaMKII-dependent diastolic SR Ca2+ leak and elevated diastolic Ca2+ levels in right atrial myocardium of patients with atrial fibrillation. *Circulation research, 106*(6), 1134-1144

Nisbet, A. M., Camelliti, P., Walker, N. L., Burton, F. L., Cobbe, S. M., Kohl, P., & Smith, G. L. (2016). Prolongation of atrio-ventricular node conduction in a rabbit model of ischaemic cardiomyopathy: Role of fibrosis and connexin remodelling. *J Mol Cell Cardiol, 94*, 54-64

Nishio, S., Teshima, Y., Takahashi, N., Thuc, L. C., Saito, S., Fukui, A., . . . Saikawa, T. (2012). Activation of CaMKII as a key regulator of reactive oxygen species production in diabetic rat heart. *Journal of molecular and cellular cardiology, 52*(5), 1103-1111

Nishitani, Y., & Matsumoto, H. (2006). Ethanol rapidly causes activation of JNK associated with ER stress under inhibition of ADH. *FEBS letters, 580*(1), 9-14

Nuss, H. B., Kaab, S., Kass, D. A., Tomaselli, G. F., & Marban, E. (1999). Cellular basis of ventricular arrhythmias and abnormal automaticity in heart failure. *Am J Physiol, 277*(1 Pt 2), H80-91

O'Brien, F., Venturi, E., & Sitsapesan, R. (2015). The ryanodine receptor provides high throughput Ca2+-release but is precisely regulated by networks of associated proteins: a focus on proteins relevant to phosphorylation. *Biochem Soc Trans, 43*(3), 426-433

O'Keefe, J. H., Bhatti, S. K., Bajwa, A., DiNicolantonio, J. J., & Lavie, C. J. (2014). Alcohol and cardiovascular health: the dose makes the poison...or the remedy. *Mayo Clin Proc, 89*(3), 382-393

Oba, T., Ishikawa, T., Murayama, T., Ogawa, Y., & Yamaguchi, M. (2000). H(2)O(2) and ethanol act synergistically to gate ryanodine receptor/calcium-release channel. *American journal of physiology. Cell physiology, 279*(5), C1366-1374

Oba, T., Koshita, M., & Yamaguchi, M. (1997). Ethanol enhances caffeine-induced Ca2+-release channel activation in skeletal muscle sarcoplasmic reticulum. *Am J Physiol, 272*(2 Pt 1), C622-627

Odagiri, K., Katoh, H., Kawashima, H., Tanaka, T., Ohtani, H., Saotome, M., . . . Hayashi, H. (2009). Local control of mitochondrial membrane potential, permeability transition pore and reactive oxygen species by calcium and calmodulin in rat ventricular myocytes. *J Mol Cell Cardiol, 46*(6), 989-997

Odnoshivkina, U. G., Sytchev, V. I., Nurullin, L. F., Giniatullin, A. R., Zefirov, A. L., & Petrov, A. M. (2015). beta2-adrenoceptor agonist-evoked reactive oxygen species generation in mouse atria: implication in delayed inotropic effect. *Eur J Pharmacol, 765*, 140-153

Ohkubo, K., Watanabe, I., Okumura, Y., Takagi, Y., Ashino, S., Kofune, M., . . . Morimoto, S. (2010). Right ventricular histological substrate and conduction delay in patients with Brugada syndrome. *Int Heart J, 51*(1), 17-23

Ojeda, M. L., Barrero, M. J., Nogales, F., Murillo, M. L., & Carreras, O. (2012). Oxidative effects of chronic ethanol consumption on the functions of heart and kidney: folic acid supplementation. *Alcohol Alcohol, 47*(4), 404-412

Orabi, A. I., Shah, A. U., Muili, K., Luo, Y., Mahmood, S. M., Ahmad, A., . . . Husain, S. Z. (2011). Ethanol enhances carbachol-induced protease activation and accelerates Ca2+ waves in isolated rat pancreatic acini. *The Journal of biological chemistry, 286*(16), 14090-14097

Overvad, T. F., Rasmussen, L. H., Skjoth, F., Overvad, K., Albertsen, I. E., Lane, D. A., . . . Larsen, T. B. (2013). Alcohol intake and prognosis of atrial fibrillation. *Heart, 99*(15), 1093-1099

Pachon, R. E., Scharf, B. A., Vatner, D. E., & Vatner, S. F. (2015). Best Anesthetics for Assessing Left Ventricular Systolic Function by Echocardiography in Mice. *American journal of physiology. Heart and circulatory physiology*, ajpheart 00890 02014

Patrick, M. E., Terry-McElrath, Y. M., Kloska, D. D., & Schulenberg, J. E. (2016). High-Intensity Drinking Among Young Adults in the United States: Prevalence, Frequency, and Developmental Change. *Alcohol Clin Exp Res, 40*(9), 1905-1912

Payne, M. E., Fong, Y. L., Ono, T., Colbran, R. J., Kemp, B. E., Soderling, T. R., & Means, A. R. (1988). Calcium/calmodulin-dependent protein kinase II. Characterization of distinct calmodulin binding and inhibitory domains. *J Biol Chem, 263*(15), 7190-7195

Peart, J. N., Pepe, S., Reichelt, M. E., Beckett, N., See Hoe, L., Ozberk, V., . . . Headrick, J. P. (2014). Dysfunctional survival-signaling and stress-intolerance in aged murine and human myocardium. *Exp Gerontol, 50*, 72-81

Pellicena, P., & Schulman, H. (2014). CaMKII inhibitors: from research tools to therapeutic agents. *Front Pharmacol, 5*, 21

Peter, R. H., Gracey, J. G., & Beach, T. B. (1968). A clinical profile of idiopathic atrial fibrillation. A functional disorder of atrial rhythm. *Annals of internal medicine, 68*(6), 1288-1295

Petrich, B. G., Gong, X., Lerner, D. L., Wang, X., Brown, J. H., Saffitz, J. E., & Wang, Y. (2002). c-Jun N-terminal kinase activation mediates downregulation of connexin43 in cardiomyocytes. *Circ Res, 91*(7), 640-647

Picht, E., DeSantiago, J., Huke, S., Kaetzel, M. A., Dedman, J. R., & Bers, D. M. (2007). CaMKII inhibition targeted to the sarcoplasmic reticulum inhibits frequency-dependent acceleration of relaxation and Ca2+ current facilitation. *J Mol Cell Cardiol, 42*(1), 196-205

Ploia, C., Antoniou, X., Sclip, A., Grande, V., Cardinetti, D., Colombo, A., . . . Borsello, T. (2011). JNK plays a key role in tau hyperphosphorylation in Alzheimer's disease models. *J Alzheimers Dis, 26*(2), 315-329

Pluteanu, F., Hess, J., Plackic, J., Nikonova, Y., Preisenberger, J., Bukowska, A., . . . Kockskamper, J. (2015). Early subcellular Ca2+ remodelling and increased

propensity for Ca2+ alternans in left atrial myocytes from hypertensive rats. *Cardiovascular research, 106*(1), 87-97

Pogwizd, S. M., & Bers, D. M. (2004). Cellular basis of triggered arrhythmias in heart failure. *Trends Cardiovasc Med, 14*(2), 61-66

Pogwizd, S. M., & Corr, B. (1992). The contribution of nonreentrant mechanisms to malignant ventricular arrhythmias. *Basic Res Cardiol, 87 Suppl 2*, 115-129

Pogwizd, S. M., Hoyt, R. H., Saffitz, J. E., Corr, P. B., Cox, J. L., & Cain, M. E. (1992). Reentrant and focal mechanisms underlying ventricular tachycardia in the human heart. *Circulation, 86*(6), 1872-1887

Pohorecky, L. A. (1982). Influence of alcohol on peripheral neurotransmitter function. *Fed Proc, 41*(8), 2452-2455

Priori, S. G., & Chen, S. R. (2011). Inherited dysfunction of sarcoplasmic reticulum Ca2+ handling and arrhythmogenesis. *Circ Res, 108*(7), 871-883

Puglisi, J. L., Yuan, W., Bassani, J. W., & Bers, D. M. (1999). Ca(2+) influx through Ca(2+) channels in rabbit ventricular myocytes during action potential clamp: influence of temperature. *Circ Res, 85*(6), e7-e16

Purohit, A., Rokita, A. G., Guan, X., Chen, B., Koval, O. M., Voigt, N., . . . Anderson, M. E. (2013). Oxidized Ca(2+)/calmodulin-dependent protein kinase II triggers atrial fibrillation. *Circulation, 128*(16), 1748-1757

Qin, Y., Zhang, Z., Chen, J., Ding, X., Tong, S., & Song, Z. (2011). Ca(2)(+) disorder caused by rapid electrical field stimulation can be modulated by CaMKIIdelta expression in primary rat atrial myocytes. *Biochemical and biophysical research communications, 409*(2), 287-292

Qiu, B., Luczak, S. E., Wall, T. L., Kirchhoff, A. M., Xu, Y., Eng, M. Y., . . . Liang, T. (2016). The FKBP5 Gene Affects Alcohol Drinking in Knockout Mice and Is Implicated in Alcohol Drinking in Humans. *Int J Mol Sci, 17*(8)

Ren, J., Wold, L. E., Natavio, M., Ren, B. H., Hannigan, J. H., & Brown, R. A. (2002). Influence of prenatal alcohol exposure on myocardial contractile function in adult rat hearts: role of intracellular calcium and apoptosis. *Alcohol Alcohol, 37*(1), 30-37

Ren, Z., Wang, X., Xu, M., Yang, F., Frank, J. A., Ke, Z. J., & Luo, J. (2016). Binge ethanol exposure causes endoplasmic reticulum stress, oxidative stress and tissue injury in the pancreas. *Oncotarget*

Ren, Z., Yang, F., Wang, X., Wang, Y., Xu, M., Frank, J. A., . . . Luo, J. (2016). Chronic plus binge ethanol exposure causes more severe pancreatic injury and inflammation. *Toxicol Appl Pharmacol, 308*, 11-19

Respress, J. L., van Oort, R. J., Li, N., Rolim, N., Dixit, S. S., deAlmeida, A., . . . Wehrens, X. H. (2012). Role of RyR2 phosphorylation at S2814 during heart failure progression. *Circulation research, 110*(11), 1474-1483

Rich, E. C., Siebold, C., & Campion, B. (1985). Alcohol-related acute atrial fibrillation. A case-control study and review of 40 patients. *Archives of internal medicine, 145*(5), 830-833

Rich, M. W. (2009). Epidemiology of atrial fibrillation. *J Interv Card Electrophysiol, 25*(1), 3-8

Rintoul, G. L., & Baimbridge, K. G. (2003). Effects of calcium buffers and calbindin-D28k upon histamine-induced calcium oscillations and calcium waves in HeLa cells. *Cell Calcium, 34*(2), 131-144

Rodrigo, R., & Rivera, G. (2002). Renal damage mediated by oxidative stress: a hypothesis of protective effects of red wine. *Free Radic Biol Med, 33*(3), 409-422

Rokita, A. G., & Anderson, M. E. (2012). New therapeutic targets in cardiology: arrhythmias and Ca2+/calmodulin-dependent kinase II (CaMKII). *Circulation, 126*(17), 2125-2139

Rose, B. A., Force, T., & Wang, Y. (2010). Mitogen-activated protein kinase signaling in the heart: angels versus demons in a heart-breaking tale. *Physiol Rev, 90*(4), 1507-1546

Rosenkranz, S. (2004). TGF-beta1 and angiotensin networking in cardiac remodeling. *Cardiovasc Res, 63*(3), 423-432

Rubart, M., & Zipes, D. P. (2005). Mechanisms of sudden cardiac death. *The Journal of clinical investigation, 115*(9), 2305-2315

Rudiger, S., Jung, P., & Shuai, J. W. (2012). Termination of Ca(2)+ release for clustered IP(3)R channels. *PLoS Comput Biol, 8*(5), e1002485

Rushworth, G. F., & Megson, I. L. (2014). Existing and potential therapeutic uses for N-acetylcysteine: the need for conversion to intracellular glutathione for antioxidant benefits. *Pharmacol Ther, 141*(2), 150-159

Sag, C. M., Wadsack, D. P., Khabbazzadeh, S., Abesser, M., Grefe, C., Neumann, K., . . . Maier, L. S. (2009). Calcium/calmodulin-dependent protein kinase II contributes to cardiac arrhythmogenesis in heart failure. *Circulation. Heart failure, 2*(6), 664-675

Said, M., Becerra, R., Valverde, C. A., Kaetzel, M. A., Dedman, J. R., Mundina-Weilenmann, C., . . . Mattiazzi, A. (2011). Calcium-calmodulin dependent protein kinase II (CaMKII): a main signal responsible for early reperfusion arrhythmias. *Journal of molecular and cellular cardiology, 51*(6), 936-944

Salling, M. C., Faccidomo, S. P., Li, C., Psilos, K., Galunas, C., Spanos, M., . . . Hodge, C. W. (2016). Moderate Alcohol Drinking and the Amygdala Proteome: Identification and Validation of Calcium/Calmodulin Dependent Kinase II and AMPA Receptor Activity as Novel Molecular Mechanisms of the Positive Reinforcing Effects of Alcohol. *Biol Psychiatry, 79*(6), 430-442

Sano, F., Ohira, T., Kitamura, A., Imano, H., Cui, R., Kiyama, M., . . . Iso, H. (2014). Heavy alcohol consumption and risk of atrial fibrillation. The Circulatory Risk in Communities Study (CIRCS). *Circulation journal : official journal of the Japanese Circulation Society, 78*(4), 955-961

Santiago, D. J., Curran, J. W., Bers, D. M., Lederer, W. J., Stern, M. D., Rios, E., & Shannon, T. R. (2010). Ca sparks do not explain all ryanodine receptor-mediated SR Ca leak in mouse ventricular myocytes. *Biophysical journal, 98*(10), 2111-2120

Santos, B. A., Roman-Campos, D., Carvalho, M. S., Miranda, F. M., Carneiro, D. C., Cavalcante, P. H., . . . Gondim, A. N. (2011). Cardiodepressive effect elicited by the essential oil of Alpinia speciosa is related to L-type Ca(2)+ current blockade. *Phytomedicine, 18*(7), 539-543

Schattenberg, J. M., & Czaja, M. J. (2014). Regulation of the effects of CYP2E1-induced oxidative stress by JNK signaling. *Redox biology, 3*, 7-15

Schulman, H., & Greengard, P. (1978). Stimulation of brain membrane protein phosphorylation by calcium and an endogenous heat-stable protein. *Nature, 271*(5644), 478-479

Schulte, J. S., Fehrmann, E., Tekook, M. A., Kranick, D., Fels, B., Li, N., . . . Muller, F. U. (2016). Cardiac expression of the CREM repressor isoform CREM-IbDeltaC-X in mice leads to arrhythmogenic alterations in ventricular cardiomyocytes. *Basic Res Cardiol, 111*(2), 15

Sengul, C., Cevik, C., Ozveren, O., Sunbul, A., Oduncu, V., Akgun, T., . . . Dindar, I. (2011). Acute alcohol consumption is associated with increased interatrial electromechanical delay in healthy men. *Cardiol J, 18*(6), 682-686

Sesso, H. D., Buring, J. E., Christen, W. G., Kurth, T., Belanger, C., MacFadyen, J., . . . Gaziano, J. M. (2008). Vitamins E and C in the prevention of cardiovascular disease in men: the Physicians' Health Study II randomized controlled trial. *JAMA, 300*(18), 2123-2133

Shah, S. A., Yoon, G. H., & Kim, M. O. (2015). Protection of the developing brain with anthocyanins against ethanol-induced oxidative stress and neurodegeneration. *Molecular neurobiology, 51*(3), 1278-1291

Shaikh, S. A., Sahoo, S. K., & Periasamy, M. (2016). Phospholamban and sarcolipin: Are they functionally redundant or distinct regulators of the Sarco(Endo)Plasmic Reticulum Calcium ATPase? *J Mol Cell Cardiol, 91*, 81-91

Shannon, T. R., Ginsburg, K. S., & Bers, D. M. (2000). Reverse mode of the sarcoplasmic reticulum calcium pump and load-dependent cytosolic calcium decline in voltage-clamped cardiac ventricular myocytes. *Biophys J, 78*(1), 322-333

Shannon, T. R., Ginsburg, K. S., & Bers, D. M. (2002). Quantitative assessment of the SR Ca2+ leak-load relationship. *Circulation research, 91*(7), 594-600

Shannon, T. R., Pogwizd, S. M., & Bers, D. M. (2003). Elevated sarcoplasmic reticulum Ca2+ leak in intact ventricular myocytes from rabbits in heart failure. *Circulation research, 93*(7), 592-594

Shao, Z., Bhattacharya, K., Hsich, E., Park, L., Walters, B., Germann, U., . . . Kilter, H. (2006). c-Jun N-terminal kinases mediate reactivation of Akt and cardiomyocyte survival after hypoxic injury in vitro and in vivo. *Circ Res, 98*(1), 111-118

Sheehan, K. A., Ke, Y., Wolska, B. M., & Solaro, R. J. (2009). Expression of active p21-activated kinase-1 induces Ca2+ flux modification with altered regulatory protein phosphorylation in cardiac myocytes. *Am J Physiol Cell Physiol, 296*(1), C47-58

Shi, L., Xu, H., Wei, J., Ma, X., & Zhang, J. (2014). Caffeine induces cardiomyocyte hypertrophy via p300 and CaMKII pathways. *Chem Biol Interact, 221*, 35-41

Shryock, J. C., Song, Y., Rajamani, S., Antzelevitch, C., & Belardinelli, L. (2013). The arrhythmogenic consequences of increasing late INa in the cardiomyocyte. *Cardiovasc Res, 99*(4), 600-611

Singh, V. P., Rubinstein, J., Arvanitis, D. A., Ren, X., Gao, X., Haghighi, K., . . . Kranias, E. G. (2013). Abnormal calcium cycling and cardiac arrhythmias associated with the human Ser96Ala genetic variant of histidine-rich calcium-binding protein. *J Am Heart Assoc, 2*(5), e000460

Siow, Y. L., Choy, P. C., Leung, W. M., & O, K. (2000). Effect of Flos carthami on stress-activated protein kinase activity in the isolated reperfused rat heart. *Mol Cell Biochem, 207*(1-2), 41-47

Skelly, A., Hashimoto, R., Al-Khatib, S., Sanders-Schmidler, G., Fu, R., Brodt, E., & McDonagh, M. (2015). Catheter Ablation for Treatment of Atrial Fibrillation.

Soeller, C., Crossman, D., Gilbert, R., & Cannell, M. B. (2007). Analysis of ryanodine receptor clusters in rat and human cardiac myocytes. *Proc Natl Acad Sci U S A, 104*(38), 14958-14963

Sokolova, O. V. (2016). [The morphological changes in the myocardial tissue after sudden cardiac death from alcoholic cardiomyopathy]. *Sud Med Ekspert, 59*(1), 3-6

Sommese, L., Valverde, C. A., Blanco, P., Castro, M. C., Rueda, O. V., Kaetzel, M., . . . Palomeque, J. (2016). Ryanodine receptor phosphorylation by CaMKII promotes spontaneous Ca(2+) release events in a rodent model of early stage diabetes: The arrhythmogenic substrate. *Int J Cardiol, 202*, 394-406

Song, Y. H., Choi, E., Park, S. H., Lee, S. H., Cho, H., Ho, W. K., & Ryu, S. Y. (2011). Sustained CaMKII activity mediates transient oxidative stress-induced long-term facilitation of L-type Ca(2+) current in cardiomyocytes. *Free Radic Biol Med, 51*(9), 1708-1716

Sossalla, S., Fluschnik, N., Schotola, H., Ort, K. R., Neef, S., Schulte, T., . . . Maier, L. S. (2010). Inhibition of elevated Ca2+/calmodulin-dependent protein kinase II improves contractility in human failing myocardium. *Circulation research, 107*(9), 1150-1161

Souza-Smith, F. M., Kerut, E. K., Breslin, J. W., & Molina, P. E. (2015). Mechanisms of Acute Alcohol Intoxication-Induced Modulation of Cyclic Mobilization of [Ca(2)(+)] in Rat Mesenteric Lymphatic Vessels. *Lymphat Res Biol, 13*(2), 93-99

Stammers, A. N., Susser, S. E., Hamm, N. C., Hlynsky, M. W., Kimber, D. E., Kehler, D. S., & Duhamel, T. A. (2015). The regulation of sarco(endo)plasmic reticulum calcium-ATPases (SERCA). *Canadian journal of physiology and pharmacology, 93*(10), 843-854

Stavermann, M., Meuth, P., Doengi, M., Thyssen, A., Deitmer, J. W., & Lohr, C. (2015). Calcium-induced calcium release and gap junctions mediate large-scale calcium waves in olfactory ensheathing cells in situ. *Cell Calcium, 58*(2), 215-225

Steiner, J. L., Pruznak, A. M., Navaratnarajah, M., & Lang, C. H. (2015). Alcohol Differentially Alters Extracellular Matrix and Adhesion Molecule Expression in Skeletal Muscle and Heart. *Alcohol Clin Exp Res, 39*(8), 1330-1340

Stevenson, W. G., Weiss, J. N., Wiener, I., & Nademanee, K. (1989). Slow conduction in the infarct scar: relevance to the occurrence, detection, and ablation of ventricular reentry circuits resulting from myocardial infarction. *Am Heart J, 117*(2), 452-467

Stokke, M. K., Briston, S. J., Jolle, G. F., Manzoor, I., Louch, W. E., Oyehaug, L., . . . Sjaastad, I. (2011). Ca(2+) wave probability is determined by the balance between SERCA2-dependent Ca(2+) reuptake and threshold SR Ca(2+) content. *Cardiovasc Res, 90*(3), 503-512

Stokke, M. K., Tovsrud, N., Louch, W. E., Oyehaug, L., Hougen, K., Sejersted, O. M., . . . Sjaastad, I. (2013). I(CaL) inhibition prevents arrhythmogenic Ca(2+) waves caused by abnormal Ca(2+) sensitivity of RyR or SR Ca(2+) accumulation. *Cardiovasc Res, 98*(2), 315-325

Suckfuell, M., Lisowska, G., Domka, W., Kabacinska, A., Morawski, K., Bodlaj, R., . . . Meyer, T. (2014). Efficacy and safety of AM-111 in the treatment of acute sensorineural hearing loss: a double-blind, randomized, placebo-controlled phase II study. *Otol Neurotol, 35*(8), 1317-1326

Sugai, Y., Miura, M., Hirose, M., Wakayama, Y., Endoh, H., Nishio, T., . . . Shimokawa, H. (2009). Contribution of Na+/Ca2+ exchange current to the formation of delayed afterdepolarizations in intact rat ventricular muscle. *J Cardiovasc Pharmacol, 53*(6), 517-522

Tachibana, H., Perrino, C., Takaoka, H., Davis, R. J., Naga Prasad, S. V., & Rockman, H. A. (2006). JNK1 is required to preserve cardiac function in the early response to pressure overload. *Biochem Biophys Res Commun, 343*(4), 1060-1066

Takahashi, N., Imai, S., Saito, F., Suzuki, K., Tanaka, H., Kushiro, T., . . . Hirayama, A. (2008). Alcohol produces imbalance of adrenal and neuronal sympathetic activity in patients with alcohol-induced neurocardiogenic syncope. *Circ J, 72*(6), 979-985

Thomas, R. C., Singh, A., Cowley, P., Myagmar, B. E., Montgomery, M. D., Swigart, P. M., . . . Simpson, P. C. (2016). A Myocardial Slice Culture Model Reveals Alpha-1A-

Adrenergic Receptor Signaling in the Human Heart. *JACC Basic Transl Sci, 1*(3), 155-167

Thornton, J. R. (1984). Atrial fibrillation in healthy non-alcoholic people after an alcoholic binge. *Lancet, 2*(8410), 1013-1015

Tonelo, D., Providencia, R., & Goncalves, L. (2013). Holiday heart syndrome revisited after 34 years. *Arquivos brasileiros de cardiologia, 101*(2), 183-189

Touze, E., & Ciocanu, D. (2014). [Stroke in atrial fibrillation patients already on oral anticoagulant: What more can be done?]. *Presse Med, 43*(7-8), 784-788

Trafford, A. W., Diaz, M. E., & Eisner, D. A. (2001). Coordinated control of cell Ca(2+) loading and triggered release from the sarcoplasmic reticulum underlies the rapid inotropic response to increased L-type Ca(2+) current. *Circ Res, 88*(2), 195-201

Trejbal, K., & Mitro, P. (2008). [ECG changes in alcoholic intoxication]. *Vnitr Lek, 54*(4), 410-414

Trial Identifier NCT0120394. *ClinicalTrials.gov*

Tsui, J., Inagaki, M., & Schulman, H. (2005). Calcium/calmodulin-dependent protein kinase II (CaMKII) localization acts in concert with substrate targeting to create spatial restriction for phosphorylation. *J Biol Chem, 280*(10), 9210-9216

Umoh, N. A., Walker, R. K., Al-Rubaiee, M., Jeffress, M. A., & Haddad, G. E. (2014a). Acute alcohol modulates cardiac function as PI3K/Akt regulates oxidative stress. *Alcoholism, clinical and experimental research, 38*(7), 1847-1864

Umoh, N. A., Walker, R. K., Al-Rubaiee, M., Jeffress, M. A., & Haddad, G. E. (2014b). Acute alcohol modulates cardiac function as PI3K/Akt regulates oxidative stress. *Alcohol Clin Exp Res, 38*(7), 1847-1864

van der Velden, H. M., Ausma, J., Rook, M. B., Hellemons, A. J., van Veen, T. A., Allessie, M. A., & Jongsma, H. J. (2000). Gap junctional remodeling in relation to stabilization of atrial fibrillation in the goat. *Cardiovasc Res, 46*(3), 476-486

van Oort, R. J., Brown, J. H., & Westenbrink, B. D. (2014). CaMKII confirms its promise in ischaemic heart disease. *Eur J Heart Fail, 16*(12), 1268-1269

Vasdev, S. C., Chakravarti, R. N., Subrahmanyam, D., Jain, A. C., & Wahi, P. L. (1975). Myocardial lesions induced by prolonged alcohol feeding in rhesus monkeys. *Cardiovasc Res, 9*(1), 134-140

Venetucci, L. A., Trafford, A. W., & Eisner, D. A. (2007). Increasing ryanodine receptor open probability alone does not produce arrhythmogenic calcium waves: threshold sarcoplasmic reticulum calcium content is required. *Circulation research, 100*(1), 105-111

Venetucci, L. A., Trafford, A. W., O'Neill, S. C., & Eisner, D. A. (2008). The sarcoplasmic reticulum and arrhythmogenic calcium release. *Cardiovascular research, 77*(2), 285-292

Vital signs: binge drinking among women and high school girls--United States, 2011. (2013). *MMWR Morb Mortal Wkly Rep, 62*(1), 9-13

Vital signs: binge drinking prevalence, frequency, and intensity among adults-U.S., 2010. (2012). *MMWR Morb Mortal Wkly Rep, 61*(1), 14-19

Voigt, N., Friedrich, A., Bock, M., Wettwer, E., Christ, T., Knaut, M., . . . Dobrev, D. (2007). Differential phosphorylation-dependent regulation of constitutively active and muscarinic receptor-activated IK,ACh channels in patients with chronic atrial fibrillation. *Cardiovasc Res, 74*(3), 426-437

Voigt, N., Heijman, J., Wang, Q., Chiang, D. Y., Li, N., Karck, M., . . . Dobrev, D. (2014). Cellular and molecular mechanisms of atrial arrhythmogenesis in patients with paroxysmal atrial fibrillation. *Circulation, 129*(2), 145-156

Wagner, A. C., Mazzucchelli, L., Miller, M., Camoratto, A. M., & Goke, B. (2000). CEP-1347 inhibits caerulein-induced rat pancreatic JNK activation and ameliorates caerulein pancreatitis. *Am J Physiol Gastrointest Liver Physiol, 278*(1), G165-172

Wagner, S., Dybkova, N., Rasenack, E. C., Jacobshagen, C., Fabritz, L., Kirchhof, P., . . . Maier, L. S. (2006). Ca2+/calmodulin-dependent protein kinase II regulates cardiac Na+ channels. *J Clin Invest, 116*(12), 3127-3138

Wagner, S., Ruff, H. M., Weber, S. L., Bellmann, S., Sowa, T., Schulte, T., . . . Maier, L. S. (2011). Reactive oxygen species-activated Ca/calmodulin kinase IIdelta is required for late I(Na) augmentation leading to cellular Na and Ca overload. *Circ Res, 108*(5), 555-565

Walden, A. P., Dibb, K. M., & Trafford, A. W. (2009). Differences in intracellular calcium homeostasis between atrial and ventricular myocytes. *J Mol Cell Cardiol, 46*(4), 463-473

Wang, Y., Su, B., Sah, V. P., Brown, J. H., Han, J., & Chien, K. R. (1998). Cardiac hypertrophy induced by mitogen-activated protein kinase kinase 7, a specific

activator for c-Jun NH2-terminal kinase in ventricular muscle cells. *The Journal of biological chemistry, 273*(10), 5423-5426

Wannamethee, G., & Shaper, A. G. (1992). Alcohol and sudden cardiac death. *British heart journal, 68*(5), 443-448

Warren, G. B., Toon, P. A., Birdsall, N. J., Lee, A. G., & Metcalfe, J. C. (1974). Reconstitution of a calcium pump using defined membrane components. *Proc Natl Acad Sci U S A, 71*(3), 622-626

Waters, D. D., Alderman, E. L., Hsia, J., Howard, B. V., Cobb, F. R., Rogers, W. J., . . . Verter, J. I. (2002). Effects of hormone replacement therapy and antioxidant vitamin supplements on coronary atherosclerosis in postmenopausal women: a randomized controlled trial. *JAMA, 288*(19), 2432-2440

Watson, T., Shantsila, E., & Lip, G. Y. (2009). Mechanisms of thrombogenesis in atrial fibrillation: Virchow's triad revisited. *Lancet, 373*(9658), 155-166

Wattigney, W. A., Mensah, G. A., & Croft, J. B. (2003). Increasing trends in hospitalization for atrial fibrillation in the United States, 1985 through 1999: implications for primary prevention. *Circulation, 108*(6), 711-716

Wei, J., Wang, W., Chopra, I., Li, H. F., Dougherty, C. J., Adi, J., . . . Webster, K. A. (2011). c-Jun N-terminal kinase (JNK-1) confers protection against brief but not extended ischemia during acute myocardial infarction. *J Biol Chem, 286*(16), 13995-14006

White, J., Lee, J. A., Shah, N., & Orchard, C. H. (1993). Differential effects of the optical isomers of EMD 53998 on contraction and cytoplasmic Ca2+ in isolated ferret cardiac muscle. *Circ Res, 73*(1), 61-70

White, S. M., Constantin, P. E., & Claycomb, W. C. (2004). Cardiac physiology at the cellular level: use of cultured HL-1 cardiomyocytes for studies of cardiac muscle cell structure and function. *American journal of physiology. Heart and circulatory physiology, 286*(3), H823-829

Wieder, N., Fink, R., & von Wegner, F. (2015). Exact stochastic simulation of a calcium microdomain reveals the impact of Ca(2)(+) fluctuations on IP(3)R gating. *Biophys J, 108*(3), 557-567

Wijffels, M. C., Kirchhof, C. J., Dorland, R., & Allessie, M. A. (1995). Atrial fibrillation begets atrial fibrillation. A study in awake chronically instrumented goats. *Circulation, 92*(7), 1954-1968

Witayavanitkul, N., Woranush, W., Bupha-Intr, T., & Wattanapermpool, J. (2013). Testosterone regulates cardiac contractile activation by modulating SERCA but not NCX activity. *American journal of physiology. Heart and circulatory physiology, 304*(3), H465-472

Wolf, P. A., Dawber, T. R., Thomas, H. E., Jr., & Kannel, W. B. (1978). Epidemiologic assessment of chronic atrial fibrillation and risk of stroke: the Framingham study. *Neurology, 28*(10), 973-977

Wolff, M. R., Buck, S. H., Stoker, S. W., Greaser, M. L., & Mentzer, R. M. (1996). Myofibrillar calcium sensitivity of isometric tension is increased in human dilated cardiomyopathies: role of altered beta-adrenergically mediated protein phosphorylation. *J Clin Invest, 98*(1), 167-176

Wu, Y., Roden, D. M., & Anderson, M. E. (1999). Calmodulin kinase inhibition prevents development of the arrhythmogenic transient inward current. *Circ Res, 84*(8), 906-912

Xiao, H. D., Fuchs, S., Campbell, D. J., Lewis, W., Dudley, S. C., Jr., Kasi, V. S., . . . Bernstein, K. E. (2004). Mice with cardiac-restricted angiotensin-converting enzyme (ACE) have atrial enlargement, cardiac arrhythmia, and sudden death. *The American journal of pathology, 165*(3), 1019-1032

Xiao, R. P., Cheng, H., Lederer, W. J., Suzuki, T., & Lakatta, E. G. (1994). Dual regulation of Ca2+/calmodulin-dependent kinase II activity by membrane voltage and by calcium influx. *Proc Natl Acad Sci U S A, 91*(20), 9659-9663

Xie, W., Santulli, G., Guo, X., Gao, M., Chen, B. X., & Marks, A. R. (2013). Imaging atrial arrhythmic intracellular calcium in intact heart. *J Mol Cell Cardiol, 64*, 120-123

Xu, D., Murakoshi, N., Igarashi, M., Hirayama, A., Ito, Y., Seo, Y., . . . Aonuma, K. (2012). PPAR-gamma activator pioglitazone prevents age-related atrial fibrillation susceptibility by improving antioxidant capacity and reducing apoptosis in a rat model. *J Cardiovasc Electrophysiol, 23*(2), 209-217

Xu, L., Lai, D., Cheng, J., Lim, H. J., Keskanokwong, T., Backs, J., . . . Wang, Y. (2010). Alterations of L-type calcium current and cardiac function in CaMKII{delta} knockout mice. *Circ Res, 107*(3), 398-407

Yan, J., Kong, W., Zhang, Q., Beyer, E. C., Walcott, G., Fast, V. G., & Ai, X. (2013). c-Jun N-terminal kinase activation contributes to reduced connexin43 and development of atrial arrhythmias. *Cardiovascular research, 97*(3), 589-597

Yan, J., Thomson, J. K., Zhao, W., Fast, V. G., Ye, T., & Ai, X. (2015). Voltage and Calcium Dual Channel Optical Mapping of Cultured HL-1 Atrial Myocyte Monolayer. *Journal of visualized experiments : JoVE*(97)

Yang, J. Y., Moulin, N., van Bemmelen, M. X., Dubuis, G., Tawadros, T., Haefliger, J. A., . . . Widmann, C. (2007). Splice variant-specific stabilization of JNKs by IB1/JIP1. *Cell Signal, 19*(10), 2201-2207

Ye, Y., Jian, K., Jaggar, J. H., Bukiya, A. N., & Dopico, A. M. (2014). Type 2 ryanodine receptors are highly sensitive to alcohol. *FEBS Lett, 588*(9), 1659-1665

Yeh, Y. H., Wakili, R., Qi, X. Y., Chartier, D., Boknik, P., Kaab, S., . . . Nattel, S. (2008). Calcium-handling abnormalities underlying atrial arrhythmogenesis and contractile dysfunction in dogs with congestive heart failure. *Circulation. Arrhythmia and electrophysiology, 1*(2), 93-102

Yu, Y., Jia, X. J., Zhang, W. P., Fang, T. T., Hu, J., Ma, S. F., & Gao, Q. (2016). The Protective Effect of Low-Dose Ethanol on Myocardial Fibrosis through Downregulating the JNK Signaling Pathway in Diabetic Rats. *J Diabetes Res, 2016*, 3834283

Yuan, W., & Bers, D. M. (1994). Ca-dependent facilitation of cardiac Ca current is due to Ca-calmodulin-dependent protein kinase. *Am J Physiol, 267*(3 Pt 2), H982-993

Yuen, M. Y., Webb, S. E., Chan, C. M., Thisse, B., Thisse, C., & Miller, A. L. (2013). Characterization of Ca(2+) signaling in the external yolk syncytial layer during the late blastula and early gastrula periods of zebrafish development. *Biochim Biophys Acta, 1833*(7), 1641-1656

Zarain-Herzberg, A., MacLennan, D. H., & Periasamy, M. (1990). Characterization of rabbit cardiac sarco(endo)plasmic reticulum Ca2(+)-ATPase gene. *J Biol Chem, 265*(8), 4670-4677

Zhang, J., Chen, B., Zhong, X., Mi, T., Guo, A., Zhou, Q., . . . Chen, S. R. (2014a). The cardiac ryanodine receptor luminal Ca2+ sensor governs Ca2+ waves, ventricular tachyarrhythmias and cardiac hypertrophy in calsequestrin-null mice. *The Biochemical journal, 461*(1), 99-106

Zhang, J., Chen, B., Zhong, X., Mi, T., Guo, A., Zhou, Q., . . . Chen, S. R. (2014b). The cardiac ryanodine receptor luminal Ca2+ sensor governs Ca2+ waves, ventricular tachyarrhythmias and cardiac hypertrophy in calsequestrin-null mice. *Biochem J, 461*(1), 99-106

Zhang, J., Zhou, Q., Smith, C. D., Chen, H., Tan, Z., Chen, B., . . . Chen, S. R. (2015). Non-beta-blocking R-carvedilol enantiomer suppresses Ca2+ waves and stress-induced ventricular tachyarrhythmia without lowering heart rate or blood pressure. *Biochem J, 470*(2), 233-242

Zhang, R., Khoo, M. S., Wu, Y., Yang, Y., Grueter, C. E., Ni, G., . . . Anderson, M. E. (2005). Calmodulin kinase II inhibition protects against structural heart disease. *Nature medicine, 11*(4), 409-417

Zhang, R. H., Gao, J. Y., Guo, H. T., Scott, G. I., Eason, A. R., Wang, X. M., & Ren, J. (2013). Inhibition of CYP2E1 attenuates chronic alcohol intake-induced myocardial contractile dysfunction and apoptosis. *Biochimica et biophysica acta, 1832*(1), 128-141

Zhang, T., Guo, T., Mishra, S., Dalton, N. D., Kranias, E. G., Peterson, K. L., . . . Brown, J. H. (2010). Phospholamban ablation rescues sarcoplasmic reticulum Ca(2+) handling but exacerbates cardiac dysfunction in CaMKIIdelta(C) transgenic mice. *Circ Res, 106*(2), 354-362

Zhang, T., Maier, L. S., Dalton, N. D., Miyamoto, S., Ross, J., Jr., Bers, D. M., & Brown, J. H. (2003). The deltaC isoform of CaMKII is activated in cardiac hypertrophy and induces dilated cardiomyopathy and heart failure. *Circulation research, 92*(8), 912-919

Zhang, X., Dong, F., Li, Q., Borgerding, A. J., Klein, A. L., & Ren, J. (2005). Cardiac overexpression of catalase antagonizes ADH-associated contractile depression and stress signaling after acute ethanol exposure in murine myocytes. *Journal of applied physiology, 99*(6), 2246-2254

Zhang, X., Klein, A. L., Alberle, N. S., Norby, F. L., Ren, B. H., Duan, J., & Ren, J. (2003). Cardiac-specific overexpression of catalase rescues ventricular myocytes from ethanol-induced cardiac contractile defect. *Journal of molecular and cellular cardiology, 35*(6), 645-652

Zhao, Z., Wen, H., Fefelova, N., Allen, C., Baba, A., Matsuda, T., & Xie, L. H. (2012). Revisiting the ionic mechanisms of early afterdepolarizations in cardiomyocytes: predominant by Ca waves or Ca currents? *Am J Physiol Heart Circ Physiol, 302*(8), H1636-1644

Zhou, W., Lin, L., Majumdar, A., Li, X., Zhang, X., Liu, W., . . . Evans, S. M. (2007). Modulation of morphogenesis by noncanonical Wnt signaling requires ATF/CREB family-mediated transcriptional activation of TGFbeta2. *Nat Genet, 39*(10), 1225-1234

Zhu, L. J., Klutho, P. J., Scott, J. A., Xie, L., Luczak, E. D., Dibbern, M. E., . . . Grumbach, I. M. (2014). Oxidative activation of the Ca(2+)/calmodulin-dependent protein kinase II (CaMKII) regulates vascular smooth muscle migration and apoptosis. *Vascul Pharmacol, 60*(2), 75-83

Zima, A. V., & Blatter, L. A. (2004). Inositol-1,4,5-trisphosphate-dependent Ca(2+) signalling in cat atrial excitation-contraction coupling and arrhythmias. *J Physiol, 555*(Pt 3), 607-615

VITA

Jiajie Yan was born on Feburary 23, 1986 to Bangkuan Yan and Wenyan Wang in Tianjin, China. Jiajie graduated from Department of Bioengineering, Zhejiang University in 2008 and came to United States for graduate studies. She later graduated from Department of Biomedical Engineering, University of Alabama at Birmingham with MS degree in 2012. She was co-adviced by Dr. Xun Ai and Dr. Pogwizd while working on a project focused on conduction alterations in a non-ischemic heart failure canine model. During this time, she started working with Dr. Xun Ai on projects related to the reetry and non-reentry mechanisms in atrial fibrillation and developed many experimental skills crutial for her PhD studies.

By the end of 2012, Jiajie moved with Dr. Xun Ai to Loyola University Chicago and started working as a research specialist in the Ai Lab. In 2013, she entered the Integraduated Program in Biomedical Sciences and later joined the physiology track. Jiajie's research in the Ai Lab during the PhD study focus on the Ca^{2+} mishandling in various atrial arrhythmia models including the aging model and binge alcohol-induced arrhythmia. She presented multiple abstracts at international conferences and published multiple manusciprts during this period of time. Upon completion of her PhD, Jiajie will work in Ai Lab at Rush University as a post-doctoral scholar to continue her scientific training while prepare to apply to medical school.

Publications

Yan J, Thomson JK, Zhao W, Fast VG, Ye T, Ai X. Voltage and calcium dual channel optical mapping of cultured HL-1 atrial myocyte monolayer. *J Vis Exp.* 2015 Mar 23;(97). doi: 10.3791/52542.

Yan J, Thomson JK, Wu X, Zhao W, Pollard AE, Ai X. Novel methods of automated quantification of gap junction distribution and interstitial collagen quantity from animal and human atrial tissue sections. *PLoS One*. 2014 Aug 8;9(8):e104357

Yan J, Kong W, Zhang Q, Walcott G, Fast V, Ai X. c-Jun N-terminal Kinase Activation Contributes to Reduced Connexin43 and Atrial Arrhythmia Development in Aged Rabbit Left Atria. *Cardiovasc Res*. 2013 1;97(3):589-97

Han C, Pogwizd SM, Killingsworth CR, **Yan J**, He B. Noninvasive Three-dimensional Cardiac Activation Imaging of Ventricular Arrhythmias in the Rabbit Heart. *Computing in Cardiology* 2010;37:125-127

Lin Q, **Yan J**, Qiu F, Song X, Fu G, Ji J. Heparin/collagen Multilayer as a Thromboresistant and Endothelial Favorable Coating for Intravascular Stent. ***J Biomed Mater Res Part A,*** 2011, 96A(1), 132-141

Manuscripts in Preparation

Under Review: **Yan J**, Zhao W, Thomson JK, Gao X, DeMarco DM, Carrillo E, Chen B, Wu X, Raguimova ON, Ginsburg KS, Bakhos M, Bers DM, Robia SL, Anderson MS, Song LS, Fill M, Ai X. Crosstalk between the stress signaling JNK2 and CaMKII underlies age-related atrial arrhythmia.

Under Review: **Yan J**, Thomson JK, Zhao W, Gao X, Wu X, DeMarco DM, Kong W, Tong M, Zhang Q, Bakhos M, Fast VG, Sun J, Liang Q, Prabhu SD, Ai X. The stress kinase JNK promotes gap junction Cx43 down-regulation and atrial fibrillation in the aged heart.

Under Review: **Yan J**, Killingsworth CR, Walcott G, Zhu Y, Litoxsky S, Huang J, Ai X, Pogwizd SM.Connexin43 downregulation and dephosphorylation both contribute to slow conduction velocity in a new irreversible arrhythmogenic canine model of nonischemic heart failure.

Under Review: Gao X, Wu X, Zhang J, Zhao W**, Yan J**, DeMarco DM, Zhang Y, Bakhos M, Mignery G, Sun J, Ai X. Isoform specific action of stress kinase JNK2 on pro-arrhythmic molecule CaMKIIδ gene expression in the aged atrium.

In Preparation: **Yan J**, Demarco DM, Zhao W, Nani A, Chen B, Song LS, Chen SR, Fill M, Ai X. Novel stress-activated JNK signaling drives atrial arrhythmic activities in repeated binge alcohol-exposed hearts.

In Preparation: Pollard AE,* **Yan J**,* (*equal roles) Thomas JK, Qin J, Barr RC, Ai X. Novel method to measure intercellular resistivity in mouse ventricles.

In Preparation: **Yan J**, Zhang Y, Thomas JK, Zhao W, Jun Sun*, Ai X. Cross-talking between Gut and Heart promotes atrial arrhythmic remodeling

In Preparation: Qin J, **Yan J**, Gao X, Zhao W, DeMarco MD, Garvey T, Ai X. Critical role of JNK1 in downregulated gap junction expression and cell-cell communication in diet-induced obese mouse hearts.

In Preparation: **Yan J**, Thomas JK, Zhao W, Silvio H Litovsky, Ai X. Chamber specific gap junctional and structural remodeling in impaired intercellular coupling in aged atria.

Selected Poster Presentations

Yan J, DeMarco DM, Zhao W, Nani A, Chen B, Song LS, Fill M, Ai X. "Novel stress-activated JNK signaling drives atrial arrhythmic activities in binge alcohol-exposed hearts" *Heart Rhythm Society 37th Annual Scientific Sessions*, San Francisco, CA, May.2016

Qin J, Gao X, **Yan J**, Zhao W, Garvey T, Ai X. "Critical role of JNK1 in downregulated gap junction expression and cell-cell communication in diet-induced obese mouse hearts." *Heart Rhythm Society 37th Annual Scientific Sessions*, San Francisco, CA, May.2016

Gao X, Wu X, Zhao W, **Yan J**, Ai X. "Critical role of JNK2 in cardiac CaMKII delta isoform gene upregulation in aged hearts" 2016 *International Society for Heart Research World Congress*, Argentina.

Yan J, Thomson JK, Zhao W, Ai X. "Novel JNK2 signaling is the predominant regulator of diastolic SR Ca leak and AF genesis in the aged heart" *Heart Rhythm Society 36th Annual Scientific Sessions*, Boston, MA, May.2015

Wu X, Zhao W, Carrillo E, Chen W, **Yan J**, Ber DM, Robia SL, Ai X. "Novel Stress Signaling JNK Regulates Pro-arrhythmic Molecular CaMKIIδ Activity and Expression in Aged Human Atrium" *American Heart Association. Scientific Sessions Chicago*, IL, Nov.2014

Yan J, Zhao W, Thomson JK, Chen B, Chen W, Carrillo E, Bers DM, Robia SL, Anderson ME, Song SL, Ai X. Featured Poster: "c-Jun N-terminal Kinase activation enhances CaMKII activation that in turn prompts atrial arrhythmias in aged hearts". *Heart Rhythm Society 35th Annual Scientific Sessions*, San Francisco, CA, May.2014

Yan J, Zhao W, Tong M, Zhang Q, Fast VG, Prabhu SD, Ai X. "c-Jun N-terminal Kinase Activation Impairs Action Potential Propagation and Enhances Arrhythmogenicity in Mouse Atria" *Basic Cardiovascular Sciences Scientific Sessions*. Las Vegas, NV, Jul.2013

Yan J, Killingsworth CR, Walcott G, Ai X, Pogwizd SM. "Connexin43 Downregulation and Dephosphorylation Contribute to Slow Conduction in a New Arrhythmogenic Canine Model of Non-ischemic Heart Failure", *Heart Rhythm Society 34th Annual Scientific Sessions*, Denver, CO, May.2013

Zhang Q, **Yan J**, Walcott G, Ai X" Activated c-Jun N-terminal Kinase Contributes to Significantly Increased Pacing-induced Atrial Fibrillation in Aged Rabbits *in vivo*" , *Heart Rhythm Society 33rd Annual Scientific Sessions*, Boston, MA, May. 2012

Yan J, Zhao W, Ai X. "c-Jun N-terminal Activation Contributes Reduced Connexin43 and Atrial Fibrillation Development in Atrium", *Hearty Rhythm Society 32nd Annual Scientific Sessions*, San Francisco, CA, May.2011

Han C, Pogwizd SM, Killingsworth CR, **Yan J**, He B. "Noninvasive Three-dimensional Cardiac Activation Imaging of Ventricular Tachycardia in the Rabbit Heart", *Circulation*. 2010:122:A17255

Kong W, **Yan J**, Zhao W, Fast VG, Ai X. "Connexin 43 Downregulation and Slowing of Conduction are Associated with Increased Inducibility Of Atrial Arrhythmias in the Aged Rabbit Left Atrium", Featured Poster, *Heart Rhythm Society 31st Annual Scientific Sessions,* Denver, CO, May.2010.

Awards

New Investigator Travel Award Winner, American Heart Association, Basic Cardiovascular Sciences, 2013

Featured Poster (ranked #1) Jiajie Yan, Weiwei Zhao, Justin Thomson, Biyi Chen, Weizhe Chen, Elena Carrillo, Donald Bers, Seth Robia, Mark Anderson, Long-sheng Song, Xun Ai. Featured Poster: "c-Jun N-terminal Kinase activation enhances CaMKII activation that in turn prompts atrial arrhythmias in aged hearts". Heart Rhythm Society 35th Annual Scientific Sessions, San Francisco, CA, May.2014

Featured Poster (ranked #9) Wei Kong, **Jiajie Yan**, Weiwei Zhao, Vladmir Fast, Xun Ai. "Connexin 43 Downregulation and Slowing of Conduction are Associated with Increased Inducibility Of Atrial Arrhythmias in the Aged Rabbit Left Atrium", Featured Poster, *Heart Rhythm Society 31st Annual Scientific Sessions,* Denver, CO, May.2010.