Mechanisms of Atrial Fibrillation in Mice with Genetic Ablation of the α1D L-type Calcium Channel

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Science
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Abstract

Atrial fibrillation (AF) is the most common supraventricular arrhythmia with a multifactorial pathophysiology. Reductions in α1 L-type calcium channel (LTCC) current (\(I_{CaL}\)) and action potential duration is commonly observed in human AF patients. As expected, mice lacking the α1D (\(Ca_{v}1.3\)) LTCC gene are more susceptible to inducible AF, albeit without a reduction in atrial refractoriness. Despite increased fibrosis and delayed conduction velocity in \(Ca_{v}1.3^{-/-}\) mice, spectral analysis of AF revealed a highly disorganized pattern consistent with electrical remodeling. Conforming to electrical remodeling, \(Ca_{v}1.3^{-/-}\) atrial myocytes displayed increased sensitivity to calcium alternans, which was alleviated with inotropic agents and absent in α1C LTCC heterozygous atrial myocytes. In addition, we observed increased electrical alternans in \(Ca_{v}1.3^{-/-}\) atria, which coupled positively to voltage. These electrical alternans were in-turn associated with AF in \(Ca_{v}1.3^{-/-}\) atria. Our findings support the conclusion that preferential loss of \(Ca_{v}1.3\) promotes calcium alternans, resulting in arrhythmogenic electrical alternans.
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<th>Description</th>
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<tbody>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>$I_{KAC}$</td>
<td>Acetylcholine activated inward-rectifying potassium current</td>
</tr>
<tr>
<td>AP</td>
<td>Action potential</td>
</tr>
<tr>
<td>APD</td>
<td>Action potential duration</td>
</tr>
<tr>
<td>AC</td>
<td>Adenyl cyclase</td>
</tr>
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<td>$\alpha$</td>
<td>Alpha</td>
</tr>
<tr>
<td>AERP</td>
<td>Atrial effective refractory period</td>
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<td>AF</td>
<td>Atrial fibrillation</td>
</tr>
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<td>AV</td>
<td>Atrioventricular</td>
</tr>
<tr>
<td>AVERP</td>
<td>Atrioventricular effective refractory period</td>
</tr>
<tr>
<td>BPM</td>
<td>Beats per minute</td>
</tr>
<tr>
<td>$\beta$</td>
<td>Beta</td>
</tr>
<tr>
<td>$\beta$-AR</td>
<td>Beta-adrenergic receptor</td>
</tr>
<tr>
<td>$\text{Ca}^{2+}$</td>
<td>Calcium</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Calcium/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CCB</td>
<td>Calcium channel blocker</td>
</tr>
<tr>
<td>CICR</td>
<td>Calcium-induced calcium release</td>
</tr>
<tr>
<td>CO</td>
<td>Cardiac output</td>
</tr>
<tr>
<td>IV</td>
<td>Current-voltage</td>
</tr>
<tr>
<td>CRU</td>
<td>Calcium release unit</td>
</tr>
<tr>
<td>CL</td>
<td>Cycle Length</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>DAD</td>
<td>Delayed afterdepolarization</td>
</tr>
<tr>
<td>$I_{so}$</td>
<td>Depolarization-activated outward potassium steady-state current</td>
</tr>
<tr>
<td>DI</td>
<td>Diastolic interval</td>
</tr>
<tr>
<td>DHP</td>
<td>Dihydropyridine</td>
</tr>
<tr>
<td>$df$</td>
<td>Dominant frequency</td>
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<tr>
<td>EAD</td>
<td>Early afterdepolarization</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>EDD</td>
<td>End-diastolic diameter</td>
</tr>
<tr>
<td>EDV</td>
<td>End-diastolic volume</td>
</tr>
<tr>
<td>ESD</td>
<td>End-systolic diameter</td>
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<td>ESV</td>
<td>End-systolic volume</td>
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<tr>
<td>$\delta$</td>
<td>Epsilon</td>
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<tr>
<td>EC</td>
<td>Excitation-contraction</td>
</tr>
<tr>
<td>FFT</td>
<td>Fast-Fourier transform</td>
</tr>
<tr>
<td>$I_{tof}$</td>
<td>Fast-transient outward potassium current</td>
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<tr>
<td>FS</td>
<td>Fractional Shortening</td>
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<tr>
<td>$I_f$</td>
<td>Funny current</td>
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<td>$\gamma$</td>
<td>Gamma</td>
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<tr>
<td>HR</td>
<td>Heart rate</td>
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<tr>
<td>HCN</td>
<td>Heterocyclic nucleotide-gated channels</td>
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<tr>
<td>$K_{ir}$</td>
<td>Inward rectifying potassium channels</td>
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<tr>
<td>$I_{K1}$</td>
<td>Inward rectifying potassium current</td>
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<tr>
<td>ISO</td>
<td>Isoproterenol</td>
</tr>
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<td>-------</td>
<td>----------------</td>
</tr>
<tr>
<td>LV</td>
<td>Left Ventricular</td>
</tr>
<tr>
<td>LVEF</td>
<td>Left ventricular ejection fraction</td>
</tr>
<tr>
<td>LVEDD</td>
<td>Left ventricular end-diastolic diameter</td>
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<tr>
<td>LVEDP</td>
<td>Left ventricular end-diastolic pressure</td>
</tr>
<tr>
<td>LVESD</td>
<td>Left ventricular end-systolic diameter</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium/calcium exchanger</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium/calcium exchanger current</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium</td>
</tr>
<tr>
<td>LTCC</td>
<td>L-type calcium channel</td>
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<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
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<tr>
<td>Eₘₗₚ</td>
<td>Membrane potential</td>
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<tr>
<td>OI</td>
<td>Organization index</td>
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<td>PSR</td>
<td>Picrosirius red</td>
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<tr>
<td>K⁺</td>
<td>Potassium</td>
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<tr>
<td>PV</td>
<td>Pulmonary vein</td>
</tr>
<tr>
<td>Iₖᵣ</td>
<td>Rapidly activating delayed rectifying potassium current</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>RAA</td>
<td>Right atrial appendage</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarcoplasmic reticulum calcium-ATPase</td>
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<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
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<td>Sodium</td>
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<tr>
<td>SDA</td>
<td>Spatially discordant alternans</td>
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<tr>
<td>SV</td>
<td>Stroke Volume</td>
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<td>TGFB</td>
<td>Transgenic Growth Factor Beta</td>
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<tr>
<td>Iₜₒ</td>
<td>Transient outward potassium current</td>
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<td>Iₛᵣ</td>
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<td>Voltage-activated L-type calcium channels</td>
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<tr>
<td>VERP</td>
<td>Ventricular effective refractory period</td>
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<td>VF</td>
<td>Ventricular fibrillation</td>
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<tr>
<td>WBCL</td>
<td>Wenckebach cycle length</td>
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Chapter 1
Introduction
1 Introduction

1.1 Atrial Fibrillation

Atrial Fibrillation (AF) is a supraventricular tachyarrhythmia, characterized on an electrocardiogram (ECG) by the replacement of regularly defined P-waves by rapid and highly irregular electrical atrial activity, which in turn causes irregular and rapid ventricular activation\(^1\).\(^2\). In 1906, *The Telecardiogram*\(^3\), in the Archives Internationales de Physiologic, was the first organized publication to characterize normal and abnormal ECGs with a string galvanometer, and they termed the nature of the atrial curves as “*pulsus irregularis perpetuus*”. Shortly thereafter, clinical encounters summarized by Thomas Lewis, further described the condition as “extremely common”\(^4\). He goes on to conclude the irregularity arises from auricular (atrial) fibrillation and the disorderly rhythm of ventricular activation is dependent on the irregular atrial movements. The rapid and irregular electrical activation of the atria over encumbers the atrioventricular (AV) node yielding the irregular contractions of the ventricle.

AF can present itself, under clinical definition, as paroxysmal, persistent, and permanent\(^5\). Paroxysmal AF, also termed intermittent AF, is defined as recurrent (two or more) episodes of AF that terminate spontaneously in less than seven days, usually less than 24 hours. Persistent AF is sustained and only terminated by pharmacological therapy or electrical cardioversion. In the clinical setting it is the most commonly encountered and significant cardiac arrhythmia and it is estimated that 350,000 Canadians are living with AF\(^6\). The prevalence of AF is rising rapidly, with over a doubling in the number of hospitalization discharges in the United States from 1984 to 1994\(^7\). In addition, the number of patients is expected to triple in the next 2-3 decades\(^8\), which can be partially attributed to the rising etiological factors of AF, such as aging, and cardiovascular disease\(^9\),\(^10\). The recent explosion of research concerning the mechanisms and therapy of AF\(^11\) parallels the ‘epidemic’ epidemiology of the condition\(^12\).

Some of the common symptoms associated with AF are palpitations, chest pain, and a reduction in exercise tolerance. Study populations from the United States\(^10\), Canada\(^9\), and United Kingdom\(^13\), present with significantly elevated age-adjusted mortality hazard ratio even in asymptomatic AF patients (comprising 15% to 30% of the total AF population)\(^14\). One of the most debilitating complications of AF is the predisposition to thromboembolic complication...
increasing the risk 4-5 fold. In the US, strokes caused by AF accounted for 15-25% of all strokes. In fact, AF is an independent risk factor for stroke and those associated with AF are found to be more debilitating, greater in magnitude, and are often fatal. During AF, loss of synchronized contraction leads to the stasis of blood and results in the formation of fibrin-rich clots. If these blood clots become dislodged and mobile, passage to the cerebrovascular system will likely result in embolization.

Since AF is not an entirely benign arrhythmia, the treatment strategies employed must be cautious of the underlying cardiovascular disease present, in addition to the temporal nature of the arrhythmia itself. Furthermore, advances in the understanding of the pathology over the past 100 years have only yielded satisfactory management approaches. Currently, the two main strategies involve cardioversion of AF to sinus rhythm, with subsequent maintenance (rhythm control), and mitigating the negative indirect effects on the ventricles during bouts of AF (rate control).

Present methods employed to cardiovert fibrillatory tissue and maintain sinus rhythm involve pharmacological agents (antiarrhythmic drugs), direct current injection (defibrillation), or cardiac surgery (catheter ablation). Pharmacological interventions usually have two primary goals, to reduce the frequency of AF incidences, or more preferably, to completely eliminate the reoccurrence of AF. The caveat to traditional antiarrhythmic drugs, such as inhibitors of potassium (K+) (class III agents) or sodium (Na+) currents (class I agents), is their increased risk of ventricular arrhythmias. Even the most effective antiarrhythmic drugs that inhibit Na+, K+, and calcium (Ca2+) currents simultaneously have increased risk of extracardiac side effects. A standard method for the electrical cardioversion of AF involves the direct current counter-shock. Methods of employment involves direct current injection via a non-invasive transthoracic or sedation with an intravenous catheter. Similar to the antiarrhythmic drugs, ventricular arrhythmias are still a risk, and dislodging a preexisting atrial thrombus is also a concern. A more recently common therapeutic alternative to antiarrhythmic drugs is catheter ablation, which is a more invasive technique with the hopes of completely eliminating the trigger for AF. The method involves using a radiofrequency catheter to physically burn selected regions of atrial tissue that appear to be the source of abnormal electrical activity. In this field, the definition of “clinical success” is variable (most often it means complete freedom from symptomatic AF) and is often difficult to assess for two reasons: asymptomatic AF is difficult to detect without proper
equipment and monitoring, and studies rely on self-reported symptomatic recurrences from the patients themselves\textsuperscript{20}. More stringent and effective monitoring using mobile cardiac outpatient telemetry post-ablation, or other similar ECG monitoring, more accurately demonstrated that recurrent AF is a common observation\textsuperscript{20}. For example, short-term studies by Vasamreddy \textit{et al} and by Senatore \textit{et al}, showed the success rate drop from 70\% to 50\% and 86\% to 72\% when asymptomatic AF episodes were included\textsuperscript{20, 21}. Overall, 28–32\% of patients at 3 months, and 50\% at 6 months experienced paroxysmal AF post-ablation, while 19\% of patients regressed to chronic AF within 3 years post ablation. These statistics, in addition to a 70\% ceiling in success (complete elimination of AF for 1-year) restricts the procedure to only extreme circumstances and repeated ablations are often a necessity.

On the other hand, rate control abandons attempts to restore sinus rhythm and focuses mainly on limiting the stimulation rates of the ventricles by using pharmacological agents that slow the conduction through the AV node and prolong the effective refractory period\textsuperscript{19}. Catheter ablation may also be used to ablate antegrade conducting accessory pathways or the node itself (which would require a pacemaker to recover the function of the AV node) to rid of any re-entry pathways\textsuperscript{17}. Complications pertaining to this method are establishing appropriate ventricular rates during sinus rhythm/exercise since ventricular function is variable between subjects. Major caveats include: failure to reduce the risk of thromboembolism since AF is not directly targeted and reduced exercise capacity.

The complexity of the underlying mechanism and the intricate multifactorial pathophysiology of AF suggest that it is unlikely a single therapeutic approach will be adequate for treating AF. Aging and underlying structural diseases lead to gradual electroanatomical “atrial remodeling”, which account for the minimal success rate in either type of treatment. Atrial remodeling is often characterized by changes in the ion channel expression and function, Ca\textsuperscript{2+} homeostasis and atrial structure\textsuperscript{22}. When the equilibrium of any one of these pathways is disturbed, promotion of the “triggers” that initiate and sustain the arrhythmia are inevitable. Also, development of AF “substrates” will help to stabilize electrical reentry. More broadly, electrical and structural remodeling can be used to classify AF triggers and substrates. Electrical remodeling refers to modifications of the normal electrophysiology parameters of the atria (wavelength reductions, ectopic beats, dispersion of refractoriness), while structural remodeling refers to physical changes to the atrial structure (fibrosis, or atrial dilatation) in addition to
cellular changes in atrial myocytes (hypertrophy, glycogen deposition, mitochondrial changes). Before these complex mechanisms of atrial remodeling will be explained, an overview of all proarrhythmic mechanisms will be discussed, because they are all interrelated and especially so when describing the initiation and perpetuation of AF.

1.2 Cardiac Arrhythmias

Cardiac arrhythmias are a heterogeneous field of conditions. Most broadly stated, a cardiac arrhythmia is an “irregular heartbeat” defined by abnormal electrical activity in the heart. Arrhythmias can arise from abnormalities in impulse generation (automatism), impulse conduction, or a combination of both\(^\text{23}\). It is important to recognize all cardiac arrhythmias, even if AF is strictly a reentry arrhythmia, because other cellular proarrhythmic mechanisms (automaticity or triggered activity) contribute to AF in a hierarchical manner\(^\text{24,25}\). For example, a process termed “kindling”, initiates spiral wave reentry, whereby appropriately timed sequences of ectopic foci facilitate the interaction of wavefront-wavetail interaction\(^\text{26-28}\) (explained in more detail in reentry arrhythmias).
Cardiac arrhythmias are a large and heterogeneous group of conditions, although their pathophysiological origin can be assigned two three broad categories: reentry arrhythmias, triggered activity (delayed and early after-depolarizations), and abnormal impulse formation (normal automaticity or abnormal automaticity). All of these adverse outcomes are the consequence of abnormal physiological processes. These three events are not mutually exclusive, and the pattern of cooperation will determine the cardiac arrhythmia that surfaces. For example, reentry can be initiated by triggered activity or abnormal impulses in a hierarchal manner.
1.2.1 Abnormal impulse formation and automaticity

Pacemaker activity or automaticity in cardiac myocytes is present in specialized cells in the SA (sinoatrial) node, the AV node, and the His-Purkinje system, in addition to few cells in both atria. These cells possess the ability to spontaneously generate action potentials (APs) in the absence of external cues. The depolarizing “funny” current ($I_f$) (contributed partly by the heterocyclic nucleotide-gated channels (HCN) HCN2 and HCN4) in these cells is activated at very negative membrane potential ($E_m$) ($-40$ to $-60$ mV), and is responsible for the initiation of the gradual spontaneous decrease in transmembrane potential during diastole. Normal conduction through the heart is a hierarchical process with the intrinsic firing rate of the SA nodal cells greatest (60–100 beats per minute (bpm)) compared to the firing rate of other ‘subsidiary’ cells (40–60bpm in the AV junction and 20–40bpm in the Purkinje system). Overdrive suppression is a mechanism in place to maintain this hierarchical pattern of activation in pacemaker cells. Sinus rhythm is maintained by continuously inhibiting the activity of subsidiary pacemaker cells. Build up of intracellular Na$^+$ during high pacing rates drives the Na$^+$-K$^+$ pump during diastole and generates a hyperpolarizing current ($I_p$) (three Na$^+$ ions are extruded while two K$^+$ ions are transported intracellularly) counteracting phase 4 depolarization. When a spontaneously active pacemaker is driven at a frequency greater than its intrinsic rate, the termination of the driver will lead to temporary quiescence (“overdrive suppression”).

Clinical arrhythmias often arise when there is enhancement or suppression of activity in these particular cell types (enhanced normal automaticity) or when normal contractile atrial or ventricular cells acquire spontaneous activity (abnormal automaticity). When tissues (i.e. SA node, AV node, HIS-Purkinje fibers) that are capable of undergoing automaticity and exhibit increased activity, it is referred to as “enhanced automaticity”. This can result from three factors: an increase in voltage threshold (the voltage threshold becomes more negative) a decrease in the maximum diastolic potential (the $E_m$ becomes less negative), and an increase in the slope of phase 4 depolarization. Phase 4 depolarization is modulated by the autonomic nervous system, and is particularly sensitive to endogenous agents, pharmacological interventions, and electrical stimulation (overdrive pacing).
Stimulation of the vagus nerve causes release of ACh (acetylcholine) and activation of the muscarinic receptor-mediated channels\textsuperscript{41}. Subsequent activation of the inward-rectifying K\textsuperscript{+} current (\(I_{\text{K}_\text{ACh}}\)) then produces a potent negative chronotropic effect\textsuperscript{42}. The rising K\textsuperscript{+} permeability\textsuperscript{41,43} increases the maximum diastolic potential (greater distance between diastolic and threshold potential) and prolongs the duration of Phase 4 depolarization. ACh is also known to negatively influence \(I_f\) and L-type Ca\textsuperscript{2+} channel (LTCC) current (\(I_{\text{CaL}}\)) thereby reducing the slope of phase 4 depolarization\textsuperscript{44,45} and phase 0 upstroke of the AP. These effects are mediated by muscarinic M2 receptor activation of the inhibitory G protein \(\alpha\)-subunit (\(\alpha_i\)), leading to negative regulation of cAMP production (negatively couples to adenylate cyclase (AC) activity), and positive regulation of cAMP hydrolysis\textsuperscript{46,47}. In patients with a supraventricular tachyarrhythmia, due to enhanced automaticity, ACh is commonly used as a diagnostic and therapeutic tool to suppress activity.

In contrast, activation of the sympathetic nervous system leads to \(\beta\)-adrenergic (\(\beta\)-AR) stimulation, subsequent activation of AC and an increase in cAMP\textsuperscript{48,49}. Protein kinases (PKA)\textsuperscript{50}, activated via allosteric modulation by cAMP, will then phosphorylate LTCCs (Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3)\textsuperscript{51,52}, and positively influence the conductance of \(I_{\text{CaL}}\). The net inward current of \(I_f\) is also influenced by \(\beta\)-AR stimulation\textsuperscript{32,53,54}. Small changes in the intracellular cAMP concentration increase the open probability of HCN and cause a positive shift in the activation curve of \(I_f\)\textsuperscript{48,55}. The combination of these effects results in an increased firing rate of pacemaker cells.

In addition to overdrive pacing, the Na\textsuperscript{+}-K\textsuperscript{+} pump plays an electrogenic role in cellular pacemaking by contributing a net outward current \(I_p\). Under physiological conditions \(I_p\) can lead to suppression of automaticity by hyperpolarizing the membrane\textsuperscript{56-58}. However, under conditions of hypoxia (such as ischemia\textsuperscript{59}) when the Na\textsuperscript{+}-K\textsuperscript{+} pump becomes inhibited or under low extracellular K\textsuperscript{+} concentration\textsuperscript{58}, the slope of phase 4 increases and accelerates pacemaking.

Some cardiac arrhythmias, such as sinus tachycardia, are the result of enhanced normal automaticity and are associated with exercise, fever, or thyrotoxicosis when they are secondary to sympathetic stimulation\textsuperscript{23}. Normal enhanced automaticity can be distinguished from abnormal automaticity, because the former is more sensitive to overdrive suppression\textsuperscript{60}. Bradycardia, and tachyarrhythmia can also be due SA node dysfunction, often associated with developed cardiac
conditions (heart failure, ischemia, cardiomyopathy, or administration of antiarrhythmic drugs\textsuperscript{61-63} or have an underlying genetic abnormality (HCN4\textsuperscript{64-66}, Ca\textsubscript{v}.1.3\textsuperscript{67}, or Na\textsubscript{v}.1.5\textsuperscript{68}).

Arrhythmias may also arise in cardiac muscle fibers of the atria and ventricles, where automaticity is normally absent, but may be brought about by abnormal depolarization of the membrane (between -60mV to -10mV)\textsuperscript{38,69}. Diastolic depolarization can be induced in quiescent Purkinje fibers under a number of abnormal conditions which include: a reduction in the extracellular K\textsuperscript{+} concentration\textsuperscript{70}, dropping the pH and concentration of oxygen in the bathing solutions, adding tetraethylammonium or barium\textsuperscript{71}, or by applying a constant depolarizing current\textsuperscript{38,40}. An ionic mechanism to explain the state of acquired automaticity, in otherwise quiescent cells, is a gain in a net depolarizing current with a decrease in K\textsuperscript{+} conductance. Generally depolarization is maintained $E_m$ where Na\textsuperscript{+} channels become inactivated, and APs are almost exclusively elicited by $I_{Ca,L}$. Common abnormal automaticity arrhythmias include: ectopic atrial tachycardias, accelerated idioventricular rhythms and ventricular tachycardias (24–72 hours post experimental myocardial infarction (MI)). Abnormal automaticity is often observed post-MI\textsuperscript{72}, and is explained by the local rise in extracellular K\textsuperscript{+} or catecholamine concentration and “injury currents”. Regions of the infarct zone directly exposed to the rise in extracellular K\textsuperscript{+} provide a depolarizing electrotonic effect on surrounding cells (with normal K\textsuperscript{+} concentrations)\textsuperscript{73}.

1.2.2 Triggered activity

Following a normal AP, oscillations in the $E_m$ (the trigger) have the capacity to elicit a new AP if the depolarizing currents exceed threshold. These “triggered” responses can stimulate new APs, and under ideal conditions can lead to self sustained foci of triggered activity. Two types of triggered activity can occur depending on the temporal activation of membrane oscillations: “early” afterdepolarizations (EADs) and “delayed” afterdepolarizations (DADs).

If the membrane oscillation occurs after the triggered AP has fully repolarized it is called a DAD. Conditions that favor the genesis of DADs are processes that promote Ca\textsuperscript{2+} overload (ischemia, β-AR overstimulation, low extracellular K\textsuperscript{+} and tachycardia\textsuperscript{74,75}) and drive the activity of the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger (NCX). The electrogenic NCX current ($I_{NCX}$) is depolarizing and can drive the $E_m$ to threshold. DADs are then dependent on the rate of the triggering rhythm.
Faster pacing rates (i.e. during tachycardia) will promote increase cytosolic Ca\(^{2+}\) and NCX activity leading to faster episodes of self-sustaining DADs\(^74\).

Membrane depolarizations that occur during the repolarization phases of the AP (Phase 2 or 3) are considered EADs. Reduced repolarization reserve, due to either a reduction in outward current, an increase in inward current, or a combination, provides an environment for EADs to spawn. One of the major currents responsible for generating an EAD upstroke is \(I_{\text{cal}}\) “Ca\(^{2+}\) window currents”. The voltage threshold for inactivation and activation for LTCC overlap\(^76,77\). If this period allows for the transformation of the channel from inactivation to closed and open states, there is the potential for a new AP upstroke via \(I_{\text{cal}}\). Ca\(^{2+}\) window currents also induce further release of Ca\(^{2+}\) from the SR, which can cooperate synergistically with NCX\(^78\). The accelerated rise in cytosolic Ca\(^{2+}\) concentration will drive NCX and produce a further depolarizing current (\(I_{\text{NCX}}\)). The converse is also possible, where spontaneous release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) activates \(I_{\text{NCX}}\), prolonging repolarization, and allowing for reactivation of \(I_{\text{cal}}\). These two factors alone however, are not sufficient to elicit an EAD. The AP plateau must remain in a threshold voltage range (just below 0mV) for an adequate duration to allow for reactivation of inward currents.

The autonomic nervous system can accelerate the previously described mechanisms of EAD genesis. Enhanced sympathetic tone can lead to EADs through the downstream actions of β-AR stimulation. Accumulation of Ca\(^{2+}\) in the cytosol stimulates spontaneous release of Ca\(^{2+}\) from the SR and increases the \(I_{\text{NCX}}\) depolarizing current. Dual activation of the parasympathetic and sympathetic nervous system occurring during late phase 3 of the AP can also promote EADs\(^79\). After a sufficient pause in diastole, loading of Ca\(^{2+}\) in the SR (sympathetic) causes a massive Ca\(^{2+}\) transient that exceeds the short AP duration (APD) (parasympathetic). As described previously, the high cytosolic Ca\(^{2+}\) concentration during a negative \(E_m\) (repolarization) generates a strong driving force for the depolarizing \(I_{\text{NCX}}\) and produces a late EAD. Late phase 3 EADs differ from other triggered activity due to the fact that the elicited Ca\(^{2+}\) transient is due to physiological mechanism of excitation-contraction (EC) coupling, while other triggered activity relies on spontaneous Ca\(^{2+}\) release from the SR.

After cardioversion of AF, the heart is known to be in a super vulnerable phase, with reinitiation of AF occurring in 56% of patients\(^80\). Ultra short refractory periods and Ca\(^{2+}\)
overload during this phase facilitate late phase 3 EADs and reinitiation of AF. Furthermore, prolongation of APD can initiate electrical heterogeneity by causing dispersion of refractoriness, which is a favorable substrate for reentry and will be described in the next section.

1.2.3 Reentry

The initiation and subsequent perpetuation of AF requires an excitable tissue for wavefront reentry sparked by tissue discordance, an anatomical barrier or triggered/automaticity foci. A number of mechanisms exist to explain the mechanism of reentry, and include: circus movement reentry, leading circle concept, spiral wave reentry, and multiple wavelet hypothesis.

Using rings of excitable tissue cut from jelly fish *Medusa Cassiopeia*, Mayer was the first to demonstrate *circus movement reentry* (Fig. 1.2.3, top left), which was further characterized in rings of dog cardiac tissue by Mines. For reentry to occur, a depolarized wavefront can only re-activate excited tissue that has recovered from its refractory period. The shorter the recovery time after activation (refractory period) and the slower speed of that wavefront (conduction velocity (CV)), the more likely that a circus movement reentry will occur. The product of the refractory period and the CV, known as the wavelength, then determines the theoretical minimum pathlength allowed for reentry. A “temporal excitable gap” occurs when the wavelength is shorter than the path of the circuit, effectively resulting in a fixed duration for previously excited tissue to recover from refractory. The region of tissue in the circuit recovered from excitation is termed the “spatial excitable gap”, and the magnitude of this area can be determined as the product of the CV and the temporal excitable gap.

Circus movement reentry was believed to require a clearly defined anatomical structure. However, in 1924, Garrey demonstrated that a physical barrier was not necessary. Turtle cardiac muscle was used to generate a sustained excitation wave rotating around a stimulation electrode. Although, speculation of the experimental design suggested that the stimulation site acted as an obstacle to conduction. It wasn’t until 1973 when Alessie et al. used left atrial rabbit atria to truly demonstrated with experimental observations that reentry does not need an physical obstacle. These observations led to the establishment of the concept *leading circle reentry* (Fig. 1.2.3, top right). The name stems from fact that reentry is sustained by propagation of a leading wave of depolarization in a circuit around a circle with “critical dimensions”. Instead of a physical barrier used to generate a temporal excitable gap, the core is relatively unexcitable
tissue, which can not reach threshold for excitability due to electrotonic depolarization. In this model of reentry, unidirectional block occurs when the wavefront moves centripetally away from the core of the circle (dimensions of the core are dependent on wavelength) and consequently conduction is only permitted around this region, and centripetally outwards to activate the rest of the myocardium.

The *spiral wave theory* (Fig. 1.2.3, bottom left) was proposed to try and modify or update the leading circle concept to more adequately describe arrhythmia behavior in excitable tissue. Inspiration for the theory originates from the Belousov-Zhabotinsky chemical reactions and computational studies of cellular electrical activity, particularly the Fitzhugh-Nagumo (FHN) model. In order to grasp the spiral wave theory, it is crucial to recognize the fundamental relationship between the balance of “source” and “sink” depolarizing currents in cardiac tissue. Efficient propagation of depolarizing current through an excitable tissue requires a depolarizing wavefront (source) to carry enough charge to bring the voltage of the surrounding cells downstream (sink) above threshold to elicit an AP. The geometry of the source is crucial to CV and the fate of electrical propagation through the tissue. A convex wavefront indicates a greater sink, since the area of exposed excitable tissue is great (source<sink). More current is required to depolarize the surrounding cells and as a result conduction is delayed. In contrast, concave geometries have a greater number of depolarized cells contributing current to a smaller area of tissue downstream (source>sink), and CV is much greater in these regions.

In the most rudimentary form, spiral wave reentry involves an activation front traveling at a constant angular velocity, around a fixed central zone (core) and propagates radially outward. The consequence of this type of propagation produces a leading front that is curved (convex), in contrast to leading circle reentry, which becomes important for the dynamics associated with this type of reentry. At the core of the spiral wave, the degree of the curvature is the greatest (source<sink), such that CV becomes so reduced block occurs. This means at the core of the tissue there is excitable tissue present, but never becomes excited in spiral wave reentry. Instead the unexcited core acts as a sink for depolarizing current (electrotonic effect) and enhances the voltage difference across this region. The difference in potential accelerates repolarization, reducing APD and further stabilizes reentry at the core. The geometric boundaries of the unexcited spiral wave core is confined by a point where repolarization and depolarization occur simultaneously (phase singularity). Despite the comprehensive explanation of spiral wave
reentry for understanding AF in cardiac tissue, this type of reentry has yet to be observed in human patients\textsuperscript{94}. Although, experimental evidence in both atrial\textsuperscript{97} and ventricular tissue\textsuperscript{98} have described reentry events typical of spiral wave.

The final theory of reentry describes an “anarchical” organization of AF, where no localized source (ectopic foci, mother rotors) is driving the fibrillation\textsuperscript{22}. The \textit{multiple wavelet theory} (Fig. 1.2.3, bottom right), first described by Gordon Moe\textsuperscript{99}, was proposed to explain the stability and perpetuation of AF when the trigger ceases to exist (circus movement reentry or ectopic foci). According to his theory, multiple interactions of wavetails and wavefronts lead to wavebreak-up and the creation of new wavefronts. The arrhythmia will sustain itself through these various interactions, and will only terminate if block, collision, or fusion of wavefronts lead to a decline in wavelets below some critical level\textsuperscript{100, 101}. The multiple wavelets are stabilized by reductions in refractoriness, heterogeneity of refractoriness, decreases in CV, and increase tissue mass. In 1985, canine atria exposed to ACh provided the first evidence for the existence of multiple wavefronts\textsuperscript{102}. 
Figure 1.2.3 Electrical reentrant mechanisms.

Top left: circus movement reentry, dependent on the size of the anatomical obstacle, CV, and refractory period. Top right: leading circle reentry, the refractory period and CV (wavelength) will determine magnitude of the reentry path since no anatomical obstacle exists. Time (ms), denoted by the numbers in the isochrones, describes the duration of activation. Bottom left: spiral wave reentry, a mathematical model predicts the wave will rotate (path indicated by black dashed line) around an excitable, but unexcited core. The white arrows illustrate radial propagation, and membrane potential is represented visually with isochrones (blue; -90mV, red; 0mV). Bottom right: multiple wavelet hypothesis, no single source entrains the fibrillation, but multiple wavelets fuse and block to form a chaotic activation pattern. Asterisks denote transmural conduction breakthrough within the mapped area, solid black lines symbolize conduction block, and solid black arrows describe the pathway for the wavefront. Reproduced/adapted with permission from Schotten et al\textsuperscript{22} (doi: 10.1152/physrev.00031.2009).
1.3 Mechanisms Underlying Sustained Atrial Arrhythmias

In the section above I review the role of “triggers” in arrhythmia generation, originating from tissue with normal automaticity (SA node, and AV node), abnormal automaticity (atrial contractile fibers), or from triggered activity (EADs and DADs), which favor the initiation of electrical reentry (kindling). However, without the proper “substrates” to help stabilize reentry, AF will quickly terminate. The idea that reentry solely perpetuates AF is still a controversial idea, and AF driven in a hierarchal manner (via triggers) is still a generally accepted concept. Therefore, in the following section, the most common source of triggered activity sustaining AF will be described, in addition to the common substrates facilitating reentry. Specifically, how these substrates promote reentry by negatively impinging on wavelength (delay CV, and reduce APD) or promote dispersion of refractoriness will be described.

1.3.1 Pulmonary vein ectopy

Pulmonary vein (PV) ectopy, although its mechanisms are a controversial field of study, is due abnormal impulse formation (automaticity), triggered activity (EADs or DADs), or microreentry due to the abnormal tissue structure of the PVs. In a group of 45 patients, Haïssaguerre et al established the first evidence of PVs as a source of ectopic activity contributing to the initiation of AF\textsuperscript{103}. They found 94% of the ectopic foci responsible for initiating AF originated from the PVs. Awareness of PV automaticity actually predates to their involvement in the initiation of paroxysmal AF\textsuperscript{104,105}. Evidence supporting automaticity in PVs comes from embryonic development, where markers, such as HNK-1\textsuperscript{106,107}, are expressed in areas destined to become SA node, as well as the PV region. However, abnormal cell types with pacemaking properties, harbored in the PV region of adult tissue remains to be established\textsuperscript{108-111}. If the ectopic focus drove the rest of the tissue, the following nodal characteristics are required: a gradual transition of electrical coupling from the central to the peripheral region of the node (protect spontaneously firing cells from electrotonic inhibition), and a distributed anisotropy (allows a smaller input current from the focus to facilitate propagation)\textsuperscript{112,113}. Interestingly, Nguyen et al\textsuperscript{114} identified abnormal PV myocytes expressing HCN4 in chronic AF patients compared to sinus rhythm patients. A high degree of fibrosis and inflammatory infiltrates separated this population of cells and in effect provided an anisotrophic conductance medium for propagation of the driver\textsuperscript{115}. Therefore, an ectopic foci will become a sustained rapidly firing
entity if propagation from a cluster of abnormal cells in the PV\textsuperscript{104,105,114}, initiated by either EADs\textsuperscript{116} or pacemaker activity\textsuperscript{117}, combine with non-uniform anisotrophic conduction (fibrosis), moderately short refractory periods\textsuperscript{118}, and AP morphology heterogeneity in the PVs\textsuperscript{116} to enable a microreentry substrate.

### 1.3.2 Atrial refractoriness

As described in mechanisms of reentry, the wavelength (APD*CV) determines the theoretical minimum pathlength allowed for reentry. Therefore, a general reduction in refractoriness will shorten the wavelength and reduce the pathlength to promote reentry. Atrial refractoriness is synonymous with APD, and it is the temporal sum of the ion channel currents. Consequently, their expression and function are often associated with reduced atrial refractoriness and a sufficient background on the ionic constituents comprising the AP is then required to comprehend their role in reentry.

#### 1.3.2.1 The atrial action potential and ionic constituents

The nature of the atrial AP is a slave to the biophysical properties of the ion channels (i.e. activation/inactivation kinetics, rectification, voltage dependence, their respective current contributions, etc.) within the myocyte. The temporal activation of the ion channels is also significant to the morphology of the atrial AP and is characterized by 4 phases: Phase 0 corresponds to the AP upstroke (rapid depolarization) once the cell reaches threshold, Phase 1 represents the initiation of brief, but rapid repolarization following the upstroke. Phase 2 is characterized by a leveling off of the AP into the “plateau level”\textsuperscript{23}. Repolarization over this period slowly progresses until Phase 3 where rapid repolarization succeeds and levels off into the resting $E_m$ of Phase 4.

The $K^+$ current ($I_K$) provides the major membrane conductance at rest, but once the cell reaches threshold (approximately -65mV) activation of the voltage-gated Na$^+$ channels ($Na_v$) leads to a large, but brief influx of Na$^+$ ions (Phase 0). The inward Na$^+$ current ($I_{Na}$) depolarizes the $E_m$ to approximately +40mV and initiates the rapid activation of the transient outward K$^+$ current ($I_{to}$) (phase 1). The rapidly inactivating K$^+$ conductance causes a short-lived repolarization of the $E_m$. In addition, other voltage-dependent membrane channels are activated during the upstroke, but have slower activation kinetics. The balance between the inward $I_{CaL}$
and the delayed rectifier K\textsuperscript{+} outward current (\(I_{Kr}\)) determine the plateau shape (Phase 2) and has the greatest modulatory effect on AP duration (APD). Eventually, inactivation of the voltage-activated LTCCs and progressive activation of \(I_K\) (Phase 3) force the \(E_m\) towards the reversal potential of K\textsuperscript{+} (Phase 4). The Ca\textsuperscript{2+} ions built up in the cell during this phase, via the actions of \(I_{CaL}\) and release from intracellular stores, must be extruded via the actions of the NCX. For every single Ca\textsuperscript{2+} ion that is extruded, three Na\textsuperscript{+} ions are brought in, contributing a small depolarizing current (\(I_{NCX}\)) further delaying the repolarization. The \(E_m\) rests around -70mV to -80mV, and is maintained by the inward rectifying K\textsuperscript{+} current (\(I_{K1}\)). The characteristics of the human atrial AP and its ionic components are summarized in Figure 1.3.2.1.
Figure 1.3.2.1 Ionic constituents of the atrial action potential.

Top right inset: comparison of APs obtained at 37°C at cycle lengths near sinus rhythm from different species. Top left inset: Simulated human AP using a Courtemanche model\textsuperscript{119}. Directly below are the time course, and main ionic currents responsible for the AP and its ionic constituents (bottom). Black shaded regions correspond to inward (depolarizing) current, while gray shaded areas represent outward (repolarizing) current. Bottom right, are the ionic currents, with the alpha ($\alpha$) subunit of the channel and their respective genes. Reproduced/adapted with permission from Schotten et al\textsuperscript{22} (doi: 10.1152/physrev.00031.2009).
1.3.2.2 Comparison of human and murine ionic constituents

Clearly illustrated in the top right inset of Figure 1.3.2.1 is the interspecies variation in the atrial AP. Specifically, the difference between the murine and human AP appears quite drastic, yet there are a number of similarities in the underlying ionic constituents responsible for the AP morphology. These major similarities in the ion channels and their function are sufficient to allow the mouse AP a suitable model for study.

The same depolarizing $I_{Na}$ drives the upstroke of the atrial AP in both humans and mice. All of the following $I_K$ components have been shown to have similar functionality in the atrial AP in both the human and mouse: $I_{K1}$; the inward rectifying current accountable for the resting $E_m^{121}$, $I_{to,f}$ and $I_{to,s}$; the rapidly inactivating and slow inactivating component of $I_{to}$ respectively$^{122}$, $I_{Kr}$; a prominent ultra-rapid activating delayed rectifier K$^+$ current (encoded by the voltage-gated K$^+$ channel (K$_v$) K$_v$1.5 in both species$^{121,122}$) driving early repolarization, and $I_{Kr}$; the inward rectifying current involved in late repolarization$^{123}$. Despite these similarities mouse atrial myocytes have additional $I_K$ components such as, $I_{kslow}^{122,124}$, which underlie the peak outward currents in mouse atrial cells. It was found to be expressed at much lower concentrations and have lower current densities compared to their ventricular counterparts ($I_{kslow}$ was present in only 40% of atrial myocytes). However, statistically rigorous designed methods used for separating and quantifying various kinetic components of $I_K$ in the mouse myocardium revealed that $I_{kslow}$ is composed of two components ($I_{kslow1}$ and $I_{kslow2}$) and in fact may be present in all atrial myocytes$^{125}$. Voltage-clamp studies on mouse atrial myocytes demonstrated the presence of another kinetically distinct Ca$^{2+}$-independent, depolarization-activated outward K$^+$ steady-state current ($I_{ss}$). $I_{ss}$ is partially encoded, approximately 50%, by the K$_v$2.1 and is a slowly activating, non-inactivating current important in late repolarization. The ACh activated $I_{KAcch}$, encoded by the inward rectifying K$^+$ channels (K$_{ir}$) K$_{ir}$3.1 and K$_{ir}$3.4$^{126}$, are responsible for APD shortening during vagal activity in both humans and mice$^{127}$. The voltage-activated $\alpha 1$ LTCCs (Ca$_v$) $\alpha 1C$ (Ca$_v$1.2) together with $\alpha 1D$ (Ca$_v$1.3) constitute the main depolarizing current present during the plateau phase of the atrial AP and share similar current-voltage relations in both humans and mice$^{52,128,129}$. Although the role of $I_{Cal}$ in Phase 2 is similar in the two species, the magnitude of $I_{Cal}$ is reduced in mouse atrial myocytes, accounting for the ‘dome’ (in contrast to a plateau) shaped morphology in the mouse atrial AP.
Despite differences in the novel $I_K$ between mice and humans (i.e. $I_{K,\text{slow}}$ and $I_{Ks}$) their influence to the APD are negligible compared to $I_{CaL}$ and $I_K$ ($I_{Kr}$ and $I_{Ks}$). The fine balance between $I_{CaL}$ and $I_K$ densities are crucial to maintaining the plateau phase of the atrial AP. Therefore, it is easy to conceive that reductions in $I_{CaL}$ and increases in $I_K$ densities will ultimately account for the greatest changes in APD.

### 1.3.2.3 L-Type calcium channels

The fundamental role of the high-voltage activated LTCCs is in EC coupling of cardiomyocytes, skeletal muscle and vascular smooth muscle cells\textsuperscript{130-132}. In addition, excitation-secretion coupling properties of osteoblasts\textsuperscript{133}, pancreatic beta cells\textsuperscript{134-137}, thyrotophs\textsuperscript{138}, corticotrophs\textsuperscript{139}, gonadotrophs\textsuperscript{140, 141} and parathyroid-hormone-producing cells of the parathyroid gland\textsuperscript{142} are modulated by LTCCs. In neuronal cells LTCCs are preferentially expressed on the membranes of cell bodies and neuronal cells where they mediate neurotransmitter release, gene transcription and cell signaling\textsuperscript{143}.

Biochemical characterization of high-voltage activated Ca\textsuperscript{2+} channels reveal a common homology of complexes and include: a pore-forming $\alpha$ subunit, a transmembrane domain, a disulfide-linked complex of $\alpha 2$ and epsilon ($\delta$) subunits, an intracellular beta ($\beta$) subunit, and in some cases, such as in LTCCs, a transmembrane gamma ($\gamma$) subunit\textsuperscript{131}. The pore forming $\alpha 1$ subunit can be used to distinguish the LTCCs encoded by the Ca\textsuperscript{v}1 (encoded by the CACNA gene) family which include: Ca\textsuperscript{v}1.1 ($\alpha 1S$), Ca\textsuperscript{v}1.2 ($\alpha 1C$), Ca\textsuperscript{v}1.3 ($\alpha 1D$) and Ca\textsuperscript{v}1.4 ($\alpha 1F$). The pore-forming $\alpha$ subunit gene of the LTCCs encodes for a protein containing four transmembrane-associated motifs (I-IV) and each motif is comprised of six membrane-spanning domains (S1-S6)\textsuperscript{130, 131, 144}. The $\alpha$ subunit of the LTCCs defines the unique electrophysiological and pharmacological properties of Ca\textsuperscript{2+} flux across the pore. For example, the different high- ($\alpha 1S$, $\alpha 1C$) and low-voltage activation ($\alpha 1D$, $\alpha 1F$), large single-channel conductance of Ca\textsuperscript{2+}, slow voltage-dependent inactivation of the channel, distinct regulation of current by cyclic adenosine mono-phosphate (cAMP)-dependent protein phosphorylation pathways and specific inhibition by Ca\textsuperscript{2+} antagonist drugs such as dihydropyridines (DHP), phenylalkylamines and benzothiazepines\textsuperscript{130, 131, 144}.

Extensive studies on the biophysical properties of cardiac $I_{CaL}$ have given insight into the activation, inactivation and recovery profile of Ca\textsuperscript{2+} channels and so a general overview of these
characteristics will be defined. The channels are rapidly activated by membrane depolarization, reaching a peak in approximately 2 to 7ms, depending on the temperature and $E_m$. Ca$^{2+}$ channel inactivation has a similar dependence on $E_m$, but is also time and intracellular Ca$^{2+}$ concentration–dependent. However, the main physiological mechanism of $I_{Ca,L}$ inactivation, on the time scale of an AP, depends on the fast Ca$^{2+}$-dependent inactivation. This phenomenon provides a negative feedback mechanism to limit the Ca$^{2+}$ entry into the cell. The mechanism of Ca$^{2+}$-dependent inactivation has been demonstrated clearly to be due to calmodulin (CaM). At the C-terminal tail of the $\alpha$1 subunit (near the ‘IQ’ motif) CaM is prebound to the Ca$^{2+}$ channel at rest (before channel activation/rises in intracellular Ca$^{2+}$ concentration). It is postulated to serve as a constituent calcium sensor and upon channel opening, Ca$^{2+}$ moving through the channel immediately binds to high-affinity Ca$^{2+}$-binding sites on the C-terminal lobe of CaM. Ca$^{2+}$ binding induces a conformational change in CaM and relieves the EF-hand–mediated inhibition of the I-II linker. The I-II linker is now free to occlude the pore of the channel and initiate inactivation. Once the Ca$^{2+}$ channel inactivates, recovery from inactivation also depends on intracellular Ca$^{2+}$– (recovery is delayed by high cytosolic Ca$^{2+}$ concentrations at a given $E_m$) and $E_m$-dependent (upon AP repolarization recovery becomes rapid). Ca$^{2+}$ channels also experience a unique process of voltage facilitation during increasing beating frequencies, whereby a progressive increase in $I_{Ca,L}$ amplitude and a prominent slowing of inactivation accumulates over subsequent beats. The exact molecular mechanism of $I_{Ca,L}$ facilitation has not been fully elucidated, however it is believed that Ca$^{2+}$/CaM-dependent protein kinase II (CaMKII) modulates this process by phosphorylation of the N and C termini of the $\alpha$1C subunit, as well as on the accessory $\beta$2 subunit.

In the heart, the two major LTCCs currents are through Ca$_v$1.2 and Ca$_v$1.3. The relative $I_{Ca,L}$ contribution of each LTCC isoform was proven to be a difficult task since both isoforms are extremely difficult to distinguish pharmacologically. Ca$_v$1.3 is only slightly less sensitive to DHPs than Ca$_v$1.2. However, transgenic mice, such as specific gene knockout of Ca$_v$1.3 (CACNA1D), selective elimination of the high DHP sensitivity within the Ca$_v$1.2 $\alpha$1C subunit (replacement of threonine 1066 in helix IIIS5 with a tyrosine residue) or genetic knockout of a single Ca$_v$1.2 allele (CACNA1C), have allowed for the determination of the relative $I_{Ca,L}$ contribution of each LTCC isoform.
Throughout the heart Ca\textsubscript{v}1.2 is highly expressed in both the ventricles and the atria, as well as in the specialized cell types comprising the conduction system of the heart including the SA node, AV node and Purkinje fibers\textsuperscript{130,136}. The role of Ca\textsubscript{v}1.2 within the working myocardium of the heart is to act as a trigger for EC coupling and initiate contraction of the ventricles\textsuperscript{130,131}. Studies in transgenic mice generated with a selective mutation to the DHP sensitivity region of the $\alpha1C$ subunit of the Ca\textsubscript{v}1.2 (Ca\textsubscript{v}1.2\textsuperscript{DHP-/-})\textsuperscript{161,165}, and heterozygous mice for Ca\textsubscript{v}1.2 (Ca\textsubscript{v}1.2\textsuperscript{+/-})\textsuperscript{162} revealed the predominant ionotropic role and EC coupling properties of Ca\textsubscript{v}1.2 in the working myocardium of the ventricles. Cells isolated from the ventricles of Ca\textsubscript{v}1.2\textsuperscript{+/-} mice display reductions in $\text{Ca}^{2+}$ handling, represented by significantly reduced electrically and caffeine evoked $\text{Ca}^{2+}$ transients, which was associated with a 25\% reduction in peak $I_{\text{CaL}}$ density\textsuperscript{162}. Associated with impaired $\text{Ca}^{2+}$ handling were significant reductions in myocyte shortening, ventricular fractional shortening, as well as cardiac contractility measured ex vivo.

The localization pattern of Ca\textsubscript{v}1.3 in the heart has been described to be limited to the cardiac conduction system (including the SA node, AV node and Purkinje fibers) as well as in the working myocardium of both atria, but not in the ventricles\textsuperscript{159,161,166}. Studies utilizing the transgenic knockout mouse of Ca\textsubscript{v}1.3 (Ca\textsubscript{v}1.3\textsuperscript{-/-}) revealed the critical role of pacemaker activity in the SA node\textsuperscript{52,167}. Ca\textsubscript{v}1.3\textsuperscript{-/-} mice display a dramatic cardiac phenotype, characterized by severe bradycardia, sinus arrest, supraventricular and ventricular extrasystoles, delayed AV conduction, varying degrees of AV node block, a decoupling of heart rate (HR) and PR interval duration, prolonged PR intervals, prolonged P-wave durations and an increased vulnerability to AF\textsuperscript{52,160,167-169}. Spontaneous APs recorded from cells isolated from the SA node of Ca\textsubscript{v}1.3\textsuperscript{-/-} mice showed a decrease in beating frequency, prolonged cycle lengths and reductions in the rate of diastolic depolarization (most notably at more depolarized potentials), suggesting a dysfunction intrinsic to the SA node\textsuperscript{52}. Whole-cell $I_{\text{CaL}}$ in SAN myocytes isolated from Ca\textsubscript{v}1.3\textsuperscript{-/-} mice presented significant reductions in $I_{\text{CaL}}$ density, a prolongation of APD with respect to 80\% repolarization, and a significant right-ward depolarization shift in activation\textsuperscript{136,167}. In mouse SA node cells, unlike Ca\textsubscript{v}1.2, Ca\textsubscript{v}1.3 undergoes voltage-dependent facilitation and co-localizes with ryanodine receptor (RyR) type 2 in sarcomeric structures\textsuperscript{170}. These properties of Ca\textsubscript{v}1.3 can improve recovery of pacemaking after pauses and stabilize SAN pacemaking during excessively slow HRs. Taken together, these findings suggest that Ca\textsubscript{v}1.3 plays a fundamental role in cardiac pacemaking as $\alpha1D$ LTCCs drive automaticity of the SA node by activating at more negative
potentials (compared to Ca\textsubscript{v}1.2) and accelerating the slope of the diastolic potential (range from -65 and -40 mV\textsuperscript{52,136,160}.

Outside of the SA node, atrial myocytes isolated from Ca\textsubscript{v}1.3\textsuperscript{−/−} mice displayed reduced intracellular Ca\textsuperscript{2+} transients and delayed times to peak cytosolic Ca\textsuperscript{2+} levels following field stimulation\textsuperscript{167}. Further investigation of Ca\textsubscript{v}1.3\textsuperscript{−/−} right atrial myocytes displayed diminished \(I_{\text{CaL}}\) density represented by a reduced \(I_{\text{CaL}}\) peak density at +10 mV\textsuperscript{167}. Also, a depolarization shift (13 mV) in the \(I_{\text{CaL}}\) peak density of the current-voltage (IV) plot verified the loss of Ca\textsubscript{v}1.3, and unmasked the contribution of the Ca\textsubscript{v}1.2 current\textsuperscript{52,167}.

These unique electrophysiological and biophysical properties of Ca\textsubscript{v}1.3 will be important in describing the specific role of Ca\textsubscript{v}1.3 \(I_{\text{CaL}}\) loss, compared to Ca\textsubscript{v}1.2 \(I_{\text{CaL}}\), in development of an AF substrate. Therefore, studies carefully dissecting Ca\textsubscript{v}1.3 \(I_{\text{CaL}}\) from Ca\textsubscript{v}1.2 \(I_{\text{CaL}}\) with the above-described transgenic animals will discern their exclusive contribution to the physiology of the atria and potentially give insight into how these disrupted pathways can lead to a pathological substrate for AF.

1.3.2.4 Ionic mechanisms of reduced atrial refractoriness

The duration of the plateau phase is governed by the opposing current densities supplied by \(I_{\text{CaL}}\) and \(I_{\text{K}}\), and ultimately has the greatest influence on APD. The APD of an atrial myocyte is almost synonymous with the refractoriness of that cell, and the APD\textsubscript{80} (APD at 80\% repolarization) describes most accurately the refractoriness of a myocyte\textsuperscript{171}. Chronic or persistent AF will lead to structural and electrical remodeling in the atria, which preserve the mechanism of reentry, hence the term ‘AF begets AF’. AF is generally associated with reductions in depolarizing inward current (\(I_{\text{Na}}, I_{\text{CaL}}, I_{\text{NCX}}\)), and/or increases in repolarizing outward currents (\(I_{\text{K}}\)), which result in abbreviations in the APD\textsuperscript{172-177}.

Specifically, patients with persistent AF display consistent and relatively rapid electrophysiological changes associated with progression of paroxysmal to persistent AF\textsuperscript{178-180}. Persistent AF in humans has been associated with reductions in atrial APDs, with persistent AF patients having shorter APDs (during sinus rhythm) compared to patients with sinus rhythm. Human right atrial cells from these patients had a significant reduction (~70\%) in \(I_{\text{CaL}}\) density\textsuperscript{129,180,181}, and were associated with a reduction in mRNA\textsuperscript{182-184} and protein\textsuperscript{184} levels of the \(\alpha_1\) pore
forming subunit of the LTCCs. Similarly, in rapidly paced canine atria, calcineurin/NFAT-dependent reductions in the α1C pore forming subunit (Ca\textsubscript{v}1.2)-based \( I_{\text{CaL}} \) were associated with AF\textsuperscript{185,186}. This evidence combined suggested APD abbreviations in human AF were likely due to reduced Ca\textsubscript{v}1.2 \( I_{\text{CaL}} \). In addition, genetic ablation of Ca\textsubscript{v}1.3 was linked to AF in mice\textsuperscript{51,52,67,187}. Ca\textsubscript{v}1.3\textsuperscript{-/-} mice were more susceptible to AF when induced with a programmed electrical stimulation \textit{in vivo}\textsuperscript{136} as well as in Langendorff-perfused hearts\textsuperscript{167}. Furthermore, human mutations in the gene encoding the multifunctional scaffolding protein, ankyrin B, caused reductions in Ca\textsubscript{v}1.3 \( I_{\text{CaL}} \), which was associated with AF in humans\textsuperscript{188-190}. Although this association with Ca\textsubscript{v}1.3 and AF has been made, the underlying mechanisms for this observation are unknown. For example, the atrial effective refractory period was indifferent between Ca\textsubscript{v}1.3\textsuperscript{+/-} and Ca\textsubscript{v}1.3\textsuperscript{-/-} mice\textsuperscript{136}, suggesting that a simple reduction in \( I_{\text{CaL}} \) will not invariably lead a reduction in refractoriness. These mice however, did display a slower time to peak of the Ca\textsuperscript{2+} transient, a reduced Ca\textsuperscript{2+} transient amplitude, a decreased SR Ca\textsuperscript{2+} content, and a diminished fractional Ca\textsuperscript{2+} release (reduced). All of this evidence would suggest a disruption in Ca\textsuperscript{2+} homeostasis, which through a number of mechanisms (EADs or DADs, Ca\textsuperscript{2+} alternans, altered biochemical Ca\textsuperscript{2+} signaling) could provide a trigger or substrate for AF.

A reduction in the depolarizing inward current \( I_{\text{Na}} \) can also abbreviate the APD, and a number of genetic mutations in the Na\textsuperscript{+} channel subunits were associated with AF\textsuperscript{191-193}. In contrast, a wide and large variety of increases in repolarizing K\textsuperscript{+} currents have been documented in AF\textsuperscript{194-199}. These findings propose an ionic mechanism for the abbreviation in APD predisposing to reentry\textsuperscript{40,178}, however dispersion of refractoriness throughout atrial tissue (heterogeneity of APD) can also promote reentry\textsuperscript{200-203}.

Regional-specific reductions in APD throughout the atria have been associated with the repolarizing outward current, \( I_{\text{KACb}} \), and are mediated by vagal stimulation (parasympathetic nerve endings and/or M\textsubscript{2}-cholinergic receptors are spatially heterogeneous) or pacing\textsuperscript{200,204-206}. The increase in the dispersion of refractoriness produces a favorable substrate for spiral wave reentry\textsuperscript{207}, and inhibition of \( I_{\text{KACb}} \) with tertiapin terminated AF in the dog\textsuperscript{208}. These effects are restricted to the atrial myocardium, since the pore-forming Kir3.x α-subunits are exclusively expressed in the atrial myocardium\textsuperscript{209}. It has long been proposed that AF is promoted by cholinergic activity, and clinical\textsuperscript{210,211} and experimental\textsuperscript{204,212} studies have solidified this association. Although, recent studies suggest that vagal stimulation, in combination with
autonomic activity (as described in section 1.3.2), may have an even greater stabilizing effect on AF via late phase 3 EADs\textsuperscript{213,214}. It is also conceivable that sympathetic activation synergistically amplifies the effect of the parasympathetic nervous system\textsuperscript{215}.

1.3.3 Atrial fibrosis

Considered to be one of the most important factors in the generation of a substrate for AF is atrial fibrosis\textsuperscript{216,217}. Biopsies from patients with AF have confirmed the presence of atrial fibrosis\textsuperscript{114,218}, and post-operative AF\textsuperscript{219} or the recurrence of AF\textsuperscript{220} has been correlated with the degree of atrial fibrosis and fibrogenic activity. Many experimental and clinical studies show the development of the AF substrate can be hindered if atrial fibrosis is prevented\textsuperscript{94}, although atrial fibrosis is only partly reversible\textsuperscript{221}. These findings, together with strong indications that animals models also confirm atrial fibrosis to be proarrhythmic\textsuperscript{222,223}, make preventative strategies targeting fibrosis critical.

Cardiac fibrosis is the product of fibrous tissue in a normal physiological process, but when an imbalance favoring the extracellular matrix deposition over degradation, the disproportionate buildup of extracellular proteins in the interstitial space ensues. Normally in the myocardium, these structural networks of matrix proteins reinforce structural integrity to adjoining myocytes and also help to coordinate their fractional shortening\textsuperscript{224}. The matrix proteins are highly organized in structure and architecture, and are composed of collagen type I and type III (collagen type III is more prominent in the heart). Matrix protein aggregation form two distinct fibrous networks: perimysial sheaths, to surround whole muscle fibers to group them together into bundles, and endomysial fibrous tissue, to support and connect individual myocytes within these bundles respectively\textsuperscript{225}. Perimysial fibers are composed of broad wavy strands orientated perpendicular to the long axis of the muscle, while endomysial fibers form fine coil-like structures running parallel to the muscle bundles. Atrial fibrosis can present itself in various degrees and forms. Increased transverse fiber separation, via deposition of collagen type I bundles\textsuperscript{226}, is often observed with fibrosis associated with structural remodeling of heart disease and normal ageing\textsuperscript{227,228}. In animal models of atrial dilatation\textsuperscript{229,230} and congestive heart failure\textsuperscript{231} a more profound process of structural remodeling occurs, called ‘replacement fibrosis’, whereby large depositions of collagen are needed to replace lost function myocardium secondary to tissue damage and cell death. The degree of fibrosis will determine conduction disturbances in
the tissue, but before this connection can be made, anistropic conduction in a functional syncytium must be entertained.

Anisotropy, the property of being directionally dependent, applies to the myocardium since there is a bias pathway for conduction, which is inherent to the parallel arrangement of elongated cells\textsuperscript{232}. In the atria, propagation of conduction presents \textit{uniform} fixed anisotropy, defined by rapid conduction in one direction (longitudinal) and more slowly in another (transverse). Stable propagation of a depolarized wavefront occurs when the cell supplies enough current (source) to depolarize itself and charge the capacitance of the neighboring tissue (sink)\textsuperscript{217}. An electrical "load" is placed on the activated tissue because of the capacitance and membrane resistance of the downstream tissue. Recurrent discontinuities within the cellular interconnections will alter the apparent electrical load the wavefront experiences and as a result, propagation can fail in one direction with greater ease than in another (unidirectional conduction slowing). An example of an amplified electrical load is deposition of collagenous septa, because it significantly increases the membrane resistance between adjacent myocytes\textsuperscript{233}. This loss of side-to-side fiber connection leads to slowed CV and \textit{non-uniform} anistrophic conduction ("zig-zag conduction")\textsuperscript{234}. If the discontinuities in conduction are large enough, it can lead to unidirectional block, and allow for reentry in relatively small circuits\textsuperscript{94}.

1.3.4 Cardiac alternans and dispersion of refractoriness

Another important factor contributing to the development of reentry and pathogenesis of arrhythmias, are electromechanical cardiac alternans, characterized by the cyclic, beat-to-beat variations in APD (electrical alternans) and contraction (Ca\textsuperscript{2+} transient) amplitude (mechanical or Ca\textsuperscript{2+} alternans)\textsuperscript{235}. The first clinical sign of cardiac alternans (in the form of arterial pulsus alternans) was presented in a patient with alcoholic cardiomyopathy\textsuperscript{236} and was soon after categorized as an ECG T wave abnormality\textsuperscript{237}. In fact, instances where predisposing factors to arrhythmias are common (acute myocardial ischemia, genetic channelopathies, and drug and electrolyte disturbances), experimental findings demonstrated the presence of electrical and mechanical alternans\textsuperscript{238}. This association between arrhythmia risk and cardiac alternans (in the form of T-wave alternans) was later confirmed in human clinical trials\textsuperscript{239,240}. Convincing evidence in animal models\textsuperscript{241,242}, numerical simulations\textsuperscript{243}, and directly in humans\textsuperscript{244-247}, have shown that cardiac alternans may induce AF or facilitate the transition from flutter to fibrillation.
Studies by Narayan et al\textsuperscript{246} demonstrated rate maladaptations leading to APD alternans were involved in the progressive disorganization of atrial flutter to fibrillation. The absence of APD alternans was associated with patients where AF failed to transition. Therefore, the evidence supporting cardiac alternans as a prerequisite to fibrillatory events, mediated by conduction block and wavebreak, is strong. However, before the arrhythmogenic potential of cardiac alternans at the tissue level can be understood, dynamic oscillations at the single cell level, developing into each type of alternan (electrical vs. mechanical) must first be described.

1.3.4.1 Electrical alternans

In a number of diverse conditions, the alternation in the AP has been illustrated in ECG from cardiac tissue in both human and animals\textsuperscript{248-252}. During regular rhythmic activity, the transmembrane APs are extremely constant, but when the beating rate is suddenly changed or even one perturbation in the cycle length occurs, AP variation will ensue\textsuperscript{249, 252}. Adjacent electrical complexes will begin to reciprocally alternate in voltage, duration, and shape. In the seminal 1968 paper by Nolasco and Dahlen\textsuperscript{253} on cardiac alternans, the relationship between alternation and rate was first explicitly defined. Utilizing mathematical principles, they devised a graphic model to predict how a change in rate would affect APD in electrical circuits. They generated “restitution curves” by plotting the APD against its preceding diastolic interval (DI). Generally, APD decreases monotonically with shortening DIs, and can be fit to a monotonic or mono-exponential equation. The basic assumption of the restitution process is that on any given beat, the APD is strictly determined by the duration of repolarization of the previous beat (DI). The standard experimental method for generating the restitution curve uses an interpolated beat (S2) to interrupt a steady-state pacing train (S1) with a set cycle length and pulse number\textsuperscript{254-256}. The interval between S1–S2 (synonymous with DI) is progressively decreased until loss of capture.

Using their graphical model, Nolasco and Dahlen\textsuperscript{253} demonstrated that sustained APD alternans occurred at the DIs were the slope was >1 (on the mono-exponential curve) at a given cycle length. At slopes >1, small changes in the DI lead to significantly greater changes in the resultant APD and at this point a dynamic instability (bifurcation) follows and causes APD to alternate. Amplified APD alternans will result in either stable wavelength oscillations or if the APD alternans amplitude continue to expand, 2:1 conduction block is inevitable\textsuperscript{257}. Studies in
dog Purkinje muscle fibers, by Saitoh et al\textsuperscript{258}, established the relationship between the steepness of the restitution curve to the magnitude of APD alternans. By using lidocaine to adjust the slope of the restitution curve, they found APD alternans magnitude increased proportionally to the steepness of the restitution curve slope. Pharmacological agents, such as verapamil and procainamide, eliminated APD alternans in canine hearts by selectively flattening the restitution curve\textsuperscript{259}. These findings suggest the restitution hypothesis is a good predictor of APD alternans susceptibility, but by no means does a slope $>1$ imply a certainty for APD alternans. For example, studies by Hall et al\textsuperscript{260} showed that when the slope reached significantly $>1$, APD alternans were absent. The opposite was found in ischemic hearts, where alternans formed even though the restitution curve never reached a slope $>1$\textsuperscript{261}. Furthermore, APD alternans in the epicardium of guinea hearts showed no correlation with restitution curve steepness\textsuperscript{262}.

Physiologically, electrical restitution is an important process, because at high HRs a reduction in the duration of systole allows adequate filling of the ventricles during diastole\textsuperscript{256}. This effect has been attributed to the actions of the sympathetic nervous system in the intact animal\textsuperscript{263}, but experimental studies on isolated tissue have demonstrated the intrinsic rate-dependent adaptation of the AP abbreviation\textsuperscript{249}. The preceding DI has a large influence on the APD, due to the fine balance between a number of inward and outward currents maintaining the AP plateau\textsuperscript{264}. Many channels are sensitive to the previous DI because of their biophysical time-dependent recovery from inactivation kinetics and a number of studies have attempted to explain the ionic mechanisms responsible for APD restitution. Recovery from inactivation of the LTCC at most cycle lengths has been shown to be an important factor in the restitution process. Gettes and Reuter\textsuperscript{265} revealed the governing role of LTCC inactivation kinetics in the restitution curve shape of mammalian myocardial fibers. Studies at physiological temperatures in rabbit ventricular myocytes by Goldhaber et al\textsuperscript{266}, further illustrated the dependence of restitution curve steepness on $I_{Ca,L}$ recovery kinetics. At short DIs, incomplete recovery from inactivation of the LTCC reduces $I_{Ca,L}$ peak density and accelerates repolarization (short APD)\textsuperscript{264}. At even shorter DIs, Na$^{+}$ channel inactivation begins to impinge on $I_{Ca,L}$ because diminished $I_{Na}$ will lead to less activation of $I_{Ca,L}$\textsuperscript{267}. APD restitution curves with a non-monotonic relationship have been observed in some cases\textsuperscript{268} and can be explained by these activation and deactivation kinetics of the ion channels influencing restitution\textsuperscript{267}. 
The fact that only the ion channel kinetics during the previous DI affects APD is an oversimplification, and the cycle length (S1 interval) also influences the restitution process. Experimental methods that varied the cycle length resulted in different restitution curves and were explained by the cells sensitivity to its pacing history. In the literature this phenomenon is referred to as “cardiac memory” or “APD accommodation”, and it will directly impact the APD at a future beat. Specifically, incomplete deactivation of $I_{Ks}$, especially the delayed inward rectifiers $I_{Kr}$ and $I_{Ks}$, incomplete reactivation of $I_{to}$ and incomplete reactivation of $I_{CaL}$ will affect the APD of the test potential (S2)\textsuperscript{273-276}. Tolkacheva et al\textsuperscript{277} and Kalb et al\textsuperscript{278}, formulated other generalized mathematical models to account for arbitrary amounts of memory and to predict the onset of alternans.

As previously stated, the slope of the restitution curve can determine the degree of APD alternans, which are the prerequisite for wavebreak and a substrate for reentry. Consistent with this, the induction and stabilization of ventricular fibrillation (VF) were mitigated with drugs that flattened the restitution curve\textsuperscript{268, 279-282}. In addition, restitution curves obtained during VF revealed a slope >1 and created dynamic APD heterogeneities facilitating conduction block and wavebreak\textsuperscript{259, 268, 283}. A steep restitution curve as a determinant for AF has also revealed a strong relationship. Patients with chronic or paroxysmal AF had a steeper restitution slope compared to control subjects\textsuperscript{284}. Factors increasing the slope of the curve, such as rapid atrial pacing or isoproterenol (ISO), increased the incidence of AF in both humans\textsuperscript{285} and canine\textsuperscript{242}. Further, non-uniform restitution kinetics in the PVs\textsuperscript{247}, and other localized regions throughout atrial tissue\textsuperscript{286} created local gradients or dispersion of repolarization contributing to the substrate for AF. However, skepticism in the field suggests APD restitution obtained during VF or AF fails to represent true APs capable of propagation\textsuperscript{287}. In actual fact they may represent graded potentials or electrotonic potentials due to conduction block and result in misleadingly steeper curves.

Even with all of these corrections to the restitution hypothesis, the equations to model the process is dependent on the membrane voltage history of the cell, and ignores the intrinsic nonlinear dynamics of calcium cycling within the cell. Ca\textsuperscript{2+} and voltage are bidirectionally coupled through Ca\textsuperscript{2+} sensitive ion channels (LTCCs) and exchangers (NCX)\textsuperscript{288}. Experiments by Chudin et al\textsuperscript{289} revealed Ca\textsuperscript{2+} transient alternans could be sustained independent of $E_m$ by clamping the voltage with an AP waveform. Therefore, both the restitution process and the dynamic intracellular Ca\textsuperscript{2+} fluxes must be appreciated in order to conceptualize alternans in a
coupled nonlinear system. These dynamic calcium fluxes mediate the physiological process of EC coupling in all cells, although this process is also inherently susceptible to unstable oscillations (Ca$^{2+}$ alternans), especially at high heart rates or with an underlying cellular pathology.

1.3.4.2 Mechanical (calcium) alternans

In this section, the mechanisms of Ca$^{2+}$ alternans will be described, but not before an in depth explanation of EC coupling in atrial myocytes is given, since this process is fundamental to Ca$^{2+}$ alternan development and stability.

1.3.4.2.1 Excitation-contraction coupling in atrial myocytes

Cardiac function is integral to Ca$^{2+}$ because it bridges the membrane depolarization of cardiomyocytes with contraction (EC coupling)$^{130}$. Cardiomyocytes contain structures, referred to as “dyadic” or “triadic” clefts$^{290}$, and are characterized by an interface of the sarcolemma membrane with the intracellular membrane of the SR (separated by ~10nm of space). The SR contains the internal Ca$^{2+}$ stores and within the SR membrane themselves are Ca$^{2+}$-sensitive Ca$^{2+}$ RyRs, with RyR type 2 as the predominant isoform in cardiac cells$^{291}$. Upon cellular depolarization, activation of LTCCs leads to a small influx of Ca$^{2+}$ into the cleft and causes a much larger release of Ca$^{2+}$ (from the SR) through the RyRs via the process Ca$^{2+}$-induced Ca$^{2+}$ release (CICR)$^{292,293}$. Recent studies by Christal et al$^{170}$ have identified Ca$_{v}1.3$ to be more intimately coupled with RyR2 in sarcomeric structures, compared to Ca$_{v}1.2$, of SA nodal cells from mice. Contraction of the cell is then initiated when Ca$^{2+}$ binds to troponin C, activating the actin and myosin contractile filaments. Initiation of relaxation (diastole) involves the decline of the cytosolic Ca$^{2+}$ mainly by resequestration of Ca$^{2+}$ into the SR by an ATP-dependent enzyme, known as the sarcoendoplasmic reticulum Ca$^{2+}$-ATPase (SERCA), and extrusion into the extracellular space via NCX$^{294,295}$.

It has been suggested that ~100 RyRs cluster with 10-25 LTCCs on the SR and sarcolemma membrane respectively and form a local SR Ca$^{2+}$ release unit (CRU) called the junction (or couplon)$^{296}$. A Ca$^{2+}$ spark, or spontaneous local Ca$^{2+}$ transient, is Ca$^{2+}$ release from spatially discrete individual clusters of RyRs and was recorded using confocal microscopy with Ca$^{2+}$ imaging$^{297}$. Whole cell transients are achieved when stimulation of the cell with an AP
activates $I_{\text{Ca,L}}$, leading to the spatial-temporal recruitment of these elementary events ($\text{Ca}^{2+}$ sparks)\textsuperscript{298}. A graded $\text{Ca}^{2+}$ transient can be achieved due to the independent activation of couplons upon a varying degree of membrane depolarization. The local control model, first described by Micheal Stern in 1996\textsuperscript{299}, and advances in subcellular $\text{Ca}^{2+}$ imaging\textsuperscript{297}, confirmed that the $\text{Ca}^{2+}$ response can be graded. Upon membrane depolarization independent activation of couplons, and not adjacent couplons, is achieved by localized fluxes of $\text{Ca}^{2+}$ through their respective LTCCs. The whole cell $\text{Ca}^{2+}$ transient then becomes the sum of discretely activated couplons upon varying degrees of membrane depolarization. $\text{Ca}^{2+}$ sparks are considered “primary” when they are triggered by $\text{Ca}^{2+}$ entering through the LTCCs, or spontaneous opening of RyRs at a couplon, while secondary sparks are diffusion of $\text{Ca}^{2+}$, or $\text{Ca}^{2+}$ waves, leading to activation of distal couplons (often under conditions of intracellular $\text{Ca}^{2+}$ load)\textsuperscript{300,301}.

Atrial myocytes have a different ultrastructure compared to ventricular myocytes, and the consequence is a modification in the spatiotemporal control and pattern of $\text{Ca}^{2+}$ release\textsuperscript{302}. Compared to ventricular cells, atrial cells either do not contain any appreciable T-tubules or they have very simple and irregular transverse T-tubule networks\textsuperscript{290,302,303}. Specifically, in smaller mammals, such as the rat\textsuperscript{304} and the mouse\textsuperscript{305}, a T-tubular network is occasionally detected, however it is far less complex than its ventricular counterpart. These T-tubular structures are known as transverse axial tubules (formed from SR membrane and not the sarcolemma) and run along the long-axis of the cell\textsuperscript{306}. Depending on whether these structures contain LTCCs or become depolarized during an AP will determine the heterogeneity of the systolic rise in cytosolic $\text{Ca}^{2+}$ concentration\textsuperscript{307}. In general, atrial myocytes contain two populations of RyRs: the junctional RyRs lying just beneath the sarcolemma (in close proximity to the LTCCs) and the non-junctional RyRs deeper inside the cell\textsuperscript{300,307-309}. The consequence of this organization is initiation of primary sparks only at the periphery of the cell, since LTCCs are only present on the sarcolemma in atrial myocytes, which forms a shell of elevated $\text{Ca}^{2+}$ around the cell\textsuperscript{309,310}. Whether a centripetal wave of $\text{Ca}^{2+}$ propagation will continue into the center of the cell will depend on “positive inotropic” factors, or the $\text{Ca}^{2+}$ content of the SR\textsuperscript{311}. Under basal conditions, the failure of centripetal $\text{Ca}^{2+}$ propagation is due to the combination of the following phenomena: the distance between junctional and non-junctional SR (~2µm) is too great to facilitate a secondary spark\textsuperscript{309-312}, the junctional RyRs are much more sensitive to CICR since they are physically coupled to the LTCCs (via the carboxyl terminal of the LTCC)\textsuperscript{313-317}, and buffers of
Ca\textsuperscript{2+}, such as SERCA, the mitochondria, and contractile myofilaments, impede diffusion of the signal\textsuperscript{311,318-320}. Refer to Figure 1.3.4.2.1 for a visual interpretation of atrial ultrastructure.
Figure 1.3.4.2.1 Atrial myocyte ultrastructure with calcium signaling machinery.

Note the arrangement of the LTCCs coupled with the RyRs only at the sarcolemmal membrane (junctional SR) with ‘orphaned’ RyRs in the central regions of the cell (non-junctional SR). The green band represents the boundary that the subsarcolemmal Ca\(^{2+}\) signal must transcend in order to recruit central RyRs and trigger maximal contraction. Reproduced/adapted with permission from Mackenzie et al\(^{311}\) (doi: 10.1242/jcs.01559).
Under conditions of increased physical activities or stress, the contractile forces of the atria must adequately refill the ventricles. Under basal conditions EC coupling in atrial myocytes is insufficient to provide such a force of contraction, and consequently enhanced centripetal propagation of Ca\(^{2+}\) throughout the myocyte must occur. This is achieved by using systems that enhance the influx of Ca\(^{2+}\) through the LTCCs, increase the diastolic Ca\(^{2+}\) concentration of the SR, and increase the sensitivity of the RyRs to CICR\(^{302,311,317}\). This can be accomplished through the sympathetic nervous system action on the heart by stimulation of β-ARs on the sarcolemma membrane\(^{130}\). The CICR “gain” (ratio of the resultant Ca\(^{2+}\) spark compared with the trigger Ca\(^{2+}\) concentration) can be increased by the multiple molecular mechanisms provided by PKA phosphorylation of LTCCs, phospholamban, and RyRs\(^{321}\). LTCC phosphorylation intensifies the trigger of Ca\(^{2+}\) influx by increasing the mean channel open time and/or channel open probability\(^{322}\). Negative regulation of SERCA sequestration activity by phospholamban is relieved by PKA phosphorylation, which increases the lumenal Ca\(^{2+}\) concentration\(^{323}\). The sensitivity of RyRs to CICR has a steep non-linear effect with respect to lumenal Ca\(^{2+}\) concentration\(^{324}\). Saturation of the Ca\(^{2+}\)-binding protein, calsequestrin, relieves negative allosteric regulation of RyR opening and promotes a more substantial Ca\(^{2+}\) flux\(^{325\text{-}328}\). RyR sensitivity to Ca\(^{2+}\) is further enhanced by the direct phosphorylation of the channel by PKA\(^{329}\). Therefore, all of these pathways promote the centripetal propagation of the Ca\(^{2+}\) signal, which allows the non-junctional SR to act as a positive inotropic reserve when cellular contraction is required.

Ca\(^{2+}\) transients restricted to the periphery of the atrial myocyte under basal conditions, and the global homogenous calcium transient during inotropic responses represents only the two extremes of Ca\(^{2+}\) signals in atrial myocytes. However, the response is a spatially graded continuum of Ca\(^{2+}\) release, which ultimately determines the degree of contraction. Studies by Mackenzie et al\(^{311}\) in rat atrial myocytes demonstrated the smooth graded response of Ca\(^{2+}\) release by increasing the Ca\(^{2+}\) concentration from 1mM to 10mM. The transient increase in extracellular Ca\(^{2+}\) concentration enhanced influx of Ca\(^{2+}\) through the LTCCs, which provided a sufficient trigger to allow central release of Ca\(^{2+}\). The positive inotropic effect of elevated extracellular Ca\(^{2+}\) was attributed solely to Ca\(^{2+}\) influx through the LTCCs, and not by an increased SR Ca\(^{2+}\) content. Graded responses may also be coordinated by the rudimentary transverse T-Tubule systems and their complexity depends on the species and the origin of the
atrial myocyte (left versus right atrial appendage (LAA, and RAA respectively))\textsuperscript{330}. Under basal conditions, a rapid \(\text{Ca}^{2+}\) release component in central regions of atrial myocytes was demonstrated\textsuperscript{304, 331}. Electrically elicited \(\text{Ca}^{2+}\) sparks originating from multiple independent regions in the cell were attributed to atrial myocytes with rudimentary T-tubules.

Accordingly, the unique ultrastructure of atrial myocytes (junctional versus non-junctional SR and transverse T-tubule system) in addition to all the processes governing the EC coupling ‘gain’ (increased LTCC influx, or increased RyR sensitivity to CICR) will ultimately determine the temporal and spatial graded \(\text{Ca}^{2+}\) responses. In the next chapter, it will be evident how perturbing the factors responsible for maintaining steady-state EC coupling, such as an immediate change in HR, will play a pivotal role in the initiation of \(\text{Ca}^{2+}\) alternans.

### 1.3.4.2.2 Mechanisms underlying calcium alternans

Since their first description as alternating arterial pulse pressure magnitudes\textsuperscript{236}, the mechanisms and clinical manifestations underlying mechanical alternans has been a persistent area of research\textsuperscript{332, 333}. Consensus in the field has hypothesized that repolarization alternans is initiated by the beat-to-beat SR \(\text{Ca}^{2+}\) release or \(\text{Ca}^{2+}\) alternans\textsuperscript{238, 257, 262, 323, 334, 335}. Although there has been no definitive experiment to prove \(\text{Ca}^{2+}\) alternans are the cause of repolarization alternans, there are is a wide range of evidence to suggest it is probable. It was shown that APD alternans could be inhibited or suppressed by using agents that interfered with cellular \(\text{Ca}^{2+}\) cycling, such as verapamil\textsuperscript{336, 337}, caffeine\textsuperscript{258, 337}, BayK8644\textsuperscript{338}, nisoldipine\textsuperscript{338}, and ryanodine\textsuperscript{258}. More conclusive experiments demonstrated that by clamping the AP waveform, \(\text{Ca}^{2+}\) alternans could occur independently or in the presence of APD alternans\textsuperscript{289, 339}. During mechanical alternans, the direct measurements of \(I_{\text{Cal}}\) have demonstrated peak \(I_{\text{Cal}}\) remains unchanged\textsuperscript{340}. In \emph{ex vivo} experiments, using confocal microscopy, \(\text{Ca}^{2+}\) alternated out of phase between adjoining cells\textsuperscript{341}. Since electrotonic properties force AP to synchronize over greater regions, it was proposed the AP could not have any significant discontinuities at the scale of two adjoining cells. Therefore, at least one of the cells was alternating independent of the voltage and modeling studies have confirmed this phenomenon\textsuperscript{342}. If then \(\text{Ca}^{2+}\) alternans are likely the cause of repolarization alternans, what then are the underlying mechanisms leading to oscillations in \(\text{Ca}^{2+}\) transients and how do they translate to APD alterations?
The susceptibility to mechanical alternans was first identified as a fundamental property in mammalian ventricular muscle when paced at rapid driving frequencies\textsuperscript{343}. A critical threshold cycle length was determined in experimental studies that varied the pacing cycle length of a wide range\textsuperscript{344-347}. Driving at this particular cycle length will produce transient alternans, while surpassing the threshold will increase the magnitude in the oscillations of the contraction strength. The cycle length defining the boundary of stability is different across mammalian species\textsuperscript{347, 348}, and factors that increase the cycle length threshold (lower heart rate) include: hypothermia\textsuperscript{349}, hypocalcemia\textsuperscript{335, 344, 348, 350}, hypercapnic acidosis\textsuperscript{339, 351}, ischemia\textsuperscript{352-355}, hypertrophy\textsuperscript{356}, and congestive heart failure\textsuperscript{357, 358}. In contrast, factors known to reduce the cycle length threshold (higher heart rate) include: β-AR agonists\textsuperscript{340, 344, 345, 348, 359, 360}, hypercalcemia\textsuperscript{344, 348}, digitalis\textsuperscript{344} and Ca\textsuperscript{2+} channel antagonists\textsuperscript{336-338, 352}. Two possible explanations could account for this phenomenon, either it is purely mechanical and is based on the Frank-Starling relationship, or it involves the process of myocardial excitation by Ca\textsuperscript{2+}. The first mechanism suggests a strong beat forces a large volume from the ventricles leaving a residual end-systolic volume, and on the next beat the resultant small end-diastolic volume reduces the force of contraction on the next beat. Experimental protocols have confirmed this observation \textit{in vivo}, and with isolated hearts have shown at high HRs the previously described process was enhanced due to shorter diastolic filling times\textsuperscript{361-364}. Load-independent environments\textsuperscript{339, 365}, such as isolated papillary muscles\textsuperscript{235, 338, 347, 348, 366}, ventricular myocytes\textsuperscript{339, 365, 367}, or atrial myocytes\textsuperscript{340, 368-370}, could sustain mechanical alternans on their own, and alluded to the intracellular Ca\textsuperscript{2+} transient as the origin. Early studies indicated the mechanism for this observation was insufficient time between successive transients for Ca\textsuperscript{2+} uptake into the SR, and as a result smaller SR Ca\textsuperscript{2+} loads and release. Further investigation into this idea revealed two important factors maintaining the stability of Ca\textsuperscript{2+} cycling between contractions.

Fractional Ca\textsuperscript{2+} release from the SR (dependent on diastolic SR Ca\textsuperscript{2+} load)\textsuperscript{323, 371-373}, and the efficiency of beat-to-beat cytosolic Ca\textsuperscript{2+} sequestration (dependent on Ca\textsuperscript{2+} re-uptake via SERCA, extrusion via NCX and plasmalemmal Ca\textsuperscript{2+}-ATPase, cytosolic buffering, mitochondrial uptake, and diastolic SR Ca\textsuperscript{2+} leak)\textsuperscript{370, 374, 375} are two dynamic interacting parameters critically important to the regulation of intracellular Ca\textsuperscript{2+} on a beat-to-beat basis. A steep fractional release curve was first proposed by Eisner et al\textsuperscript{373} (later established by computer simulations\textsuperscript{330, 376, 377} and theoretical studies\textsuperscript{257, 378, 379}) to explain an increased susceptibility to Ca\textsuperscript{2+} alternans. The
theory suggests a positive nonlinear relationship between the concentration of the Ca\textsuperscript{2+} in the SR and the amount or fraction of Ca\textsuperscript{2+} released from the SR via CICR (larger fractional release associated with a greater SR Ca\textsuperscript{2+} content\textsuperscript{321}). The phenomenological parameter of Ca\textsuperscript{2+} sequestration describes the net efficiency of cytosolic buffering of Ca\textsuperscript{2+} and is a key additional factor cooperating with fractional release to determine vulnerability to Ca\textsuperscript{2+} alternans\textsuperscript{378}. In general, factors that promote increases in Ca\textsuperscript{2+} SR load or fractional release will stimulate alternans. These factors depend critically on SR Ca\textsuperscript{2+} sequestration rate. While under reduced sequestration activity, modest SR Ca\textsuperscript{2+} loads and fractional release can induce alternans\textsuperscript{301}. In contrast, increasing the efficacy of Ca\textsuperscript{2+} sequestration can be protective towards alternans, and larger diastolic SR Ca\textsuperscript{2+} concentrations or a larger fractional release are required to overcome this state to induce alternans.

Evidence supporting these ideas has been provided by experimental procedures exploring the molecular correlates to Ca\textsuperscript{2+} sequestration, or factors that modify diastolic SR Ca\textsuperscript{2+} load. For example, SERCA2a overexpression\textsuperscript{380-382} or reduced expression\textsuperscript{334} suppresses or enhances Ca\textsuperscript{2+} alternans respectively. Similarly, promoting Ca\textsuperscript{2+} extrusion from the cell via NCX (computer simulation)\textsuperscript{383} or increasing luminal Ca\textsuperscript{2+} buffers (mice hearts)\textsuperscript{384} suppressed Ca\textsuperscript{2+} alternans. Reducing SR Ca\textsuperscript{2+} content by increasing RyR2 open probability with caffeine or ryanodine also suppress alternans\textsuperscript{258,332,337,382}. Therefore, distinct from electrically driven alternans in which only the APD restitution slope determines alternan stability, Ca\textsuperscript{2+} driven alternans can depend on two equally important factors related to SR Ca\textsuperscript{2+} handling. It is important to note however that the boundary between stability and instability (alternans) of Ca\textsuperscript{2+} cycling is non-linear\textsuperscript{370}, such that, at low sequestration rates (shallow boundary) this balance is dominated by fractional release properties of the SR, whereas at high sequestration rates (steep boundary) slight changes in Ca\textsuperscript{2+} sequestration determine alternans.

Experiments directly measuring the dynamic Ca\textsuperscript{2+} concentration of the SR content revealed insignificant diastolic SR Ca\textsuperscript{2+} fluctuations in the presence of Ca\textsuperscript{2+} alternans\textsuperscript{335}. These findings suggest that the beat-to-beat feedback control of SR content is not the only mechanism underlying susceptibility to pacing-induced Ca\textsuperscript{2+} alternans. Instead, it was proposed the kinetics of restitution of SR Ca\textsuperscript{2+} release or the time-dependence of refractoriness of SR Ca\textsuperscript{2+} release was the primary mechanism underlying Ca\textsuperscript{2+} alternans\textsuperscript{335}. The idea of SR refractoriness was elegantly exemplified in atrial myocytes of a recent study by Shykrl \textit{et al}\textsuperscript{370}. This mechanism has been
supported in experiments exploring the proteins involved in SR refractoriness. The role of RyR refractoriness in Ca\(^{2+}\) release is still under debate\(^{385}\), although experimental studies on the recovery kinetics of Ca\(^{2+}\) transients and sparks have supported this possibly. Experiments that impinged on the refractoriness of RyR Ca\(^{2+}\) release, by increasing pacing frequency, induced Ca\(^{2+}\) alternans\(^{386-388}\). In fact, instabilities in Ca\(^{2+}\) release may be due to delayed recovery of CICR or refractoriness of RyR-mediated Ca\(^{2+}\) release\(^{389}\). Refractoriness of RyR is modulated by calsequesterin\(^{390}\), and calsequesterin knockout\(^{375}\) or overexpression\(^{391}\) mice suppress or promote alternans respectively. Ca\(^{2+}\) binding to calsequesterin changes the structure of the protein, and relieves negative inhibition from the RyRs. Furthermore, inhibition of glycolysis in cat atrial and ventricular myocytes increased susceptibility to alternans and was attributed to the time-dependent recovery of SR Ca\(^{2+}\)-release mechanism\(^{340}\).

The ability of fractional Ca\(^{2+}\) release, cytosolic Ca\(^{2+}\) sequestration or the restitution and refractory kinetics of the SR Ca\(^{2+}\)-release mechanism to modulate Ca\(^{2+}\) alternans lead to the 3R theory\(^{392,393}\) to unify these molecular mechanisms. Both numeric simulations and mean-field representations can be used to illustrate how coupled stochastic excitable elements (a couplon network) can lead to a period-doubling bifurcation (alternans) when subject to global periodic forcing (rapid pacing)\(^{394}\). A deviation from the stable equilibrium between the Randomness of Ca\(^{2+}\) sparks (LTCC mediated primary sparks via RyR CICR), Recruitment of a Ca\(^{2+}\) spark by a neighboring CRU (secondary sparks mediated by Ca\(^{2+}\) waves), and the Refractoriness of a CRU (LTCC or RyR) will contribute to the development of Ca\(^{2+}\) alternans\(^{392,393}\). Ultimately, the theory provides a framework to explain how these three complex interactions lead to Ca\(^{2+}\) alternans.

The theory also helps rationalize conflicting results under certain pharmacological interventions. For example, Ca\(^{2+}\) alternans has been induced by partially blocking LTCCs in some experiments\(^{323,372,395}\), while in others it could be suppressed\(^ {396}\). If the probability for recruitment is high, then either a very high or low primary spark probability, induced by either LTCC agonist BayK8644 or antagonist nisodipine, will suppress alternans\(^{338}\). The opposite would be true if the probability for recruitment was low\(^{382,397}\).

Although the effects of Ca\(^{2+}\) channel blockers (CCBs) on alternan genesis are contradictory due to differences in the underlying physiological conditions, CCBs will certainly
decrease coupling fidelity (low probability of RyR opening in response to LTCC opening) and will introduce spatial non-uniformities of primary sparks. This was demonstrated by lowering LTCC open probability by clamping at more negative potentials (-20mV) or by partial block with LTCC antagonists. In order for non-uniform Ca\textsuperscript{2+} sparks, provided by reduced primary spark probability, to generate stable Ca\textsuperscript{2+} alternans, a large enough recruitment probability of couplons must be maintained. Ca\textsuperscript{2+} waves activating peripheral CRUs (secondary sparks), are facilitated by high recruitment probabilities (high intracellular Ca\textsuperscript{2+} concentration and increased SR sensitivity or load) and can accelerate the development of Ca\textsuperscript{2+} alternans by resetting the phase of SR Ca\textsuperscript{2+} release and refractoriness. This phenomenon was observed when high intracellular Ca\textsuperscript{2+} concentrations (high recruitment probability), which promoted Ca\textsuperscript{2+} waves and led to stable Ca\textsuperscript{2+} alternans when LTCC open probability was reduced (via voltage clamping at less depolarized potentials).

When Ca\textsuperscript{2+} alternans develop, they can either be spatially in phase throughout different regions of the myocyte on a given beat, or described in many experiments, out of phase, known as spatially discordant Ca\textsuperscript{2+} alternans. The cause of subcellular Ca\textsuperscript{2+} alternans still remains uncertain, however many different mechanisms have been proposed. The underlying mechanism for the development of the alternans themselves is probably intimately linked to the type of alternans that ensue. Shiferaw and Karma have suggested a dynamical pattern-forming instability (a Turing instability) as the cause of discordant Ca\textsuperscript{2+} alternans, and was later confirmed in experimental studies by Gaeta et al. The theory requires negative Ca\textsuperscript{2+}-to-voltage coupling (large Ca\textsuperscript{2+} transient causes a short APD via Ca\textsuperscript{2+}-induced inactivation of LTCCs) fluctuations at the single cell level to cause long-range voltage inhibition at distal sites in the tissue.

Spatially discordant Ca\textsuperscript{2+} alternans have also been found when Ca\textsuperscript{2+}-to-voltage coupling was positive, implying other potential mechanisms exist. Disparities of Ca\textsuperscript{2+}-cycling properties across the cell might explain the origins of spatially discordant Ca\textsuperscript{2+} alternans. Simulation studies by Aistrup et al., modeled regional differences in the density of LTCCs, NCX, or SERCA channel density across the cell, which led to the formation of phase-mismatched Ca\textsuperscript{2+} alternans. Experimental studies confirmed this theory by decreasing the open probability of RyR (tetracaine or intracellular acidification). They found spatially discordant Ca\textsuperscript{2+} alternans depended on SR Ca\textsuperscript{2+} release, propagated as waves, and inducing local resetting of SR refractoriness. The idea
that local phase resetting by Ca\(^{2+}\) waves can cause subcellular Ca\(^{2+}\) alternans was further illustrated by Xie et al.\(^{97}\). Atrial myocytes are especially more vulnerable to pacing-induced Ca\(^{2+}\) alternans,\(^{310, 340}\), and in particular spatially discordant Ca\(^{2+}\) alternans are often more pronounced\(^{340, 403}\). In atrial myocytes, underdeveloped T-tubular system, resulting in a unique Ca\(^{2+}\) release mechanism during EC coupling (previously described in EC coupling)\(^{309-311, 314, 317, 405}\), was found to facilitate the genesis of subcellular Ca\(^{2+}\) alternans, and the propagation of Ca\(^{2+}\) waves\(^{403}\). More recently, the unique EC coupling properties of atrial myocytes was incorporated into a simulation by Li et al.\(^{330}\), while varying parameters of LTCC influx, RyR sensitivity, SR content, and SERCA inhibition. These studies confirmed the inherent susceptibility of the Ca\(^{2+}\) release mechanism in atrial myocytes to discordant Ca\(^{2+}\) alternans, especially so when LTCC Ca\(^{2+}\) conductance was reduced. The arrhythmogenic potential of spatially discordance Ca\(^{2+}\) alternans can be dampened if the alternating out of phase regions are equal in magnitude\(^{406}\). The consequence is an invariant whole cell Ca\(^{2+}\) transient and a less evident oscillation in the APD.

As previously mentioned, the information flow between Ca\(^{2+}\) and voltage is bidirectional. This means the AP can influence the SR Ca\(^{2+}\) loading and consequently dynamics of Ca\(^{2+}\) cycling within the cell via the transmembrane Ca\(^{2+}\) fluxes through the LTCCs and NCX (Fig. 1.3.4.3). Similarly, the whole cell Ca\(^{2+}\) transient can feed back on the shape of the AP through the Ca\(^{2+}\) sensitive ionic current LTCC and NCX. Positive coupling can occur when a large Ca\(^{2+}\) transients increases gain through NCX leading to a depolarizing current (1Ca\(^{2+}\) for 3Na\(^{+}\)) and APD prolongation\(^{22}\). On the other hand, a large Ca\(^{2+}\) transient can yield a shorter APD, due to increased Ca\(^{2+}\) induced inactivation of LTCCs\(^{266, 407}\).

Theoretical studies by Shiferaw et al.\(^{288}\), explored how voltage-driven and Ca\(^{2+}\)-driven instabilities cooperate with each other to generate alternans. When voltage \(\rightarrow\) Ca\(^{2+}\) and Ca\(^{2+}\) \(\rightarrow\) voltage coupling are both positive (longer APD generates a larger Ca\(^{2+}\) transient, while a larger Ca\(^{2+}\) transient prolongs APD) then the synergistic interaction promotes the onset of alternans quicker. However, when voltage \(\rightarrow\) Ca\(^{2+}\) coupling is positive and Ca\(^{2+}\) \(\rightarrow\) voltage coupling is negative (long APD increases Ca\(^{2+}\) transient, but the larger Ca\(^{2+}\) transient shortens APD) then there is a dynamic equilibrium and alternans will only ensue if an imbalance forms. If voltage driven alternans predominate, alternans are electromechanically concordant (in phase), but if Ca\(^{2+}\) drives the system the alternans are electromechanically discordant (out of phase). If both these instabilities equally balance each other then a quasiperiodic patterns forms (both APD and
calcium transient oscillate in both magnitude and degree of electromechanical concordance/discordance), which has been observed in Purkinje fibers. Therefore, if at the cellular level calcium-driven alternans predominate, then APD will follow suit and oscillate secondarily.

It is likely in atrial myocytes that coupling between calcium alternans and voltage is positive. Experimental and computational studies have provided evidence in rabbit atrial myocytes that the late phase of the atrial AP (early repolarization) was modulated by a net extrusion of calcium through NCX. At Em around -40mV, a “foot-like” projection of depolarization is observed in atrial myocyte and is proportional to the magnitude of the calcium transient. The density of IK1 is 5- to 10-fold smaller in atrial myocytes compared to ventricular myocytes, and consequently the membrane resistant of atrial myocytes is much greater. This means that the small depolarizing current afforded by INCX can generate large changes in voltage to the AP.

1.3.4.3 The link to arrhythmogenesis

Like calcium alternans, cardiac electrical alternans may either be spatially concordant or discordant, the latter associated with a greater risk of arrhythmogenesis. For a given beat, the APD may alternate in phase homogenously throughout the tissue, known as concordant alternans. In contrast, the APD may oscillate out-of-phase and heterogeneously throughout the tissue (long-short-long pattern in one region, whereas short-long-short pattern in another), termed spatially discordant alternans (SDA). The out-of-phase regions create a nodal line, where no alternans are present. These regions are highly susceptible to localized conduction block because the spatial gradient in the APD are the greatest at the nodal line. Whole heart mapping studies have revealed SDA to be a precursor to reentry arrhythmias, since they enhance the gradients and dispersion of repolarization. SDA has essentially provided a mechanistic link of how cardiac alternans develop into VF and AF. Additionally, calcium waves, often a consequence of spatially discordant calcium alternans and higher heart rates, can pose an arrhythmogenic threat on their own. The inward INCX and calcium-activated nonselective cation currents can translate the high diastolic calcium (due to the wave) to rises in Em and elicit an AP (DAD) if the threshold for INa is met. Changes that increase the “diastolic calcium-voltage coupling gain”, such as β-AR stimulation (enhance CICR), and heart failure (reduced couplon spacing or increased INCX and decreased IK1), will enhance
Ca\textsuperscript{2+} waves and predispose to DADs\textsuperscript{425}. Furthermore, rises in diastolic $E_m$ can impede conduction via reductions in $I_{Na}$ resulting in delayed or blocked conduction.
Figure 1.3.4.3 Calcium to voltage coupling and spatially discordant action potential duration alternans.

Left: Positive (NCX depolarizing $I_{\text{NCX}}$) and negative (LTCC Ca$^{2+}$-induced inactivation) Ca$^{2+}$ to voltage ($V_m$) coupling. Right: Arrhythmogenic spatially discordant APD alternans. In region “a” (red tissue), a long-short pattern for APD alternans is observed, whereas in region “b” (blue tissue) a short–long pattern of APD alternans is present. The white region of tissue represents the nodal line separating the two region and with no APD alternans. If a ectopic foci (*) occurs in the region with a short APD, it can initiate figure-eight region by blocking as it propagates across the nodal line into the long APD region, while propagating laterally until the long APD region recovers. Reproduced/adapted with permission from Weiss et al$^{257}$ (doi: 10.1161/01.RES.0000224540.97431.f0)
Two different mechanisms have been characterized to explain the transition from concordant to SDA. The first requires an intrinsically heterogeneous tissue, caused by structural, electrophysiological, and/or intracellular Ca$^{2+}$ cycling heterogeneities. Anatomical barriers in the heart can promote electrical uncoupling of neighboring cells, and promote local repolarization gradients. This was demonstrated with surgical induced structural barriers on the ventricle of guinea pig hearts, which promoted SDA through electrical isolation of the myocardium. Similarly, structural barriers mediated by healed infarcts in canines were associated with wavebreak, reentry excitation, and VF. These observations were explained by gap junction remodeling, fibrosis, and delayed Na$^+$ channel recovery at the epicardial border zone of the infarct, which led to increased asymmetry of DI distribution. Local electrophysiological variance in the myocardium may also promote the transition from concordance to discordance. Differences in the ionic currents across the tissue (i.e. from base to apex of ventricles) can lead to electrical uncoupling of cells (normally synchronized by electrotonic forces), followed by unique localized APD restitution properties, stimulating SDA, and leading to AF and VF. However, this process is counteracted by axial current (through gap junctions) between distal cells from the source current which tends to attenuate dispersion of repolarization during APD restitution. Similar to the spatial organization of ionic currents across the membrane, Ca$^{2+}$ cycling proteins display similar transmural heterogeneity. In guinea pig and canine ventricles, endocardial expression of RyR2 and SERCA2a were significantly reduced and were associated with a greater susceptibility to Ca$^{2+}$ alternans. Since Ca$^{2+}$ bidirectionally couples to voltage, regional Ca$^{2+}$ cycling can influence APD restitution properties and underlie spatially heterogeneous APD alternans.

The second mechanism permitting the transition to SDA can occur even in a completely homogenous tissue, but involves more dynamic processes, such as CV restitution (in combination with APD restitution), negative electromechanical coupling and ectopic foci. Similar to APD restitution, CV is also sensitive to the previous DI, since incomplete recovery from inactivation of Na$^+$ channels is challenged at shorter DIs, and slows conduction. Simulations of homogenous cardiac tissue and rabbit hearts have proposed steep CV restitution to predispose to SDA. At steep slopes in the CV restitution curve (during high pacing rates), small changes in DI produce large delays in conduction. The slow propagation of conduction allows the DI at distal sites to become slightly lengthened and as a consequence the
APD becomes prolonged. If this process continues, it will self amplify over subsequent beats and will evolve into stable SDA\textsuperscript{257}. The opposite is true for SDA generated by ectopic foci, where SDA are only a transient event. Simulations in 1 and 2D cardiac tissue have demonstrated the dispersion of DI by the invasion of an ectopic foci and can result in SDA\textsuperscript{433}. An ectopic wave also has the potential to reverse the phase of electromechanical alternans in cat ventricular myocytes\textsuperscript{365}. It was proposed that regional changes in electromechanical phase elicited by a premature beat could result in SDA. A simulation study demonstrated that out of phase electromechanical alternans (negative coupling), driven by Ca\textsuperscript{2+}, could alone form SDA and in the absence of CV restitution\textsuperscript{342}. The electrotonic coupling of voltage between cells allows small differences between them to become amplified only when coupling was negative. Therefore, spatial inhomogeneity in the tissue is not a requisite for SDA, but more dynamic processes are required to provoke spatial non-uniformities.

1.3.5 Characterization of atrial fibrillation substrates: frequency domain analysis

A common and evolving technique to characterize the electrical activation during bouts of AF and to improve the efficacy of ablation therapies, use a high-resolution spectral analysis\textsuperscript{434-437}. Researchers have employed spectral techniques to illustrate that although AF appears to be seemingly random and chaotic, there are phases of regular activation and spatiotemporal periodicity depending on the underlying substrate. The analysis demonstrates a hierarchy with appreciable frequency gradients across the atria. Signals obtained in the time domain, such as the ECG, display time in the x-axis, and voltage in the y-axis, whereas frequency domain plots display frequency in the x-axis instead. In order to convert signals between the time (or spatial) domain to signals in the frequency domain, the mathematical fast Fourier Transform (FFT) is employed. Signals can be decomposed into a sum of weighted sinusoidal functions if the signal obtained is continuous or discreet (digital acquisition). The y-axis in a frequency domain plot directly represents the magnitude of the sinusoidal amplitude and is derived from the function constituting the signal\textsuperscript{435}. The frequency with the largest magnitude is referred to as the “dominant frequency” in the literature, and is often used in the clinical setting to approximate atrial activation rates\textsuperscript{435}. The frequency components with lower magnitudes either represent secondary component frequencies or multiples of the dominant/secondary frequencies, termed “harmonics”. An advantage of using the FFT analysis to dissect the ECG signals obtained during
AF is the ability to determine the “organization” of the AF\textsuperscript{434, 436, 437}. Most interesting was the observation that different organizations were correlated with specific underlying AF substrates. For example, electrically remodeled atria, primarily due to a reduction in the atrial effective refractory period, were found to be more disorganized with multiple frequency components and poor periodicity\textsuperscript{434}. On the other hand, completely structurally remodeled atria, consisting of interstitial fibrosis and hypertrophy of the atria, were described to be more organized with only a single dominant frequency\textsuperscript{434}. Therefore, the organization index obtained from an ECG signal can give valuable insight into the potential substrate governing the reentry arrhythmia.
1.4 Synopsis

Advances in the understanding of AF over the past 100 years have only yielded satisfactory management approaches, and is highlighted by the limited treatment strategies currently available\textsuperscript{18,19}. Therefore, full understanding of the underlying pathophysiology of AF is a critical undertaking. The importance of $I_{\text{Ca,L}}$ in the development of AF has been investigated previously in rapidly paced atria of dogs\textsuperscript{186,438}, and these studies proposed reductions in $I_{\text{Ca,L}}$, presumably as a result of reduced Ca\textsubscript{v}1.2 expression in response to tachypacing\textsuperscript{438,439}, led to a reduced APD and atrial refractoriness. Support for this hypothesis was provided by the observation that human right atrial cells from patients with AF had a significant reduction (~70\%) in $I_{\text{Ca,L}}$ density\textsuperscript{129,180,181}, and were associated with a reduction in mRNA\textsuperscript{182-184} and protein\textsuperscript{184} levels of the α1 LTCCs. These observations would be consistent with the idea that AF with genetic ablation of Ca\textsubscript{v}1.3 could arise from reduced APDs in atrial myocytes. However, intracellular AP recordings in Ca\textsubscript{v}1.3\textsuperscript{−/−} isolated SA node myocytes demonstrated that APDs are in fact prolonged, contrary to what would be expected\textsuperscript{51}. Consistent with this observation, standard pacing protocols to obtain \textit{in vivo} electrophysiological parameters in Ca\textsubscript{v}1.3 mice, revealed no difference in the atrial effective refractory period between the groups. Here then potentially lies a novel role of Ca\textsubscript{v}1.3 $I_{\text{Ca,L}}$ in the development of a substrate for AF. Another important factor contributing to the development of reentry and pathogenesis of arrhythmias are cardiac alternans. Ca\textsuperscript{2+} alternans, at the cellular level, is an essential player in enhancing the vulnerability to electrical re-entry at the tissue level\textsuperscript{301}. The role of $I_{\text{Ca,L}}$ in physiological context of Ca\textsuperscript{2+} cycling has been attributed to EC coupling fidelity. Spatially non-uniform and decreased magnitude Ca\textsuperscript{2+} transients ensue if the $I_{\text{Ca,L}}$ trigger is reduced with pharmacological agents\textsuperscript{399,400}, by voltage protocols\textsuperscript{398} or simulations\textsuperscript{440}. This heterogeneity of Ca\textsuperscript{2+} release will spawn discordant Ca\textsuperscript{2+} alternans if there is sufficient Ca\textsuperscript{2+} in the SR to sustain wave propagation\textsuperscript{323,330,341,372,401}. Ca\textsubscript{v}1.3\textsuperscript{−/−} atrial myocytes display: diminished $I_{\text{Ca,L}}$ density, and reduced intracellular Ca\textsuperscript{2+} transients, and delayed times to peak cytosolic Ca\textsuperscript{2+} levels following field stimulation\textsuperscript{167}. In SA nodal cells, Ca\textsubscript{v}1.3 was found to more intimately co-localize with RyR2 compared to Ca\textsubscript{v}1.2. This evidence combined suggests a specific role of Ca\textsubscript{v}1.3 in EC coupling fidelity and I therefore hypothesized:

\textbf{AF in Ca\textsubscript{v}1.3\textsuperscript{−/−} mice is primarily due to Ca\textsuperscript{2+} alternans, since knockout of Ca\textsubscript{v}1.3 reduces the $I_{\text{Ca,L}}$ trigger for EC coupling, and results in spatially non-uniform release of Ca\textsuperscript{2+}.}
Technical Contributions and Acknowledgements

I would like to acknowledge the following individuals for contributing to my MSc project:

1. Dr. Roozbeh Aschar-Sobbi

   Dr. Sobbi has been involved intimately with this project. Intellectually, Dr. Sobbi and I worked together in planning many of my studies. He performed the in vivo intracardiac electrophysiological recordings, and surgical implantation of pressure and ECG telemetry units. Any technical and intellectual expertise I have acquired over the course of my MSc has been credited primarily due to his patience and willingness to instruct me.

2. Farzad Izaddousdtar

   Farzad contributed experimentally to generate the early optical mapping, anesthetized surface ECG recordings, and unanesthetized telemetry ECG recordings in the Ca\textsubscript{1.3} study. He also developed the method of representing ECG FFT signaling processing in origin, and preformed a portion of my in vivo intracardiac electrophysiological recordings. He also contributed intellectually to the planning of my experiments and technically in all of the histological experimentation and analysis.

3. Huiyan Zha

   Huiyan preformed all of the single cell electrophysiology, and generated all of the data pertaining to the simultaneous AP and Ca\textsuperscript{2+} transient recordings from Ca\textsubscript{1.3} mice.

4. Wallace Yang

   Wallace wrote the program used to average the action potentials used for the microelectrode data analysis, a JavaScript plugin for imageJ used to generate moving frequency maps of optical mapping images, a JavaScript plugin for imageJ to slice frames, a macro for excel to analyze the APD at 90% repolarization of a sequence of APs, and analysis of my optical mapping, ECG and microelectrode correlation studies.

5. Nazar Polidovitch

   Nazar contributed experimentally to preform some of the echocardiogram recordings on aged Ca\textsubscript{1.3} mice.

I was responsible for planning, and primary data collection of: Ca\textsuperscript{2+} transients from isolated atrial myocytes, surface ECG measurements, microelectrode recordings, atrial isolations, optical mapping, all histological experiments, invasive hemodynamics recordings (left and right ventricle), in vivo intracardiac electrophysiological recordings, devising the Ca\textsuperscript{2+} alternan protocol, devising the APD restitution protocol, devising the analysis for quantification of temporal and spatial variance of Ca\textsuperscript{2+} alternans. I was also responsible for all analysis of data, and generation of all figures.
Chapter 2

Materials and Methods
2 Materials and Methods

2.1 Experimental Animals

All experiments were conducted on 8-12 week old male mice with Ca,1.3 ablation (generation of the a1D KO has been previously described\textsuperscript{160}) provided generously by Dr. J. Striessnig (University of Innsbruck, Innsbruck, Austria), or Ca,1.2 ablation (generation of the a1D KO has been previously described\textsuperscript{162}) provided generously by Dr. JD. Molkentin (University of Cincinnati, Ohio, USA). Mice were generated in a mixed CD1/C57B6 (50/50) background in our animal care facility by breeding heterozygous males to heterozygous females. All mice were housed in temperature and humidity controlled rooms with 12h light cycles, at the Department of Comparative Medicine, University of Toronto. All Experimental protocols conformed to the standards of the Canadian Council on Animal Care.

2.2 Atrial Myocyte Isolation

Male Ca,1.3 or Ca,1.2 (7-10 weeks) mice were administrated 200\(\mu\)L IP injection of heparin (1000 IU/ml) to prevent blood clotting and were given 5min for sufficient adsorption. They were subsequently sacrificed under constant isoflurane (via a 50mL conical tube with a lightly doused isoflurane tissue inside and exposed to the animals airways). Midsternal incision was used to open the thorax and the heart was rapidly excised and transferred to a bath of modified horizontal Langendorff apparatus in cold (~4\(^\circ\)C) Ca\textsuperscript{2+}-free Tyrode solution containing (in mmol/L) 130 NaCl, 5.4 KCl, 0.4 NaH\textsubscript{2}PO\textsubscript{4}, 0.5 MgCl\textsubscript{2}, 22 D-glucose, 25 HEPES with pH adjusted to 7.4 with NaOH. In bath aorta was cannulated on a blunted and smoothed 20-gage needle attached to a bubble trap and water jacket heated perfusion system. Hearts were immediately perfused retrogradely with warm (35.0\(^\circ\)C) Ca\textsuperscript{2+}-free Tyrode solution upon successful cannulation on the horizontal Langendorff apparatus. After hearts were perfused at a constant flow rate of 3.5 ml/min, for 10 minutes with the Ca\textsuperscript{2+}-free solution, 1 mg/ml type II collagenase (225 U/ml, Worthington Biochemical Corporation, Lakewood, NJ, USA) and 0.3U/ml of elastase was added to the Ca\textsuperscript{2+}-free Tyrodes and perfused for approximately 7-8min. During digestion hearts were gently massaged with blunt forceps to ensure adequate perfusion. After complete digestion of the atrial tissue, the LAA was removed and gently dissociated in a high K\textsuperscript{+} solution (modified Kraft-Brüe (KB)) containing (in mmol/L) 100 K\textsuperscript{+}-glutamate, 10 K\textsuperscript{+}-aspartate, 25 KCl, 10 KH\textsubscript{2}PO\textsubscript{4}, 2MgSO\textsubscript{4}, 20 taurine, 5 creatine, 0.5 EGTA, 20 D-glucose, 5
HEPES, and 0.1% BSA, with pH adjusted to 7.4 with KOH. Atrial myocytes were stored in KB solution at ~4°C (in a weigh boat on ice) until used in Ca²⁺ imaging studies.

### 2.3 Calcium Transient Measurements and Calcium Alternan Induction

Calcium transients were measured with a Yokogawa fluorescence laser scanning confocal microscope with a disk speed of 9351 revolutions/sec. Ca²⁺ transients of atrial cells were recorded using a high speed EMCCD-camera (Cascade Evolve: 128; Photometrics, AZ, USA), which captures at 510 frames/sec with custom relay housing interfaced with MetaMorph® advance (Version 7.7.0.0) for Olympus software resulting in an image resolution of 16-bit. 60x oil immersion objective was used to visualize the transients.

To visually record transients, cells were loaded with 1.1µM of the Ca²⁺ indicator Fluo-3 AM (AnaSpec; CA, USA) for 20 minutes at room temperature. Cells were superfused for 15 min continuously at 32-34°C with Tyrodes solution containing (in mmol/L) 140 NaCl, 5.4 KCl, 1 MgCl₂, and 1.0 CaCl₂, 10 D-glucose and 10 HEPES, with pH adjusted to 7.4 with NaOH to allow for deesterification of the dye. To maintain a constant temperature around 32-34°C, cells were superfused with a HPRE2 (Cell MicroControls) inline pre-heater (set at 40°C) in heated dish with a negative feedback automatic temperature controller (Warner Instruments Corporation). Fluo-3 AM was excited with a 491 nm intensity laser and fluorescence was captured through a high-pass 510nm filter. Two field platinum electrodes attached to a Grass S44 stimulator (Grass instrument Co, MA, USA) were used to electrically stimulate (2.5ms pulse duration) and pace atrial myocytes. Atrial myocytes were then paced at baseline frequency of 1Hz to determine minimal voltage threshold and viability of cells. To induce Ca²⁺ alternans, cells were then paced at 1.5X the magnitude of minimal threshold at 3Hz, 4Hz, 5Hz, 6Hz, 7Hz, and 10Hz, meanwhile dropping back to a baseline of 3Hz between each recording for at least 30s. At each frequency 5000 frames or ~10 seconds of paced Ca²⁺ transients were acquired.

For experiments at higher extracellular Ca²⁺ concentrations, a Tyrodes solution with 1.5mM CaCl₂ was continuously superfused at 32-34°C. The effect of β-AR stimulation on alternan susceptibility, in atrial myocytes, was studied using the non-selective agonist ISO. Cells were first superperfused in Tyrodes solution (1.0mM CaCl₂) at 32-34°C for a 15min deesterification, and then switched to the same Tyrodes solution, but containing 50nM ISO. To
observe the before and after effect of ISO on the same cell, cells were first paced at 6Hz and 7Hz (to induce Ca\textsuperscript{2+} alternans) in absence of ISO, and then again in presence of 50nM ISO.

2.4 Cellular Electrophysiology

Simultaneous [Ca\textsuperscript{2+}], and AP recordings were performed on current-clamped left atrial myocytes. AP recordings were recorded with a patch-clamp amplifier 200A. Pelamp 9.0 software was used to produce the signal, collect and process the data. The resistances of the glass electrodes used were 5-8MΩ and they were filled with the pipette solution and immersed in the extracellular solution. The patch pipette solution contained (in mM): K\textsuperscript{+} aspartate 120, KCl 20, NaCl 10, *MgATP 5, HEPES 10 (pH to 7.2 with KOH). The extracellular perfusion solution was normal Tyrode’s solution containing (in mM): NaCl 140, KCl 4, MgCl\textsubscript{2} 1.0, CaCl\textsubscript{2} 1.0, HEPES 10, Glucose 10 (pH to 7.4 with NaOH). Nystatin was prepared as a 50mg/mL stock solution in DMSO, and was added into the pipette solution to a final concentration at 0.2 mg/mL when used. All pipette solutions were back filled into the pipette. The experiments were performed at 32 ºC.

2.5 Calcium Alternans Quantification of Temporal and Spatial Variance

The recordings were processed using imageJ (NIH, USA, http://rsbweb.nih.gov/ij/) to subtract the background, and to measure the stacks of the fluorescence. Ca\textsuperscript{2+} transients were then normalized using the follow equation: 

\[
\frac{\text{peak} - \text{baseline}}{\text{maximum} - \text{baseline}}
\]

Where “peak” represents the maximum value of the systolic Ca\textsuperscript{2+} transient signal, “baseline” represents the minimum diastolic Ca\textsuperscript{2+} signal in the whole acquired recording, and “maximum” represents the greatest systolic Ca\textsuperscript{2+} transient signal in the whole acquired recording.

Normalized peaks were then used to quantify the degree of temporal (difference in the systolic Ca\textsuperscript{2+} transient with another systolic Ca\textsuperscript{2+} transient in the signal) and spatial alternans (difference in the Ca\textsuperscript{2+} signal between different areas of the cell in a particular instance of time). In order to quantify temporal alternans, simply the variance of the normalized peaks from the whole cell Ca\textsuperscript{2+} transients was compared between each group (Fig. 2.4): 

\[
\frac{\Sigma(x - \bar{x})^2}{n-1}
\]
Where “$\bar{x}$” represents the average of the normalized peaks throughout the acquired recording, and “$n$” are the number of peaks in the recording.

For temporal alternans, the cell was divided into 4 separate equal areas, and the variance of the Ca$^{2+}$ signal between these areas at a particular time was calculated (Fig. 2.4). The variance between the 4 areas was calculated at every time point in the acquired signal to generate “variance curves” (Fig. 2.4). The “integ1” X-function on OriginPro (Version 6.8.0) (OriginLab, Northampton, MA, U.S.A) was used to integrate the “variance curves” and obtain mathematical areas. Mathematical areas from the integral of the curves were then used to compare between the groups.
Figure 2.1 Spatial and temporal alternans analysis.

Stacks were measured (illustrated by the raw traces on the right) from four different locations (represented by the optical images from a peak Ca$^{2+}$ transient) from a single left atrial myocyte at 7Hz. The top whole cell trace is an average of the Ca$^{2+}$ signal from the whole cell (represented by the white border around the whole cell on the left). The next 4 traces are the average Ca$^{2+}$ signal from the 4 separate areas (highlighted by the white boarders in the representative image of the cells on the left). The variance was calculated at each time point between the 4 areas to generate the variance curve at the bottom. The integral of the variance curve was used to obtain mathematical areas to compare the degree of spatial alternans between the groups. The analysis displays the heterogeneity throughout 4 different areas of the cell at a particular point in time (note the largest variance between the areas at the systolic point of the Ca$^{2+}$ signal).
2.6 Atrial Isolations

Mice were IP injected with 0.2 ml heparin (1000IU/ml, Leo Pharma, Thornhill, ON, Canada) to prevent blood clotting and given 5 minutes for absorption. Mice were then subsequently sacrificed under constant isoflurane (via a 50mL conical tube with a lightly doused isoflurane tissue inside and exposed to the animals airways). The thorax was opened by midsternal incision, and excised hearts were transferred into Tyrode’s solution (35°C) consisting of (in mmol/L) 140 NaCl, 5.4 KCl, 1.2 KH2PO4, 1 MgCl2, 1.8 CaCl2, 5.55 D-glucose, 5 HEPES, and 10 IU/ml heparin with pH adjusted to 7.4. The heart was pinned to a Sylgard coated petri dish and atria were separated from the ventricles with by cutting along the AV connective tissue. Atria were then pinned and opened up by cutting along the superior and inferior vena cava (Fig. 2.5). As much excessive fat and connective tissue was removed from the tissue before experimentation.
Figure 2.5 Isolated atria preparation

Lead I and II refer the placement of the platinum needle electrodes inserted in the Sylgard petri-dish to record ECG. Left atrial appendage (LAA); right atrial appendage (RAA); superior vena cava (SVC); inferior vena cava.
2.7 Optical Mapping and Atrial Fibrillation Induction in Isolated Atrial Preparations

Immediately following isolation, atria were incubated with 1µM AminoNaphthylethenylPyridinium (Di-4-ANEPPS) in 35°C Tyrode’s solution for 10 min. Di-4-ANEPPS, is an aphiphilic compound with two hydrocarbon chains that allow it to anchor into the plasma membrane. When bound to the membrane, the chromophore aligns perpendicularly to the membrane/aqueous interface and undergoes reduction in emission intensity (~10% per 100 mV depolarization) when excited at 540 nm and monitored at 680 nm emission. Atria were transferred and continuously perfused using 35°C carbogenized (95% O₂ & 5% CO₂) Krebs solution composed of (in mmol/L) 118 NaCl, 4.2 KCl, 1.2 KH₂PO₄, 1.8 CaCl₂, 1.2 MgSO₄, 2.3 NaHCO₃, 20 D-glucose, 2 Na-pyruvate. The voltage across the tissue was excited using a mercury light source (X-Cite Exacte, Lumen Dynamics, Mississauga, ON, Canada) with a 543±22nm band-pass filter. A 645±75 nm band-pass filter was used to illuminate fluorescence. A high speed EMCCD camera (Cascade 128+, Cascade Evolve, Photometrics, Tucson, AZ, U.S.A) was used to capture optical images of depolarization. Frames were captured at 934 fps, at 2x2 binning and 64x64 pixels resolution, using Image Pro Plus (Media Cybernetics, Rockville, MD, U.S.A) software.

Field recordings of isolated atria were obtained using platinum needle electrodes inserted in the Sylgard petri-dish (F-E7, Grass Technologies, West Warwick, RI, USA) in lead II arrangement (Fig. 2.5), connected to a Gould ACQ-7700 amplifier with Ponemah Physiology Platform acquisition software (Data Sciences International, New Brighton, MN, USA).

Vulnerability to AF was assessed by programmed stimulation using a platinum electrode connected to Pulsar 6i & 6b (FHC Inc, Bowdoinham, ME, U.S.A). Capture threshold was determined at a cycle length (CL) of 100ms (10Hz) and subsequent pulses were delivered that 1.5X the threshold amperage. AF vulnerability was assessed using the following rapid stimulation: 27 pulses at 40ms (25Hz) down to 20ms (50Hz) by 2ms decrements, and 20 pulses at 20ms (50Hz) 20 times. Each protocol was repeated 2X on the LAA and RAA and AF was defined as rapid, chaotic atrial activation (discernable on the ECG as ‘noisy’ electrical signal or multiple sites of activation on a optical map) lasting ≥10 s in duration and was reproducible (Fig. 2.6).
To estimate epicardial CV, atria were retrograde paced at the LAA, with a CL of 100ms (10Hz), and a sequence of 5000 frames was captured. The image sequence was analyzed using Scroll software (courtesy of Sergey Mrinov) and was also used to generate isochronal maps. A vector velocity map was used to guide region of interest (ROI) analysis. Regions with uniform conduction, at 3 separate sites were calculated for a minimum of 3 activations and averaged.

Moving frequency maps of the optical images were created using the “Yang STFT” plugin, a java-script created by the technician Wallace Yang, for the software ImageJ. Optical signals obtained from di-4-ANEPPS loaded atria were Fourier transformed with a 512 point moving window, with 256 points of overlap. The organization index (OI) was obtained from the area under the peak of the dominant frequency (df), divided by the total area under the Fourier transform plot from 0–70 Hz. The dominant frequency from the optical images frequency maps was defined as the frequency with the greatest area encapsulating the atria.
Figure 2.6 Representative electrocardiogram and optical mapping montages of sinus rhythm followed by atrial fibrillation.

A, Representative ECG field recording of an isolated atria in sinus rhythm (SR) (beginning), with a rapid stimulation protocol (middle), followed by atrial fibrillation (end). Note the single well-defined SR wave of activation, but the random, “noisy”, and high frequency activation during AF. B, Optical mapping montages of an isolated atria stained with the voltage sensitive dye, di-4-ANEPPS. White numbers represent the order of occurrence in time. B, Left, isolated atria in SR, note the hierarchal initiation of depolarization from the SA node, followed by synchronized and coordinated propagation across each atrial appendage (white arrows). B Right, isolated atria in AF, note the activation begins as a reentry in LAA followed by propagation to the rest of the tissue (white arrows).
2.8 Signal Processing and Analysis

The FFT algorithm is used to convert discrete signals (i.e. digital) to a discrete frequency spectrum. The organization and frequencies of AF can be characterized using frequency domain analysis. Briefly, the $df$ is the sinusoidal component of a continuous signal with the largest amplitude, whereas the smaller amplitude harmonics are multiples of the $df$. Dynamic non-stationary signals cannot be effectively analyzed with the classic FFT. The short-time FFT, a similar time frequency analysis, can assess the major frequency changes over time. An short-time FFT allows for tracking of $df$, as well as organization of AF through time, without the averaging effect of performing a FFT of long discrete signals.

Isolated atrial (leads I and II) ECGs obtained during AF were analyzed using the short-time FFT function in OriginPro software. A 4096-point (0.8192 s) moving window (rectangular) with 2048 points of overlap was Fourier transformed and plotted on a 3D surface map (Fig. 2.7). A similar method employed by Everett et al., was used to quantify the organization of AF. ECG segments were processed with a 2nd order Butterworth band-pass filter (80–500 Hz) followed by a 2nd order Butterworth low-pass filter (40 Hz) in MatLab (MathWorks, Natick, MA, U.S.A) (Fig. 2.7). Using OriginPro, an FFT function, with a Hanning window, was subsequently applied to the filtered signals.

Discrete Fourier transfer plots were generated by applying the FFT function, in OriginPro, to 2-second segments from the middle, beginning and end of an AF signal. The area under the peak of the $df$ was expressed as a ratio of the total area of the discrete Fourier transfer plot. If an AF signal is very disorganized, multiple frequency components will exist, and as a result decrease the ratio between areas under the $df$ and the total plot. In contrast, if the AF tends to be more organized (i.e. a single frequency component exists), the ratio will tend to increase. The area under the $df$ of the discrete Fourier transfer plot was divided by the total area under the plot from 0–50 Hz. The ratio was averaged for each 2-second AF segment from the signal to obtain the overall OI for that AF event. At least three AF events were analyzed with this method from each isolated atria.
Figure 2.7 Discrete fast-Fourier plots of isolated atria atrial fibrillation

A, Representative AF ECG signal (acquired at 5000Hz) from a isolated mouse atria, with sinus rhythm, followed by rapid stimulation and the induction of AF. B, the AF signal was subsequently filtered, and subjected to the OriginPro short-time FFT function (moving 4096 pt rectangular window, with 2096 pt overlap) to yield the 3D surface plot. The frequency (Hz), amplitude, and time (s) are plotted in the x-, y-, and z-axis, respectively. Color scale corresponds to amplitude (from low; blue, to high; red). Note the large peak, at ~40 Hz and after 20 seconds of elapsed time, in the short-time FFT plot. Also note the secondary component at ~20 Hz and after 20 seconds of elapsed time. This secondary component likely reflects 2:1 propagation through the atria. C, A short 2-second AF segment of a mouse isolated atria. D, The signal in A was filtered and Fourier transformed, using OriginPro, to obtain the discrete Fourier transform plot. The OI (red) represents the ratio of the area under the df (yellow box) to the area under the whole plot.
2.9 Intracellular Recordings of Isolated Atrial Preparations

Pipettes were pulled from borosilicate glass (1.5mm OD, 0.75 mm ID, WPI, Sarasota, FL, U.S.A) using a Flaming/Brown pipette puller (model p-87, Sutter Instrument Company, Novato, CA, U.S.A). Pipettes were filled with 3M KCl with a resistance of ≥30 MΩ. Pipettes were positioned using course manipulators (Newport, Irvine, CA, U.S.A) and recordings were acquired using an Axopatch 200B amplifier with a MiniDigi 1A digitizer and Axoscope acquisition software (Molecular Devices, Sunnyvale, CA, U.S.A). Data was analyzed using Clampfit (Molecular Devices, Sunnyvale, CA, U.S.A) and APD at 90%, 50% and 30% repolarization (APD90; APD50; APD30 respectively) was used to compare between the groups. A minimum resting potential of -70mV, and minimum amplitude of 80mV was used as selection criteria in the integrity of recordings. APs were either recorded at 35°C with either baseline SA node beating rates or at a pacing frequency of 10Hz (100ms) to normalize for the differences in beating frequencies of the atria. APs paced at 10Hz were collected from the LAA and RAA and the epicardial platinum electrode was placed at a proximal site to the recording location on the same appendage. At least 3 recordings were obtained from each atrial appendage.

APD restitution curves were generated from APs collected from the LAA and RAA. Restitution values were obtained from a train of 30 pulses at either 10Hz (100ms) or 25Hz (40ms) (S1) followed by a varied delayed after pulse (S2). Restitution curves were generated by from the APD90 immediately following the S2 pulse, and plotting it against the corresponding S1-S2 diastolic interval (DI). OriginPro was used to fit the restitution curve to the exponential power equation “y=A1*(1-exp(-x/t1))^n+y0”’. The time constant (Tau) obtained from the equation was used then used to compare the slopes between each group.

2.10 Histology

Animals were anesthetized using isoflurane oxygen mixture (2.5%), the thorax was opened by midsternal incision, and the inferior vena cava was cut. A 30-gauge needle was used to puncture the apex of the heart for direct perfusion with 1% KCl in 0.01M PBS. After arresting in diastole the heart was directly perfused with fresh 4% paraformaldehyde in 0.01M PB through the same punctured opening. Hearts were excised and placed in 4% paraformaldehyde in 0.01M PB overnight, washed 3X with 0.01M PBS and dehydrated in a series of alcohol washes. The Toronto Center for Pharmacogenomics Histology Core services were used for paraffin
embedding, cutting (5µm thickness, 3 slices per slide at 100µm distance apart), Picrosirius red (PSR) staining. The fluorescence properties of Picrosirius red<sup>442</sup> was exploited using a spinning disk confocal microscopy system (Yokogawa CSU-X1, Calgary, AB, Canada) with 491 nm excitation laser (Cobolt, Solna, Sweden) and a 700±65 nm band-pass emission filter. Z-Stacks were acquired using a NanoScan Z stage controller (Prior Scientific, Cambridge, U.K) and a Quantem 512c EMCCD camera (Photometrics, Tucson, AZ, U.S.A). The threshold method<sup>443</sup> of quantification was used in ImageJ software.

The Ly6b.2 alloantigen (Cedar Lane, Burlington, ON, Canada), and the CD107b (mac-3) antibodies (BD Pharmingen, Mississauga, ON, Canada) were used to immunohistochemical stain neutrophils and macrophage respectively. Antigen retrieval was achieved via the tris-buffer microwave method. Streptavidin-biotin peroxidase was used for signal amplification (SK-6100, Vector Labs, Burlington, ON, Canada) with the diaminobenzidine (DAB) chromogen (SK-4100, Vector Labs, Burlington, ON, Canada). Mayer’s haematoxylin (Sigma-Aldrich, Oakville, ON, Canada) was used for counterstaining of nuclei. Total cells of the left atrium were counted in a minimum of 2 slices at a minimum of 100µm apart. Number of cells per field of view (with a minimum of 5 fields) was counted in anatomically similar locations in the left ventricle, in a minimum of 2 slices, and at a minimum of 100µm apart.

**2.11 Surface Electrocardiograms on Anesthetized Mice**

Using a Fluotec Mark 2 Vaporizer (Cyprane, Keighley, UK) to deliver and regulate an isoflurane/oxygen mixture, animals were maintained under minimal anesthesia in the supine position. The leg-twitch reflex was used to gauge level of anesthesia. Ideal anesthesia of most mice were under ~1.5% isoflurane. Platinum sub-dermal needle electrodes (F-E7, Grass Technologies, West Warwick, RI, USA) in lead II arrangement, connected to a Gould ACQ-7700 amplifier with Ponemah Physiology Platform acquisition software (Data Sciences International, New Brighton, MN, USA) were used to record surface ECGs. In order to record stable HRs for 5min a minimum equilibration period of 15min was used. Core temperature was continuously monitored using a rectal temperature probe (THM 150, Indus Instruments) and maintained between 37.0–37.4°C. HRs were derived from R-R interval.
Pharmacological blockade of autonomic input to the heart was accomplished using a combined intraperitoneal injection of 1mg/kg BW of atropine sulphate and 10mg/kg BW of propranolol hydrochloride (Sigma-Aldrich, Oakville, ON, Canada).

2.12 Surface Electrocardiograms and Pressure on Unrestrained Unanesthetized Mice

Animals were maintained under anesthesia (as described previously, but at ~2% isoflurane) and implanted with radio frequency ECG/pressure transmitters (HD-XII, Data Sciences International, New Brighton, MN, USA). Sterile conditions were maintained through the course of the surgery. To reveal the muscular layer of the abdominal cavity an incision was made along the abdomen of the skin. An additional dermal incision was made lateral to the clavicle to expose the underlying tissue. A small incision was made in the muscular layer of the abdomen and the radiofrequency emitter was inserted into the intraperitoneal cavity. The leads were guided over the muscular layer, under the skin, to the left leg and right arm (lead II configuration). Leads were anchored at various points using 7-0 prolene suture, while the skin was sutured using 6-0 silk suture. The pressure-transducing catheter was inserted into the right carotid and guided into the left ventricle. Animals were given ~2 weeks to recover. After full recovery, data was acquired and analyzed offline using Data Sciences A.R.T 4.1 software (Data Sciences International, New Brighton, MN, USA). HR, end-diastolic, and systolic pressures, and maximum and minimum dP/dT of the left ventricle were averaged over a moving 30s window using Analysis (Data Sciences International, New Brighton, MN, U.S.A) software.

2.13 Invasive Hemodynamics

Using an isoflurane oxygen mixture regulated by a Fluotec Mark 2 Vaporizer (Cyprane, Keighley, UK) animals were anesthetized in the supine position. Upon conclusion of surgical procedures, animals were maintained at minimal anesthesia at ~1.5%. A 1F pressure-transducing catheter (Scisense, London, ON, Canada) was introduced into the right carotid artery, guided through the aorta, the left atrium and into the left ventricle. Data was acquired at 5000 Hz using the Scisense ADV500 control unit (Scisense, London, ON, Canada) with Acknowledge (Biopac, Goleta, CA, U.S.A) acquisition software. A minimum equilibration period of 15 min preceded 5 min of data acquisition. Core temperature was monitored using a rectal probe (THM 150, Indus Instruments) and was maintained at 37.3–37.6°C. Aortic pressures were recorded
before entry into the left ventricle in order to calculate mean arterial pressure (MAP) using the following equation: 

$$(\text{MAP}) = \text{Diastolic Pressure} + \frac{1}{3}(\text{Systolic Pressure} - \text{Diastolic Pressure}).$$

End-diastolic left ventricular pressure was used as an indirect measure of left atrial pressure.

### 2.14 In Vivo Electrophysiological Recordings

Animals were anesthetized using an isoflurane oxygen mixture maintained at ~2.5%. The right jugular vein was isolated and a small incision was made to allow the cannulation with a 2F octapolar electrophysiology catheter (CI’BER Mouse, Numed, Hopkinton, NY, U.S.A). The catheter was guided into the right atrium via the superior vena cava and into the right ventricle. Data was acquired using a Gould ACQ-7700 amplifier with Ponemah Physiology Platform acquisition software (Data Sciences International, New Brighton, MN, USA). Each electrode ring is 0.5 mm in length and separated by 0.5 mm. The his signal was used for consistent positioning of the catheter between animals.

A 3s train was delivered using leads 78 at 10ms below the RR interval in order to determine the Wenckebach cycle length (WBCL). The CL was progressively reduced by 5ms until conduction to the ventricles was blocked, and was confirmed 3 times. To determine the AV refractory period (AVERP), a driving train ($S_1$) of 7 pulses was delivered using leads 78 at 20ms below the R-R interval followed by a single ($S_2$) extrastimulation. The initial coupling interval of $S_2$ was reduced by 5ms decrements until AV block occurred. The interval was subsequently increased by 1-2ms intervals until recapture and the obtained value was confirmed 3 times. The ventricular effective refractory period (VERP), the high and mid atrial effective refractory periods (AERP) were obtained using a similar protocol, with the exception that pulses were delivered through leads 12, 78 and 56, respectively. Specifically for atrial refractory periods, the $S_2$ coupling interval was initially delivered at below capture (~15ms) and increased by 5ms increments until atrial capture, and was then reduced by 1-2ms until loss of capture.

To assess AF vulnerability a series of stimulation protocols were delivered to the high atrium, followed by the mid atrium. The protocol consisted of the following: 27 pulses at 40ms interval, reduced to 20ms by 2ms decrements, repeated 3 times. 20 pulses at 20ms coupling interval repeated 20 times separated by 1.5s, followed by a 5 minutes break and repeated to a maximum of 3 times. The latter protocol only was used to assess VF. AF was defined as a reproducible bout of chaotic atrial activation, with irregularly regularly ventricular activation
with a duration ≥ 10s. VF was defined as a reproducible rapid chaotic ventricular activation with a duration ≥ 2s.

2.15 Statistical Analysis

Summary data are expressed as mean±SEM (standard error mean). Significance was determined using unpaired student’s t-test. The Fischer’s exact test was used to determine significance when data was categorized into groups based on a particular parameter (i.e. sustained AF vs. non-sustained AF, or stable alternans vs. no alternans). The Pearson product-moment correlation coefficient was used to measure the linear correlation (dependence) between two variables X and Y. P values <0.05 were considered statistically significant.
Chapter 3

Results
3 Results

3.1 Characterization of Ca\textsubscript{v}1.3\textsuperscript{-/-} Mice

Previous studies utilizing Ca\textsubscript{v}1.3\textsuperscript{-/-} mice showed a dramatic cardiac phenotype, characterized by sinus bradycardia, intermittent sinus arrest, supraventricular and ventricular extrasystoles, delayed AV conduction, AV node block (Type I & Type II), decoupling of HR and PR interval duration, and prolonged PR and P-wave durations\textsuperscript{34,66,74-76}. Therefore, I further characterized these electrical phenomena in surface ECG of isoflurane anesthetized Ca\textsubscript{v}1.3 mice in lead II arrangement. These preliminary studies using surface ECG recordings confirmed the presence of sinus bradycardia in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice (478±14bpm in Ca\textsubscript{v}1.3\textsuperscript{+/+}; 350±23bpm in Ca\textsubscript{v}1.3\textsuperscript{-/-}; P<<0.01), significantly prolonged PR intervals (34.67±1.4ms in Ca\textsubscript{v}1.3\textsuperscript{+/+}; 48.47±2.3ms in Ca\textsubscript{v}1.3\textsuperscript{-/-}; P<<0.01) and P-wave durations (10.06±0.54ms in Ca\textsubscript{v}1.3\textsuperscript{+/+}; 15.86±0.99ms in Ca\textsubscript{v}1.3\textsuperscript{-/-}; P<<0.01) (Fig. 3.1.1). These initial findings were further elaborated upon and confirmed using radio-frequency emitting ECG telemetry units in Ca\textsubscript{v}1.3 mice. Telemetric in vivo ECG recordings, similar to studies performed in anesthetized mice, showed RR interval prolongations in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice (120.8±12.7ms in Ca\textsubscript{v}1.3\textsuperscript{+/+}; 182.82±22.7ms in Ca\textsubscript{v}1.3\textsuperscript{-/-}; P<0.01) (Fig. 3.1.2). In addition, sinus arrhythmias as quantified using RR variance, were found to be significantly greater in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice, consistent with SA nodal dysfunction (30.64±25.9ms in Ca\textsubscript{v}1.3\textsuperscript{+/+}; 561.2±221.8ms, in Ca\textsubscript{v}1.3\textsuperscript{-/-}; P<0.05) (Fig. 3.1.2). Prior literature has identified the expression pattern of Ca\textsubscript{v}1.3 to be highly concentrated in the SA and AV node, and Ca\textsubscript{v}1.3\textsuperscript{-/-} mice have a prolonged AVERP\textsuperscript{136}. Thus, it is not surprising that our telemetry ECG data uncover 2\textsuperscript{nd} degree AV in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice\textsuperscript{160,168} (Fig. 3.1.2), and my electrophysiological parameters (attained with programmed stimulations to the mid atria of anesthetized animals using the proximal leads of the EP catheter) show a prolongation of the AVERP (51.00±1.568ms in Ca\textsubscript{v}1.3\textsuperscript{+/+}; 67.76±3.699ms in Ca\textsubscript{v}1.3\textsuperscript{-/-}; P<<0.01) (Fig. 6.1 B).

To further characterize the SA nodal dysfunction in Cav1.3/-/- mice\textsuperscript{51,52,160}, isolated atrial preparations of Ca\textsubscript{v}1.3 mice, stained with the voltage sensitive dye di-4-ANEPPS, were used to map electrical propagation throughout the atria. Interestingly, sinus arrest was often followed by ectopic foci emanating from the PV sleeves in Ca\textsubscript{v}1.3\textsuperscript{-/-} atria (Fig. 3.1.3). The ECG from Ca\textsubscript{v}1.3\textsuperscript{-/-} isolated atria revealed two different atrial activation waveforms confirming presence of ectopic foci outside of the SA node detected in optical images (Fig. 3.1.3).
In addition to the electrical abnormalities present in Ca\textsubscript{v}1.3\textsuperscript{−/−} hearts wet ventricle or atria weights standardized to tibia length showed elevations in ventricular mass, but not atrial mass, suggesting hypertrophic remodeling in ventricles of Ca\textsubscript{v}1.3\textsuperscript{−/−} mice (8.39±0.17mg/mm in Ca\textsubscript{v}1.3\textsuperscript{+/+}; 9.21 ± 0.17mg/mm in Ca\textsubscript{v}1.3\textsuperscript{−/−}; P<0.01).
Figure 3.1.1 Bradycardia and conduction abnormalities in Ca\textsubscript{v}1.3\textsuperscript{−/−} mice.

Surface ECG recordings of anesthetized Ca\textsubscript{v}1.3\textsuperscript{−/−} mice show diverse electrical abnormalities compared to Ca\textsubscript{v}1.3\textsuperscript{+/+} mice. A, Representative 1-second ECG traces from a Ca\textsubscript{v}1.3\textsuperscript{+/+} (top) and Ca\textsubscript{v}1.3\textsuperscript{−/−} (bottom) mouse. Note the prolonged RR interval in the Ca\textsubscript{v}1.3\textsuperscript{−/−} ECG tracing. B, Representative ECG “P QRS T” segments from a Ca\textsubscript{v}1.3\textsuperscript{+/+} (top) and Ca\textsubscript{v}1.3\textsuperscript{−/−} (bottom) mouse. Black dotted lines mark the beginning and end of the P wave with the respective duration above, whereas the red dotted lines mark the PR interval with the respective duration in between. Note the prolongation in both the P-wave and PR interval in the Ca\textsubscript{v}1.3\textsuperscript{−/−} tracing. C, Significantly reduced HRs were found in Ca\textsubscript{v}1.3\textsuperscript{−/−} mice. D, Ca\textsubscript{v}1.3\textsuperscript{−/−} mice display significantly prolonged PR intervals. E, P-wave duration was found to be significantly reduced in Ca\textsubscript{v}1.3\textsuperscript{−/−} mice.
Figure 3.1.2 Bradycardia, arrhythmias, sinus arrest and atrioventricular block in unanesthetized, unrestrained Ca\textsubscript{v}1.3\textsuperscript{−/−} mice

Radio-frequency emitter ECG telemetry units were implanted in Ca\textsubscript{v}1.3 mice and surface ECGs were recorded from unanesthetized, unrestrained Ca\textsubscript{v}1.3 mice in lead II configuration. \textit{A}, Representative ECG tracings from a Ca\textsubscript{v}1.3\textsuperscript{+/+} (top) and Ca\textsubscript{v}1.3\textsuperscript{−/−} (bottom) mouse with the respective RR interval given between each QRS, and arrows highlighting sinus arrest and 2nd degree AV block. \textit{B}, Ca\textsubscript{v}1.3\textsuperscript{−/−} mice exhibit significantly prolonged RR-intervals. \textit{C}, mean beat-beat variance is significantly increased in Ca\textsubscript{v}1.3\textsuperscript{−/−} mice.
Figure 3.1.3 Ectopic events in \( \text{Ca}_v1.3^{-/-} \) isolated atria

\( \text{Ca}_v1.3^{-/-} \) mice are described as having sick sinus syndrome with a variety of SA node abnormalities. To further characterize the electrical properties of the SA node and the atria, field ECG recordings and optical mapping images of di-4-ANEPPS stained isolated atrial preparations from \( \text{Ca}_v1.3 \) mice were acquired. A, Representative ECG recordings from \( \text{Ca}_v1.3^{+/+} \) (left) and \( \text{Ca}_v1.3^{-/-} \) (right) isolated atria. Note the different patterns of atrial activation in the \( \text{Ca}_v1.3^{-/-} \) ECG, while the \( \text{Ca}_v1.3^{+/+} \) waveforms remain consistent. B, Single ECG waveform of atrial activation (above) originating at the sinus node (left) and ectopic site (right) from a \( \text{Ca}_v1.3^{-/-} \) isolated atria with the corresponding isochronal schematic of the ectopic activity (below).
Figure 3.1.4 Ca\textsubscript{v}1.3\textsuperscript{-/-} mice display characteristics of ventricular hypertrophy at 12 weeks.

To characterize structural changes associated with genetic ablation of Ca\textsubscript{v}1.3\textsuperscript{-/-}, the wet weight of atria and ventricles standardized to tibia length were measured. A, Hearts were arrested in diastole and subsequently fixed for morphological comparison. A representative comparison of a Ca\textsubscript{v}1.3\textsuperscript{+/+} (left) and Ca\textsubscript{v}1.3\textsuperscript{-/-} (right) heart sliced along the vertical axis, and imaged with both halves. B, Comparison of the wet weights of the atria and ventricles standardized to tibia length. Ventricle to tibia length (v/tb) was significantly greater in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice, however there was no difference in the atria to tibia length (a/tb).
3.2 Increased Vulnerability to Atrial Fibrillation in Ca$_v$1.3$^{+/−}$ Atria

The increased susceptibility of Ca$_v$1.3$^{+/−}$ mice to induced AF is well established. *In vivo* electrophysiological studies provided the first evidence of inducible AF in Ca$_v$1.3$^{+/−}$ mice by employing various rapid stimulation protocols$^{136}$, which was extended in Langendorff perfused hearts in a more recent study$^{167}$. To confirm AF susceptibility in Ca$_v$1.3$^{+/−}$ mice (since these animals are in a different strain background), AF vulnerability was assessed both *in vivo* and *ex vivo* in isolated atria preparations. Isolated atria from Ca$_v$1.3 mice were probed for susceptibility to inducible AF with a rapid stimulation protocol applied with an epicardial electrode to the LAA and RAA. Significantly longer AF durations were observed in Ca$_v$1.3$^{+/−}$ atria, compared to controls (7.0±3.3s in Ca$_v$1.3$^{+/−}$, 50.6±14.7s in Ca$_v$1.3$^{+/−}$; $P<0.05$) (Fig. 3.2.1 B). AF was defined as reproducible episodes of rapid atrial activation with durations lasting greater than 10 seconds and a significantly greater proportion of Ca$_v$1.3$^{+/−}$ atria experienced sustained AF ($P<0.05$). Sustained AF was induced in 73% of the isolated atria from Ca$_v$1.3$^{+/−}$ mice, and only 15% of the Ca$_v$1.3$^{+/−}$ atria (Fig. 3.2.1). Interestingly, of the 73% where sustained AF was induced, 13% of the Ca$_v$1.3$^{+/−}$ atria also underwent episodes of sustained spontaneous AF. These *ex vivo* studies on isolated atrial preparations from Ca$_v$1.3$^{+/−}$ mice further confirm that Cav1.3$^{+/−}$ exhibit greater vulnerability to AF. Importantly, optical mapping has allowed for detailed characterization of the nature of AF events.

For *in vivo* studies, I introduced an octapolar electrophysiology catheter into the right jugular vein and guided the catheter into the right ventricle. By delivering a series of rapid stimulations to the atria through the proximal leads of the catheter (leads 78 or 56), I failed to induce AF in Ca$_v$1.3$^{+/−}$ mice (Fig. 6.1C). Even with the addition of the parasympathetic agonist carbachol (50ng/g) to induce a shortening in the AERP, Ca$_v$1.3$^{+/−}$ mice failed to exhibit a significant increase in AF duration compared to baseline (without carbachol). Since AF events were readily induced in isolated, denervated atrial preparations, autonomic input was blocked using pharmacological interventions (atropine (1mg/kg) and propranolol (10mg/kg)). Despite pharmacological inhibition of autonomic input to the hearts of Ca$_v$1.3$^{+/−}$ mice, sustained AF was not induced. These differences in findings could reflect a difference in methodology for *in vivo* AF induction, since Zhang et al$^{136}$ used a midline sternotomy approach.
Ca\textsubscript{v}1.3\textsuperscript{-/-} mice have been shown to be susceptible to inducible AF both \textit{in vivo} and \textit{ex vivo}. Isolated atria from Ca\textsubscript{v}1.3 mice were probed for AF vulnerability using two rapid stimulation protocols, with an epicardial platinum electrode, on both the LAA and RAA. \textit{A}, Representative ECG field recording of a Ca\textsubscript{v}1.3\textsuperscript{-/-} isolated atria in sinus rhythm (SR), followed by AF, either due to a stimulation protocol (left) or spontaneously (right). \textit{B}, Montage of a single sinus activation (left) and cycle of AF (right) obtained from a di-4-ANEPPs stained Ca\textsubscript{v}1.3\textsuperscript{-/-} atria. White arrows depict the depolarization propagation pathway, while the dotted white line represents conduction block. Bright yellow coloring depicts depolarized voltage across the tissue. \textit{C}, Maximal duration of AF for each atria was plotted, and Ca\textsubscript{v}1.3\textsuperscript{-/-} mice had significantly longer episodes of AF. \textit{D}, AF was defined as lasting greater than 10 seconds and was reproducible. A significantly greater proportion of Ca\textsubscript{v}1.3\textsuperscript{-/-} atria underwent sustained AF (Fischer’s exact test). Sustained AF was induced in 73\% (11/15) of Ca\textsubscript{v}1.3\textsuperscript{-/-} isolated atria, with 13\% (2/15) of the 73\% also undergoing spontaneous AF, while only 15\% of Ca\textsubscript{v}1.3\textsuperscript{+/-} atria exhibited sustained AF. 

\textbf{Figure 3.2.1 Increased susceptibility to AF in isolated Ca\textsubscript{v}1.3\textsuperscript{-/-} atria.}
3.3 Potential Mechanisms of Atrial Fibrillation in Ca\textsubscript{v}1.3\texttextsuperscript{-/-} Mice: Electrical Remodeling

3.3.1 Atrial Refractoriness

In general, changes in the electrophysiology of the atria that shorten the refractoriness predispose to AF\textsuperscript{144}. In human patients with persistent AF, mRNA and protein levels of the Ca\textsubscript{v}1.2 L-type Ca\textsuperscript{2+} channels, along with I\textsubscript{CaL} were reduced\textsuperscript{182-184}. Intuitively, it is conceivable that genetic ablation of Ca\textsubscript{v}1.3 would lead to reduced APDs in atrial myocytes. To determine the AERP, programmed stimulations were delivered to the high and mid atria of anesthetized animals using the proximal leads of the EP catheter. Surprisingly my electrophysiological parameters in Ca\textsubscript{v}1.3\texttextsuperscript{-/-} revealed a significant prolongation of the AERP, contrary to what would be expected (24.50±2.251ms in Ca\textsubscript{v}1.3\textsuperscript{+/+}; 39.25±2.339ms in Ca\textsubscript{v}1.3\texttextsuperscript{-/-}) (Fig. 6.1 B).

The electrophysiological consequence on the APD of the atria with genetic ablation of Ca\textsubscript{v}1.3, and independent of autonomic influences, was determined via microelectrode recordings of the isolated atria. Previous studies\textsuperscript{51, 52, 160} in SA node pacemaker cells from Ca\textsubscript{v}1.3\texttextsuperscript{-/-} mice showed a decrease in beating frequency, prolonged cycle lengths and reductions in the rate of diastolic depolarization. Therefore, I investigated the nature of SA node firing rate because differences between the groups could lead to unwanted electrical restitution or accommodation effects to the APD. My results have shown severe SA node dysfunction in isolated atria of Ca\textsubscript{v}1.3\texttextsuperscript{-/-} mice, characterized by sinus bradycardia, and erratic pacing (4.3±1.5ms in Ca\textsubscript{v}1.3\textsuperscript{+/+}; 32.8±10.7ms in Ca\textsubscript{v}1.3\texttextsuperscript{-/-}; P<0.01) (Fig. 6.2). These differences in baseline beating rates could influence the APD\textsuperscript{249, 264}, therefore, the LAA and RAA were paced with an epicardial electrode at a proximal site (a couple of millimeters away) to the microelectrode recording. Intracellular APs were recorded at pacing frequencies of 10Hz, and 25Hz (similar to frequencies observed during AF). Contrary to what was expected, pacing at 10Hz uncovered a trend towards prolongation in the APD of Ca\textsubscript{v}1.3\texttextsuperscript{-/-} atria (Fig. 6.3). Pacing at 25Hz displayed a general reduction in APD between both genotypes, however there were no significant differences between the two groups (Fig. 6.3). These results do not support a mechanism of reduced atrial refractoriness characterized by APD reductions in Ca\textsubscript{v}1.3\texttextsuperscript{-/-} mice. However, there are other types of electrical remodeling which can predispose to AF, namely cardiac alternans.
3.3.2 Action potential duration (electrical) alternans

The previous results provide evidence against traditional electrical remodeling characterized by reduced APD as the cause of increased AF vulnerability in Ca\textsubscript{v}1.3\textsuperscript{−/−} mice. Another important factor contributing to the development of reentry and pathogenesis of arrhythmias are electromechanical cardiac alternans characterized by the cyclic, beat-to-beat variations in APD (electrical alternans) and contraction amplitude (Ca\textsuperscript{2+} transient, mechanical or Ca\textsuperscript{2+} alternans\textsuperscript{235}). Whole heart mapping studies have revealed cardiac alternans, especially in the form of SDA, to be a precursor to reentry arrhythmias, since they enhance the gradients and dispersion of repolarization leading to conduction block\textsuperscript{413, 416, 417}. Accordingly, I next investigated whether APD alternans were present in Ca\textsubscript{v}1.3\textsuperscript{−/−} mice, since loss of I\textsubscript{Ca} has also been associated with the development of electrical and Ca\textsuperscript{2+} alternans. In order to answer this question, I rapidly paced isolated atria on the RAA and LAA at frequencies similar to AF (25Hz) and simultaneously recorded APD. The magnitude of APD alternans was quantified via the difference in APD at 90% repolarization of two sequential APs (|APD\textsubscript{n}−APD\textsubscript{n+1}|) across the course of the stimulation period. In the RAA of Ca\textsubscript{v}1.3\textsuperscript{−/−} a significantly greater degree of APD alternans was present compared to Ca\textsubscript{v}1.3\textsuperscript{+/+} controls (0.91±0.2ms in Ca\textsubscript{v}1.3\textsuperscript{+/+}; 3.56±0.7ms in Ca\textsubscript{v}1.3\textsuperscript{−/−}; P<0.01) (Fig. 3.3.2.1 B), with no significant differences in the LAA. Furthermore, comparing the APD at baseline and during stimulation illustrated a drastic and rapid reduction in APD (∼50%) equal in both groups upon stimulation (Fig. 3.3.2.1 A).

Taken together, the evidence suggests that APD alternans, not a reduction in APD, is a more likely candidate mechanism for increased AF vulnerability in Ca\textsubscript{v}1.3\textsuperscript{−/−} mice. Strength for this argument is provided by careful analysis of AF in Ca\textsubscript{v}1.3\textsuperscript{−/−} isolated atria. Simultaneous recordings of ECG and APs (via microelectrodes) during AF, in isolated atria, revealed that over time APD alternans begin to stabilize in the cell as the AF perpetuates (Fig. 3.3.2.2 A). Moreover, when APD alternans began to stabilize during AF they inevitably led to regional conduction block. This was characterized by a distinct peak on the isolated atria ECG, indicative of atrial activation, which was not associated with a stimulated AP in a cell of the LAA (Fig. 3.3.2.2 B). APD alternans surface due to two independent mechanisms, electrical restitution (steep APD restitution curve), or secondary to Ca\textsuperscript{2+} alternans (Ca\textsuperscript{2+}-sensitive ion channels and exchangers). Since Ca\textsubscript{v}1.3\textsuperscript{−/−} atria were more susceptible to APD alternans, the underlying source for this dynamic instability was next investigated.
Figure 3.3.2.1 Greater susceptibility to pacing induced alternans in the right atrial appendage of Ca\textsubscript{v}1.3\textsuperscript{-/-} mice.

APD alternans increase the likelihood of reentry by creating a dispersion of refractoriness, which led us to probe for vulnerability to APD alternans in isolated atria from Ca\textsubscript{v}1.3 mice. Isolated atria from Ca\textsubscript{v}1.3 mice were epicardial paced from baseline, on the RAA and LAA at 25Hz, while APs were recorded at a proximal site with a microelectrode. A, Representative plot of the APD\textsubscript{90}, with respect to the sequential beat number, in the RAA. The plot displays the sequential events of 10 baseline APs, followed by 30 stimulated APs at 25Hz (the boxes above the plot border these respective regions). Note the dynamic and rapid accommodation of APD upon stimulation, and the stabilized alternating pattern of APD in Ca\textsubscript{v}1.3\textsuperscript{-/-} atria nearing the end of the stimulation. B, The difference in APD\textsubscript{90} of the first beat followed by the APD\textsubscript{90} of the next beat (|APD\textsubscript{90}\textsubscript{n}-APD\textsubscript{90}\textsubscript{n+1}|) was used to quantify the degree of APD alternans. A significantly greater magnitude of APD alternans was observed in Ca\textsubscript{v}1.3\textsuperscript{-/-} atria when paced at 25Hz.
2.2 Evidence of action potential duration alternans during atrial fibrillation in Ca_{1.3} isolated atria.

Typical of cardiac alternans, especially in the form of SDA, is dispersion of repolarization leading to regional conduction block and reentry. Simultaneous ECG and microelectrode recordings of isolated atrial preparations allowed for monitoring of APD alternans and conduction block during AF. A, A representative example of a simultaneous ECG recording (top) and APs (bottom) from the LAA of a Ca_{1.3}^{-/-} isolated atria (both recordings are synchronized in time). Sinus rhythm (SR) is shown, followed by a rapid stimulation protocol inducing AF. Note the progression of AF in Ca_{1.3}^{-/-} atria from a stable ECG pattern with stable APs to a more complex pattern, which was associated with APD alternans. B, Similar recording as A, however a different episode of induced AF in Ca_{1.3}^{-/-} atria and excluding the portion of SR and stimulation. Note how APD alternans in the microelectrode recording (bottom) precede complex atrial activations evident in the ECG (top). Also note the regions bordered by the dashed red lines, when atrial activation in the ECG (top) fails to elicit an AP (or the opposite, AP without atrial activation), suggesting regional conduction block (CB).
3.3.3  Electrical restitution

At sinus rhythm, the transmembrane APs are constant in morphology, but when the beating rate is suddenly changed or even one perturbation in the cycle length occurs, AP variation will ensue\textsuperscript{249, 252}. The underlying mechanism for this observation has been attributed to the electrical restitution properties of the cardiac ion channels. Restitution curves are used to describe how a change in rate can affect APD, and it is often used to predict the susceptibility to conduction block leading to fibrillation\textsuperscript{253}. Specifically, recovery from inactivation of LTCCs, along with cytosolic Ca\textsuperscript{2+} concentrations, are important modulators of APD, especially during the electrical restitution process\textsuperscript{445}. At short DIs, incomplete recovery from inactivation of the LTCC reduces $I_{CaL}$ peak density and accelerates repolarization (short APD)\textsuperscript{264}. Therefore, it is conceivable that loss of Ca\textsubscript{v}1.3 $I_{CaL}$ may lead to marked differences in the dynamics of APD restitution during atrial pacing, leading to APD heterogeneity and as a result, electrical reentry.

In order to assess differences in the electrical restitution process of Ca\textsubscript{v}1.3 mice, I generated APD restitution curves (APD of each beat is plotted against the duration of the preceding DI at a specific cycle length), wherein a steep slope is an important predictive index in the stability of membrane voltage dynamics\textsuperscript{264, 301, 446}. The CL (S1 interval) influences the restitution process\textsuperscript{263, 273} because incomplete recovery from inactivation of $I_{CaL}$ will affect the APD of the test potential (S2)\textsuperscript{273-276}. Therefore, we paced isolated atria at 10Hz or 25Hz to determine the effect of “cardiac memory” on $I_{CaL}$. Also, 25Hz was chosen to reflect our rapid stimulation protocols to induce AF, whereas 10Hz was to used to potentially explain the spontaneous AF. APs were recorded with a microelectrode on the LAA and RAA (regional heterogeneity in APD restitution) at a proximal site to the stimulation electrode. However, the slope of the restitution curves fit to the monoexponential power equation (see methods) at 10Hz and 25Hz (Fig. 6.4 A) never reached a slope >1 (the point in the curve where slight perturbations in heart rate will promote APD alternans\textsuperscript{446}) in the LAA and RAA of Ca\textsubscript{v}1.3 mice (Fig. 6.4 B). Furthermore, there was no significant difference in time constant (Tau) of the curves between the groups at 10Hz or 25Hz in the RAA or LAA of Ca\textsubscript{v}1.3 atria (Fig. 6.4 C) further suggesting that the slopes of the restitution curves between the groups were similar. It is unlikely then that electrical restitution in Ca\textsubscript{v}1.3 atria is the basis for the APD alternans, which is consistent reduced $I_{Ca}$ magnitude suppressing APD alternans\textsuperscript{260, 447, 449, 487}. Another promising candidate to
explain electrical alternans is a dynamic instability in Ca\(^{2+}\)-cycling, which can then translate to voltage.

### 3.3.4 Calcium (mechanical) alternans

My electrical restitution suggests that APD alternans in Ca\(_{v}1.3^{-/-}\) atria during AF was not related to differences in the steepness of the electrical restitution curves. Although, electrical alternans can also develop secondary to Ca\(^{2+}\) alternans, and modulate voltage through Ca\(^{2+}\) sensitive ion channels (LTCCs) and exchangers (NCX)\(^{22, 23, 289, 351, 365, 448, 449}\). Ca\(^{2+}\) alternans are often the consequence of altered Ca\(^{2+}\) homeostasis, which was present in Ca\(_{v}1.3\) atrial myocytes (reduced intracellular Ca\(^{2+}\) transients and delayed times to peak in cytosolic Ca\(^{2+}\) levels following field stimulation\(^{167}\)). Accordingly, I studied for the presence of Ca\(^{2+}\) alternans in Ca\(_{v}1.3\) mice. Ca\(^{2+}\) transients were measured with a Yokogawa fluorescence laser scanning confocal microscope and Ca\(^{2+}\) transients of atrial cells were recorded using a high speed EMCCD-camera with a 491nm excitation laser. Ca\(^{2+}\) transients were evoked by field pacing individual LAA myocytes at 3Hz, 4Hz, 5Hz, 6Hz, 7Hz, and 10Hz, dropping down to a baseline frequency of 3Hz between each acquisition of 5000 frames (~10s). Pacing at supra-physiological frequencies has been shown to induce Ca\(^{2+}\) alternans in atrial myocytes\(^{314, 344, 407}\). The variance of normalized Ca\(^{2+}\) transient peaks was used to quantify the degree of temporal alternans over the ~10s recording. Atrial myocytes from the LAA of Ca\(_{v}1.3^{-/-}\) mice developed a greater magnitude of Ca\(^{2+}\) alternans at 6Hz (0.0038±0.0006 in Ca\(_{v}1.3^{+/+}\); 0.0089±0.001 in Ca\(_{v}1.3^{-/-}\); P<<0.01) and 7Hz (0.0053±0.0006 in Ca\(_{v}1.3^{+/+}\); 0.027±0.004 in Ca\(_{v}1.3^{-/-}\); P<<0.01) compared to Ca\(_{v}1.3^{+/+}\) mice (Fig. 3.3.4.1 B). Interestingly, the heterozygous Ca\(_{v}1.3^{+/+}\) LAA myocytes displayed an intermediate phenotype at 7Hz between both Ca\(_{v}1.3^{+/+}\) and Ca\(_{v}1.3^{-/-}\) (0.012±0.002 for Ca\(_{v}1.3^{+/+}\); P<0.01 between Ca\(_{v}1.3^{+/+}\) and Ca\(_{v}1.3^{-/-}\)).

It was observed that when cells developed stable Ca\(^{2+}\) alternans, the temporal alternans variance value was ≥0.01. I used this value as a threshold for categorizing stable Ca\(^{2+}\) alternans, and it was found that a greater proportion of Ca\(_{v}1.3^{-/-}\) LAA myocytes developed stable alternans at 6Hz (3% in Ca\(_{v}1.3^{+/+}\) and 22% in Ca\(_{v}1.3^{-/-}\); P<0.01) and 7Hz (10% in Ca\(_{v}1.3^{+/+}\) and 54% in Ca\(_{v}1.3^{-/-}\); P<0.01) when compared to Ca\(_{v}1.3^{+/+}\) (Fig. 3.3.4.1 C). Again the heterozygous Ca\(_{v}1.3^{+/+}\) LAA myocytes displayed an intermediate phenotype at 7Hz between both Ca\(_{v}1.3^{+/+}\) and Ca\(_{v}1.3^{-/-}\) (25% in Ca\(_{v}1.3^{+/+}\); P<0.05 against Ca\(_{v}1.3^{+/+}\) and P<0.01 against Ca\(_{v}1.3^{-/-}\)). Partial blockade of
LTCCs with CCBs has shown to influence Ca\(^{2+}\) alternans\(^{223,372,395}\), but more importantly the type of alternans that ensued were subcellular alternans or discordant. It is conceivable that loss of Ca\(_v\)1.3 is producing a similar reduction in \(I_{CaL}\) (which has been observed in Ca\(_v\)1.3\(^{-/-}\) atrial myocytes\(^{136,167}\)) resulting in Ca\(^{2+}\) alternans with a discordant nature.

When Ca\(^{2+}\) alternans develop, they can be either spatially in phase throughout different regions of the myocyte on a given beat, or as described in many experiments\(^{341,371,397,401-403}\), out of phase, known as spatially discordant Ca\(^{2+}\) alternans. The underlying mechanism for the development of the alternans themselves is intimately linked to the type of alternans that succeed. This led us to characterize the nature of Ca\(^{2+}\) alternans and to meticulously analyze the sequential process leading to alternans. Further characterization of the Ca\(^{2+}\) alternans in Ca\(_v\)1.3\(^{-/-}\) LAA myocytes revealed they were spatially discordant (Fig. 3.3.4.1 A), which was qualitatively assessed by measuring and comparing the regional Ca\(^{2+}\) signals at a particular instance (Fig.3.3.4.2 A). The cell was divided into four equal areas, and the variance of the Ca\(^{2+}\) signal between these areas was calculated at one frame. The variance between the areas was calculated throughout the 10s recording at each frame to generate variance curves (variance between 4 areas over time) (Fig.3.3.4.2 A). The spatial variance (degree of Ca\(^{2+}\) discordance) was then quantified by using the integral to obtain the area under the curves. When compared to the few Ca\(_v\)1.3\(^{+/+}\) LAA myocytes that developed Ca\(^{2+}\) alternans, Ca\(_v\)1.3\(^{-/-}\) LAA myocytes had a significantly greater magnitude of spatial alternans at 7Hz (25.24\(\pm\)4.26 for Ca\(_v\)1.3\(^{+/+}\); 46.77\(\pm\)6.34 for Ca\(_v\)1.3\(^{-/-}\); P<<0.01). Therefore, these findings are consistent with observation that reduced LTCC \(I_{CaL}\) activation also leads to discordant alternans\(^{403,404}\). Another commonly associated phenotype with discordance is Ca\(^{2+}\) waves, responsible for synchronizing subcellular Ca\(^{2+}\) gradients across the cell.
Figure 3.3.4.1 Ca$_{v}$1.3$^{-/-}$ left atrial appendage myocytes display a greater vulnerability to pacing-induced calcium alternans.

Atrial myocytes are susceptible to pacing-induced alternans, and partial blockade of LTCCs has shown to influence Ca$^{2+}$ alternans. In order to investigate for susceptibility to Ca$^{2+}$ alternans in Ca$_{v}$1.3$^{-/-}$ mice, LAA myocytes were field paced from 3 to 7Hz (1Hz steps, dropping back to a baseline of 3Hz between recordings), and 10Hz in 1.0mM Ca$^{2+}$ at 30-32°C. A, Representative optical images at the peak of the Ca$^{2+}$ transient (top) of four consecutive field stimulated (7Hz) transients from a Ca$_{v}$1.3$^{+/+}$ (left) and Ca$_{v}$1.3$^{-/-}$ (right) LAA myocyte. Bright yellow corresponds to the Ca$^{2+}$ signal intensity. Numbers in the optical images correspond to the point in time of the Ca$^{2+}$ signal tracing (below). Note the beat-to-beat oscillation in the Ca$^{2+}$ transient magnitude of the Ca$_{v}$1.3$^{-/-}$ myocyte. C, Ca$_{v}$1.3$^{-/-}$ LAA myocytes paced at 6 and 7Hz lead to a dynamic instability in Ca$^{2+}$ cycling and a significant increase in temporal alternan magnitude. The heterozygous mice display an intermediate phenotype. At least 10 cells were recorded from each animal and cells were obtained from an n=6 for Ca$_{v}$1.3$^{+/+}$, n=3 for Ca$_{v}$1.3$^{+/-}$ and n=4 for Ca$_{v}$1.3$^{-/-}$ mice. *P<0.001 between Ca$_{v}$1.3$^{+/+}$ and Ca$_{v}$1.3$^{-/-}$ at 6Hz and 7Hz. #P<0.005 between Ca$_{v}$1.3$^{+/-}$ and Ca$_{v}$1.3$^{+/-}$ at 7Hz. ϕP<0.005 between Ca$_{v}$1.3$^{+/+}$ and Ca$_{v}$1.3$^{+/-}$ at 7Hz. C, Stable alternans occurred at a temporal alternans variance of ≥0.01. Total number of experimental cells tested was included above the bars. A greater proportion of Ca$_{v}$1.3$^{-/-}$ LAA myocytes developed stable Ca$^{2+}$ alternans at 6 and 7Hz compared to Ca$_{v}$1.3$^{+/+}$ with an intermediate phenotype in Ca$_{v}$1.3$^{+/-}$ at 7Hz. Significance determined using Fischer’s exact test.
The underlying mechanism for the development of the alternans themselves is likely intimately linked to the type of alternans that ensue. The type of alternans that mature in Ca$_v$1.3$^{-/-}$ myocytes could then provide insight into the potential mechanism of how the Ca$^{2+}$ alternans develop. Accordingly, the degree of spatial Ca$^{2+}$ alternans (magnitude of subcellular or discordant alternans) was quantified by comparing the areas under the variance curve. A, Representative example of a single Ca$_v$1.3$^{-/-}$ left atrial myocyte paced at 7Hz. White boxes represent areas where Ca$^{2+}$ signals (right) were measured from optical images (left). The variance of the Ca$^{2+}$ signal between the areas was plotted against time to generate the variance curve (bottom). Note the heterogeneity of the Ca$^{2+}$ signal across the 4 areas, especially at the peak of the transient, which resulted in a large variance. The magnitude of spatial alternans (large area under variance curve) was significantly pronounced in Ca$_v$1.3$^{-/-}$ LAA myocytes compared to Ca$_v$1.3$^{+/+}$ controls.

**Figure 3.3.4.2 Calcium alternans in Ca$_v$1.3$^{-/-}$ left atrial myocytes display spatial discordance.**
Disparities of Ca\(^{2+}\)-cycling properties across the cell can explain the origins of spatially discordant Ca\(^{2+}\) alternans in Ca\(_{v}1.3^{-/-}\) atrial myocytes. Differences in the regional open probability\(^{323}\) or refractoriness\(^{370}\) of RyR, or regional cellular expression patterns of LTCCs or NCX\(^{382,397}\) promote discordance. Simulation\(^{330,341,401}\), and experimental studies\(^{371,397}\), have found spatially discordant Ca\(^{2+}\) alternans depended on SR Ca\(^{2+}\) release, propagated as waves, and inducing local resetting of SR refractoriness\(^{323,341,371,397,401}\). Atrial myocytes are particularly susceptible to subcellular alternans since they lack well-developed T-tubules. Ca\(^{2+}\) waves are essential for the efficient propagation of Ca\(^{2+}\) from periphery to activate orphaned RyRs in the central regions of the cell\(^{313-315,318,321,409}\). I then investigated whether Ca\(^{2+}\) waves were responsible for local resetting of SR release resulting in the genesis and stabilization of subcellular Ca\(^{2+}\) alternans in Ca\(_{v}1.3^{-/-}\) atrial myocytes. By dissecting single Ca\(^{2+}\) transients into a series of frames to generate montages, I was able to identify and follow Ca\(^{2+}\) waves over time (Fig. 3.3.4.3). Looking at Ca\(_{v}1.3^{-/-}\) LAA myocytes field paced at 5Hz, before the genesis of Ca\(^{2+}\) alternans, asynchronous release resulted in a portion of the Ca\(^{2+}\) signal lingering long after the initial stimulated transient, suggesting either a Ca\(^{2+}\) wave, or spontaneous Ca\(^{2+}\) release in this region (Fig. 3.3.4.3 A). On the next beat, the region invaded by the Ca\(^{2+}\) wave on the previous beat, fails to induce a large release of Ca\(^{2+}\) in that area. In another example, Ca\(^{2+}\) waves are crucial to maintaining the subcellular alternan pattern once they have already developed. The small beat was associated with a failure of propagation of the Ca\(^{2+}\) signal from the periphery to the central regions of the cell (Fig. 3.3.4.3 B). On the large beat, successful propagation of the Ca\(^{2+}\) signal to the center of the cell is mediated by diffusion of Ca\(^{2+}\), and was manifested by the succession of the Ca\(^{2+}\) signal long after the initial field stimulation. Ca\(_{v}1.3\) was found to preferentially localize with RyR2 in SA node myocytes\(^{170}\), suggesting a specific role in EC coupling. Therefore, Ca\(_{v}1.3\) may have a direct role in the EC coupling \(I_{Ca}\) trigger, and loss of Ca\(_{v}1.3\) may promote regional CICR. Asynchronous release would then provide a mechanistic link to discordant Ca\(^{2+}\) alternans in Ca\(_{v}1.3^{-/-}\) atrial myocytes, via Ca\(^{2+}\) waves.
Figure 3.3.4.3 Calcium waves are responsible for the initiation and perpetuation of calcium alternans in Ca,1.3⁻/⁻ left atrial myocytes.

Spatially discordant Ca²⁺ alternans often arise through asynchronous Ca²⁺ release, promoting Ca²⁺ waves, and inducing local resetting of SR refractoriness. Since discordant Ca²⁺ alternans are present in Ca,1.3⁻/⁻ atrial myocytes, I probed for the presence of Ca²⁺ waves before and during Ca,1.3⁻/⁻ Ca²⁺ alternans. A, Representative Ca²⁺ transients from a Ca,1.3⁻/⁻ left atrial myocyte field paced at 5Hz (top) with optical montages of two successive Ca²⁺ transients. The red box highlights the two successive Ca²⁺ transients analyzed. The numbers above the Ca²⁺ signal of the transients correspond to the montages of a sequence of events during an individual transient (below). Between each frame in the optical images represents 20ms of elapsed time (left to right). Note the presence of a Ca²⁺ signal, long after the stimulation in beat 1, suggesting diffusion of Ca²⁺ (highlighted by white dashed box in montage 1). On the 2⁰ beat, Ca²⁺ fails to release in the region invaded by the Ca²⁺ signal on the previous beat (highlighted by white dashed box in montage 2). B, same as A except at 6Hz and between each frame 17ms has elapsed. Also, yellow arrows depict direction of propagation of the Ca²⁺ signal. Note that on the small beat (2) the Ca²⁺ signal fails to invade the central region of the cell. Whereas the large beat (1), the Ca²⁺ signal efficiently diffuses from the periphery to the central region of the cell.
The role of $I_{\text{Ca}}$ in $\text{Ca}^{2+}$ cycling has been attributed to EC coupling fidelity by acting as the “trigger” of $\text{Ca}^{2+}$ release from the SR. Spatially non-uniform release of $\text{Ca}^{2+}$ can occur if the $I_{\text{Ca}}$ trigger is reduced in simulations\textsuperscript{440}, or in experiments with isolated myocytes using pharmacological agents\textsuperscript{399, 400}, or voltage protocols\textsuperscript{398}. If the reduction in $I_{\text{Cal,L}}$, due to loss of $\text{Ca}_{v1.3}$, is in fact resulting in a reduced EC coupling fidelity promoting asynchronous $\text{Ca}^{2+}$ release, then application of an inotropic agent might alleviate these effects and protect against subcellular $\text{Ca}^{2+}$ alternans. The $\beta$-AR agonist ISO has been shown to protect against pacing-induced alternans in atrial myocytes\textsuperscript{360, 370, 450}. I therefore preformed the same field pacing-protocols on $\text{Ca}_{v1.3}$ LAA myocytes, but in the presence of 50nM ISO. To illustrate the protective effects of $\text{Ca}^{2+}$ alternans in $\text{Ca}_{v1.3^{-/-}}$ LAA myocytes, the cell was paced at 6Hz and 7Hz to induce alternans, returned to a baseline of 3Hz, and then paced again at 6Hz and 7Hz in the presence of ISO. As shown in Figure 3.3.4.4 A, a representative $\text{Ca}_{v1.3^{-/-}}$ LAA myocyte was susceptible to pacing-induced alternans at 7Hz, but application of ISO reversed this phenomenon.

Using the same method to quantify the degree of temporal alternans, application of ISO shifted the susceptibility to pacing induced alternans in $\text{Ca}_{v1.3^{-/-}}$ LAA myocytes to higher frequencies (Fig. 3.3.4.4 B). The magnitude of temporal alternans was lower in $\text{Ca}_{v1.3^{-/-}}$ with ISO compared to without, at 6Hz (0.0089±0.001 in $\text{Ca}_{v1.3^{-/-}}$; 0.0017±0.0002 in $\text{Ca}_{v1.3^{-/-}}$ +ISO; P<<0.01) and 7Hz (0.027±0.004 in $\text{Ca}_{v1.3^{-/-}}$; 0.0045±0.001 in $\text{Ca}_{v1.3^{-/-}}$ +ISO; P<<0.01). In the range of frequencies tested, $\text{Ca}_{v1.3^{-/-}}$ in the presence of ISO had no significant impact on the susceptibility to pacing-induced alternans when compared to the absence of ISO. Similar results were observed when the proportion of myocytes progressing into stable alternans was considered. ISO significantly reduced the number of cells that progressed into stable $\text{Ca}^{2+}$ alternans at 6Hz (22% in $\text{Ca}_{v1.3^{-/-}}$; 0% in $\text{Ca}_{v1.3^{-/-}}$ +ISO; P<<0.01) and 7Hz (54% in $\text{Ca}_{v1.3^{-/-}}$; 7% in $\text{Ca}_{v1.3^{-/-}}$ +ISO; P<<0.01). These findings aren’t surprising since ISO has been shown to protect against pacing-induced alternans\textsuperscript{360, 467, 468}. However, the effects of $\beta$-AR stimulation are broad and have multiple protective mechanisms ($\text{Ca}^{2+}$ sequesteration or increased $I_{\text{Cal,L}}$). An inotropic agent that selectively promotes enhanced EC coupling fidelity via an increase in the $I_{\text{Ca}}$ trigger would then be ideal.
Figure 3.3.4.4 Nonselective β-adrenergic stimulation of Ca,1.3−/− left atrial myocytes with isoproterenol shifts susceptibility to Ca2+ alternans to higher frequencies.

The β-AR agonist ISO has been shown to protect against pacing-induced alternans in atrial myocytes through a multitude of mechanisms associated with increasing EC coupling fidelity. If loss of Ca,1.3 I_{Ca} is causing a deficiency in EC coupling, then application of ISO should mitigate the phenotype. Accordingly, I preformed the same field stimulation protocols on LAA myocytes to induce Ca2+ alternans, with the exception of 50nM ISO in the perfusate. A, representative raw whole cell Ca2+ signals from of a Ca,1.3−/− LAA myocyte, first paced in the absence (left), and then presence of ISO (right) at 6Hz. F and Fo represent the Ca2+ signal and the baseline signal of the cell, respectively. Note the stable Ca2+ transient peaks once ISO has bee applied to the cell.

B, A significant reduction in temporal alternans in 50nM ISO treated Ca,1.3−/− LAA myocytes compared to untreated Ca,1.3−/− LAA myocytes. No significant differences were observed between 50nM ISO treated Ca,1.3+/+/ LAA myocytes compared to untreated Ca,1.3+/+/ LAA myocytes with the tested frequency range. At least 10 cells were recorded from each animal and cells were obtained from an n=6 for Ca,1.3+/+, n=2 for Ca,1.3+/+ +ISO, n=4 for Ca,1.3−/−, and an n=4 for Ca,1.3−/− +ISO mice. *P<0.001 between Ca,1.3+/+ and Ca,1.3−/− at 6Hz and 7Hz. **P<0.005 between Ca,1.3−/− and Ca,1.3+/+ +ISO at 6Hz and 7Hz. C, Stable alternans occurred at a temporal alternans variance of ≥0.01. Total number of experimental cells tested is indicated above the bars. A significantly smaller proportion of Ca,1.3−/− +ISO LAA myocytes developed stable Ca2+ alternans at 6 and 7Hz when compared to Ca,1.3−/− without ISO. Significance determined using Fischer’s exact test.
Experimentally, increasing the external Ca\(^{2+}\) concentration has been shown to selectively increase \(I_{Ca}\) (with negligible effect on SR load) and promote Ca\(^{2+}\) release at the peripheral and central region of field-paced atrial myocytes\(^{311}\). In simulation studies with atrial myocytes, increasing the Ca\(^{2+}\) conductance through the LTCCs was found to protect against pacing-induced alternans\(^{330}\). Increasing the driving force for Ca\(^{2+}\) in the remaining LTCCs and increasing the EC coupling trigger could then potentially recover loss of Ca\(_v\)1.3 \(I_{Ca}\) in Ca\(_v\)1.3\(^{+/−}\) mice. I replicated this intervention by increasing the extracellular Ca\(^{2+}\) concentration from 1.0 to 1.5mM, and preformed the same field pacing protocols on Ca\(_v\)1.3 LAA myocytes.

Consistent with notion of elevated external Ca\(^{2+}\) acting as a positive inotropic agent, Ca\(_v\)1.3\(^{+/−}\) LAA myocytes in the presence of 1.5mM Ca\(^{2+}\) shifted their susceptibility to Ca\(^{2+}\) alternans to higher frequencies, when compared to Ca\(_v\)1.3\(^{+/−}\) LAA myocytes at 1.0mM Ca\(^{2+}\) (Fig. 3.3.4.5). This conclusion was drawn from the observation that Ca\(_v\)1.3\(^{+/−}\) LAA myocytes in the presence of 1.5mM Ca\(^{2+}\) had a significantly reduced magnitude in temporal alternans compared to Ca\(_v\)1.3\(^{+/−}\) LAA myocytes in 1.0mM Ca\(^{2+}\), at 6Hz (0.0089±0.001 in Ca\(_v\)1.3\(^{+/−}\) 1.0mM Ca\(^{2+}\); 0.0044±0.001 in Ca\(_v\)1.3\(^{+/−}\) 1.5mM Ca\(^{2+}\); P<0.05), and at 7Hz (0.027±0.004 in Ca\(_v\)1.3\(^{+/−}\) 1.0mM Ca\(^{2+}\); 0.0099 ± 0.002 in Ca\(_v\)1.3\(^{+/−}\) 1.5mM Ca\(^{2+}\); P<<0.01) (Fig. 3.3.4.5 A). In the range of frequencies tested, Ca\(_v\)1.3\(^{+/−}\) at 1.5mM Ca\(^{2+}\) had no significant effects on the susceptibility to pacing-induced alternans when compared Ca\(_v\)1.3\(^{+/−}\) at 1.0mM Ca\(^{2+}\). Comparable results were observed when the proportion of myocytes progressing into stable alternans was considered. Higher external Ca\(^{2+}\) significantly reduced the number of cells that developed into stable Ca\(^{2+}\) alternans in Ca\(_v\)1.3\(^{+/−}\) at 6Hz (22% in Ca\(_v\)1.3\(^{+/−}\) 1.0mM Ca\(^{2+}\); 7% in Ca\(_v\)1.3\(^{+/−}\) 1.5mM Ca\(^{2+}\); P<0.05) and 7Hz (54% in Ca\(_v\)1.3\(^{+/−}\); 23% in Ca\(_v\)1.3\(^{+/−}\) 1.5mM Ca\(^{2+}\); P<<0.01). However, raising extracellular Ca\(^{2+}\) concentration wasn’t as strong as an inotropic agent as ISO since a significantly greater proportion of Ca\(_v\)1.3\(^{+/−}\) at 1.5mM Ca\(^{2+}\) LAA myocytes developed Ca\(^{2+}\) alternans when compared to Ca\(_v\)1.3\(^{+/−}\) at 1.0mM Ca\(^{2+}\) and paced at 7Hz (10% in Ca\(_v\)1.3\(^{+/−}\) 1.0mM Ca\(^{2+}\) 23% in Ca\(_v\)1.3\(^{+/−}\) 1.5mM Ca\(^{2+}\); P<0.05). The results presented thus far suggest Ca\(_v\)1.3\(^{+/−}\) LAA myocytes are susceptible to pacing-induced Ca\(^{2+}\) alternans. This phenotype is suppressed in the presence of inotropic agents, which selectively increase the \(I_{Ca}\) trigger in EC coupling. In-line with these observations, addition of agent that reduces the \(I_{Ca}\) trigger, such as a CCB, should then increase the susceptibility to Ca\(^{2+}\) alternans in Ca\(_v\)1.3\(^{+/−}\) atrial myocytes.
Reduced sensitivity to pacing-induced calcium alternans in \( \text{Ca}_{1.3}^-/^- \) atrial myocytes in response to elevated extracellular calcium concentration.

Increasing extracellular \( \text{Ca}^{2+} \) concentration acts as a positive inotropic agent via preferential increase in \( I_{\text{Ca}} \) conductance. In hopes to recover lost \( \text{Ca}_{1.3} \) \( I_{\text{Ca}} \) the extracellular \( \text{Ca}^{2+} \) concentration was raised from 1.0 to 1.5mM and the field-pacing protocol was performed on \( \text{Ca}_{1.3} \) LAA myocytes. A significant reduction in temporal alternans was observed in \( \text{Ca}_{1.3}^-/^- \) LAA myocytes in 1.5mM external \( \text{Ca}^{2+} \) compared to \( \text{Ca}_{1.3}^-/^- \) at 1.0mM \( \text{Ca}^{2+} \). In the frequency range tested, no significant differences were observed between \( \text{Ca}_{1.3}^+/-/^- \) LAA myocytes 1.5mM external \( \text{Ca}^{2+} \) compared to \( \text{Ca}_{1.3}^+/-/^- \) at 1.0mM \( \text{Ca}^{2+} \).

Stable alternans occurred at a temporal alternans variance of \( \geq 0.01 \). Total number of experimental cells tested included above the bars. A significantly smaller proportion of \( \text{Ca}_{1.3}^-/^- \) LAA myocytes at 1.5mM \( \text{Ca}^{2+} \) developed stable \( \text{Ca}^{2+} \) alternans at 6 and 7Hz when compared to \( \text{Ca}_{1.3}^-/^- \) at 1.0mM \( \text{Ca}^{2+} \). A significantly greater proportion of \( \text{Ca}_{1.3}^-/^- \) LAA myocytes at 1.5mM \( \text{Ca}^{2+} \) developed stable \( \text{Ca}^{2+} \) alternans at 7Hz when compared to \( \text{Ca}_{1.3}^+/-/^- \) at 1.0mM \( \text{Ca}^{2+} \).

Significance determined using Fischer’s exact test.
Experiments using CCBs have demonstrated their impact on asynchronous release of Ca\(^{2+}\) throughout the cell\(^{399}\), in addition to their influence on the development of Ca\(^{2+}\) alternans\(^{327,376}\). Application of CCB to minimize the \(I_{\text{Ca}}\) trigger in Cav1.3\(^{+/+}\) LAA myocytes should then present a similar phenotype to Cav1.3\(^{+/−}\) with respect to susceptibility to pacing-induced Ca\(^{2+}\) alternans. The same field-pacing protocol was applied to Cav1.3\(^{+/+}\) LAA myocytes, however the CCB verapamil was added to the perfusate at 50nM, 100nM, and 200nM. Contrary to what was expected, there was not a significant increase of susceptibility to pacing-induced Ca\(^{2+}\) alternans in Cav1.3\(^{+/+}\) LAA atrial myocytes (Fig. 6.5). As expected, there was a general reduction in the Ca\(^{2+}\) transient magnitude, illustrated by the raw Ca\(^{2+}\) signals from a myocytes paced at 7Hz in the absence and then presence of 50nM verapamil (Fig. 6.5 A). No significant increase in temporal alternan magnitude was observed in Cav1.3\(^{+/−}\) mice at 6Hz or 7Hz (Fig. 6.5 B). Although this may seem to contradict the concept of reduced \(I_{\text{CaL}}\) favoring alternan genesis, it supports the preferential role of Cav1.3 \(I_{\text{CaL}}\) in the EC coupling trigger\(^{170}\). This is consistent with the elevated \(I_{\text{CaL}}\) conductance experiments (high external Ca\(^{2+}\)) where only a partial recovery to pacing-induced Ca\(^{2+}\) alternans was observed in Cav1.3\(^{−/−}\) atrial myocytes. Furthermore, experiments using CCBs to assess their role in Ca\(^{2+}\) alternans development were performed in ventricular myocytes, where Cav1.3 expression is negligible.

A general reduction in \(I_{\text{CaL}}\) (both Cav1.2 and Cav1.3) is clearly not sufficient to induce alternans in atrial myocytes. With this in mind, loss of Cav1.2 should not have a significant impact to pacing-induced Ca\(^{2+}\) alternans, compared to loss of Cav1.3. To test this hypothesis, I acquired transgenic mice heterozygous for Cav1.2 (Cav1.2\(^{+/−}\)), where a 25% reduction in \(I_{\text{CaL}}\) current density was observed in ventricular myocytes\(^{162}\). I performed the same field-pacing protocol on Cav1.2\(^{+/+}\) and Cav1.2\(^{+/−}\) LAA myocytes to assess their sensitivity to Ca\(^{2+}\) alternans. Consistent with the notion that Cav1.3 plays a more fundamental role in Ca\(^{2+}\)-cycling dynamics, Cav1.2\(^{+/−}\) LAA myocytes displayed no difference in vulnerability to pacing-induced Ca\(^{2+}\) alternans when compared to Cav1.2\(^{+/+}\) (Fig. 3.3.4.6). No significant difference in temporal alternan magnitude (Fig. 3.3.4.6 A) or in the proportion of cells that developed stable Ca\(^{2+}\) alternans was observed at any frequency tested (Fig. 3.3.4.6 B). These results, in combination with the CCB observations, further strengthen the argument for a preferential role of Cav1.3 in the LTCC trigger. Although it has yet to be clarified how this dynamic instability in Ca\(^{2+}\) at the
cellular level can then translate to arrhythmogenesis at the tissue level. An explanation lies in the voltage sensitivity of certain ion channels and exchangers to $\text{Ca}^{2+}$. 
Figure 3.3.4.6 Pacing-induced calcium sensitivity was unaffected in Ca,1.2+/− left atrial appendage myocytes.

In SA node myocytes from mice, Ca,1.3 has been suggested to play a more critical role to EC coupling compared to Ca,1.2 and loss of Ca,1.2 should have a negligible impact of Ca^{2+} cycling dynamics. Accordingly, I preformed the field pacing protocol to induce Ca^{2+} alternans in Ca,1.2+/− LAA myocytes to determine the role of Ca,1.2 I_{Ca} in Ca^{2+} cycling dynamics. A, The magnitude in temporal alternans was no significantly different between Ca,1.2+/− and Ca,1.2+/+ LAA myocytes at any frequencies. Cells were obtained from an n=1 Ca,1.2+/+, n=1 for Ca,1.2+/− mice. B, Stable alternans occurred at a temporal alternans variance of ≥0.01. No significant difference in the proportion of cells that developed stable Ca^{2+} was observed between Ca,1.2+/+ and Ca,1.2+/− LAA myocytes. Total number of experimental cells tested included above the bars.
3.4 From Cell to Heart: Link of Calcium Alternans to Arrhythmogensis

3.4.1 Translation to electrical alternans from calcium alternans

Intracellular Ca\textsuperscript{2+} transients can couple to voltage through the Ca\textsuperscript{2+} sensitive ionic currents and exchangers. Positive coupling can occur when a large Ca\textsuperscript{2+} transients increases gain through NCX leading to a depolarizing current (1Ca\textsuperscript{2+} for 3Na\textsuperscript{+}) and APD prolongation\textsuperscript{22}. On the other hand, a large Ca\textsuperscript{2+} transient can yield a shorter APD due to increased Ca\textsuperscript{2+}-induced inactivation of LTCCs\textsuperscript{266,407}. Therefore, if at the cellular level, Ca\textsuperscript{2+} transients alternate, APD will follow suit and oscillate secondarily. In order to determine whether Ca\textsuperscript{2+} alternans were translating to APD alternans and to characterize the nature of this interaction (positive vs. negative), we carried out simultaneous current-clamp patch recordings to measure APs, and fluorescent laser scanning confocal microscopy to acquire Ca\textsuperscript{2+} transients. I found that when Ca\textsuperscript{2+} alternans stabilized in either Ca\textsubscript{v}1.3\textsuperscript{+/-} or Ca\textsubscript{v}1.3\textsuperscript{-/-} LAA myocyte, the APs also displayed oscillations in duration (Fig. 3.4.1 1A). Not only did the APD oscillate with the Ca\textsuperscript{2+} transient alternans, but it also was a statistically significant positive correlation in Ca\textsubscript{v}1.3\textsuperscript{+/-} (P<<0.01), and Ca\textsubscript{v}1.3\textsuperscript{-/-} (P<0.05) mice (Fig. 3.4.1 1B). In atrial myocytes, $I_{NCX}$ can influence prolongation of APD, which is proportional to the intracellular Ca\textsuperscript{2+} transient. Therefore, it is not surprising that a positive correlation to voltage was observed during Ca\textsuperscript{2+} alternans in both genotypes. This effectively provided a mechanistic link for Ca\textsuperscript{2+} alternans to electrical alternans. Once these electrical alternans have stabilized in cardiac tissue, they can be a precursor to reentry arrhythmias via dispersion of refractoriness\textsuperscript{413,416,417}. These observations led us to characterize the electrical properties of Ca\textsubscript{v}1.3\textsuperscript{-/-} atria during AF.
Figure 3.4.1.1 Positive calcium to voltage coupling during alternans in Ca\textsubscript{v}1.3 left atrial myocytes.

In order for Ca\textsuperscript{2+} alternans to be a precursor to reentry arrhythmias at this tissue level, they must first translate to APD alternans. Thus, to determine whether Ca\textsuperscript{2+} alternans influence APD and the type of coupling that exist, simultaneous APs and Ca\textsuperscript{2+} transients were recorded. Confocal microscopy was used to visualize Fluo-3 loaded LAA myocytes, while APs were recorded in the current-clamp mode. Injecting current through the pipette tip allowed pacing of cells. A, Representative Ca\textsuperscript{2+} signal tracings (top) and the corresponding APs (bottom) of a Ca\textsubscript{v}1.3\textsuperscript{+/+} (left) and Ca\textsubscript{v}1.3\textsuperscript{-/-} (right) LAA myocyte paced at 5Hz. F and F\textsubscript{o} represent the Ca\textsuperscript{2+} signal and the baseline signal of the cell, respectively. Note the simultaneous Ca\textsuperscript{2+} transient magnitude and APD alternans. Also note that when the Ca\textsuperscript{2+} transient was large, the APD was prolonged. B, Representative plot of Ca\textsuperscript{2+} transient magnitude against the respective APD\textsubscript{90} of a Ca\textsubscript{v}1.3\textsuperscript{+/+} (left) and Ca\textsubscript{v}1.3\textsuperscript{-/-} (right) LAA myocyte during a segment of stable alternans. Dashed lines represent the 95% confidence bands for the linear regression fit, while solid line is the fit to a linear regression. A Pearson’s correlation test determined a significant relationship between F/F\textsubscript{o} vs. APD\textsubscript{90} for Ca\textsubscript{v}1.3\textsuperscript{+/+} (P<0.001), and Ca\textsubscript{v}1.3\textsuperscript{-/-} (P<0.05). Note the positive relationship between Ca\textsuperscript{2+} transient magnitude and APD\textsubscript{90} when fit to a linear model.
3.4.2 Consequences of Ca\textsuperscript{2+} alternans

APD alternans alone don’t pose an arrhythmogenic threat unless they become SDA. SDA enhance the gradients and dispersion of repolarization leading to conduction block\textsuperscript{413, 416, 417}. Additionally, Ca\textsuperscript{2+} waves can pose an arrhythmogenic threat by causing rises in diastolic E\textsubscript{m} to promote EADs/DADs\textsuperscript{397, 403, 420} or regional delays/block in conduction (via reduced available I\textsubscript{Na}). Consequently, I would expect an electrical substrate with these characteristics to produce a complex and disorganized pattern due to multiple sites of regional conduction block. To further characterize the specific nature of AF in Ca\textsubscript{v}1.3\textsuperscript{-/-} atria, optical images captured from isolated atria were used to directly interpret conduction block.

By performing STFT analysis on optically generated APs, we were able to track spatial, as well as temporal, changes of df\textsubscript{s} during AF. For comparison, electrical remodeling was pharmacologically induced in structurally/electrically normal Ca\textsubscript{v}1.3\textsuperscript{+/+} atria using the muscarinic agonist carbachol (homogenous reduction in APD shortening). Compared to electrically remodeled atria (Ca\textsubscript{v}1.3\textsuperscript{+/+}+carbachol), Ca\textsubscript{v}1.3\textsuperscript{-/-} exhibited AF with dynamic df\textsubscript{s} and multiple secondary components that were spatially heterogeneous (**Fig. 3.4.2.1 A**). The complexity of AF events can be quantified using the organizational index (OI). The OI is the ratio of the area under the df peak over the area under the discrete FFT plot from 0–70Hz, a value which increases with decreasing complexity (single component, no sub harmonics). The method for calculation of OI was then used to quantify complexity of AF in Ca\textsubscript{v}1.3 atria. The AF in Ca\textsubscript{v}1.3\textsuperscript{-/-} was much more complex, with a significantly lower OI, when compared to Ca\textsubscript{v}1.3\textsuperscript{+/+} with 50nM carbachol (0.78±0.04 in Ca\textsubscript{v}1.3\textsuperscript{+/+}+CCH; 0.59±0.04 in Ca\textsubscript{v}1.3\textsuperscript{-/-}; P<0.05) (**Fig. 3.4.2.1 B**). To determine whether the spatial gradients in frequencies observed in the Ca\textsubscript{v}1.3\textsuperscript{-/-} AF were due to conduction block, I systematically analyzed the sequence of events in the activation patterns of the LAA during AF in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice (**Fig. 3.4.2.2**). I found that when heterogeneity of the activation frequencies was observed in the LAA (evident in frequency maps) (**Fig. 3.4.2.2 A**), it was due to regional conduction block in 2:1 and 3:1 coupling patterns (**Fig. 3.4.2.2 B and C**). From this evidence it can be concluded that regional conduction block was due to either refractory tissue (due to dispersion of APD) or exhaustion of conduction (due to rises in the diastolic E\textsubscript{m}).
Figure 3.4.2.1 Atrial fibrillation in Ca\textsubscript{v}1.3\textsuperscript{-/-} atria exhibits greater spatial and temporal heterogeneity of dominant frequencies.

SDA enhance the heterogeneity of the APD across tissue resulting in localized conduction block and multiple sites of reentry, contrary to a general reduction AERP facilitating macro reentry. Frequency maps were generated from AF optical images of Ca\textsubscript{v}1.3\textsuperscript{-/-} and Ca\textsubscript{v}1.3\textsuperscript{+/+} with 50nM carbachol (CCH) to compare the consequence of different electrical substrates. Short-time FFTs were preformed on optical images with a 512 frame (~550ms) window with a 256 frame (~275ms) overlap. To quantify the spatial fragmentation of frequencies encapsulating the atria, the same method for OI calculation was used, but instead extrapolated from optical image signals. 

A, Representative frequency maps of AF optical mapping images from a Ca\textsubscript{v}1.3\textsuperscript{+/+} atria with 50nM CCH (top) and a Ca\textsubscript{v}1.3\textsuperscript{-/-} atria (bottom). Numbering represents sequence of events, with an elapsed duration of 257ms between each frame. Note the spatial heterogeneity and dynamic nature of the dfs encapsulating the Ca\textsubscript{v}1.3\textsuperscript{-/-} atria (bottom). 

B, A significantly lower OI was observed in AF of Ca\textsubscript{v}1.3\textsuperscript{-/-} atria compared to AF in Ca\textsubscript{v}1.3\textsuperscript{+/+} with 50nM CCH.
SDA is characterized by alternating phase of APD in localized regions of tissue, promoting multiple sites of conduction block and reentry. To determine whether conduction block was responsible for the fragmentation of dfs in the frequency maps of Ca\textsubscript{v}1.3\textsuperscript{−/−} AF, I analyzed the LAA, where a high degree of heterogeneity was observed. Short-time FFTs were preformed on optical images with a 512 frame (~550ms) window with a 256 frame (~275ms) overlap to create frequency maps and corresponding montages were generated from optical images. A, Representative frequency maps of AF optical mapping images from a Ca\textsubscript{v}1.3\textsuperscript{−/−} atria. Numbering represents sequence of events, with an elapsed duration of 257ms between each frame. Note the segregation of frequencies in the Ca\textsubscript{v}1.3\textsuperscript{−/−} LAA and the dynamic temporal properties of the frequencies. B, Montage of a cycle of AF obtained from a di-4-ANEPPs stained Ca\textsubscript{v}1.3\textsuperscript{−/−} atria. White arrows depict the depolarization propagation pathway, while the dotted white line represents conduction block. Numbering depicts the sequence of events, with 85ms of elapsed time between each frame. The lettering ‘a’ and ‘b’ are the location in the LAA where the voltage signal was recorded from in C. Note the wave of depolarization propagated completely (region ‘a’ to ‘b’) through the LAA on the first activation (1-5), but the failure of conduction (only activation of region ‘a’) on the next pass through the LAA (6-10). C, The voltage signal intensity was measured from region ‘a’ and ‘b’ (as depicted in montage of B) to generate raw tracings of conduction in the LAA. Note at a particular instance, the rise of voltage intensity in region ‘a’ wasn’t always correlated with a rise in voltage in region ‘b’, suggesting conduction block.
3.5 Other Potential Mechanisms of Atrial Fibrillation in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice: Structural Remodeling

Although we have already described an electrically remodeled atria in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice, AF is a multifactorial pathophysiology. Ca\textsubscript{v}1.3\textsuperscript{-/-} mice have increased ventricular mass, and cardiovascular risk factors associated with the development of AF include left ventricular hypertrophy, and left ventricular dysfunction\textsuperscript{451-453}. This led us to characterize evidence for structural remodeling in these mice. Chronic AV block in dogs induced a decrease in mean arterial pressure (MAP), a rise in left ventricular end-diastolic pressure (LVEDP), an indirect measure of left atrial pressure, and right atrial pressure\textsuperscript{454, 455}. Moreover, development of myocardial hypertrophy in dogs has been shown following complete AV block\textsuperscript{456}. In addition, atrial dilatation due to chronic AV block in the goat was associated with a greater vulnerability to induced AF paroxysms\textsuperscript{457, 458}, however a mechanism for this association has yet to be described. These observations led us to suspect sinus bradycardia, acute episodes of AV block, and supraventricular and ventricular extrasystoles, as inducers of hemodynamic stress on the atria of Ca\textsubscript{v}1.3\textsuperscript{-/-} mice. Since chronic atrial dilatation due to elevations in atrial pressure promotes stretch and atrial fibrosis\textsuperscript{459-462}, and infiltrating inflammatory cells are the source of a multitude of inflammatory cytokines implicated in tipping the balance of collagen synthesis/degradation in favor of synthesis\textsuperscript{463}, I pursued evidence for the presence of atrial fibrosis, elevated atrial pressure, and inflammatory cell infiltration.

Previous studies, as well as my results, have illustrated P-wave duration prolongation (Fig. 3.1.1), as well as a decrease in the P-wave amplitude\textsuperscript{167} in Ca\textsubscript{v}1.3\textsuperscript{-/-}, observations consistent with delayed atrial conduction. To directly assess atrial conduction, CV was measured using the optical mapping technique of atria stained with Di-4-ANEPPS. To estimate epicardial CV, atria were paced at 10Hz to control for beating rates. A sequence of 5000 frames were captured at 934 frames/s and analyzed using Scroll software (courtesy of Sergey Mironov, and see Laughner et al for detailed methods)\textsuperscript{464}. Also, to avoid recruitment of the conducting system in the atria (of which Ca\textsubscript{v}1.3 plays a significant role in) retrograde conduction of the LAA was accomplished by pacing at the edge of the LAA at 10Hz. Consistent with prolonged P-wave durations observed \textit{in vivo}, Ca\textsubscript{v}1.3\textsuperscript{-/-} mice displayed a significantly delayed conduction when paced at 10Hz (0.65±0.020m/s in Ca\textsubscript{v}1.3\textsuperscript{+/+}; 0.47±0.010m/s in Ca\textsubscript{v}1.3\textsuperscript{-/-}; P<<0.01) (Fig. 3.5.1).
Atrial fibrosis is considered to be one of the most important factors in the generation of a substrate for AF. Biopsies from patients with AF have confirmed the presence of atrial fibrosis, and post-operative AF or the recurrence of AF has been correlated with the degree of atrial fibrosis and fibrogenic activity. Atrial fibrosis promotes the loss of side-to-side fiber connections leading to slowed CV and non-uniform anistrophic conduction (“zig-zag conduction”). Also, since fibroblasts are electrically coupled to myocytes, it poses an electrical sink for the available current. If these discontinuities in conduction are large enough, it can lead to unidirectional block, and allow for reentry in relatively small circuits. In order to determine whether atrial fibrosis was present in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice, histological analysis was attained using confocal laser microscopy of PSR stained hearts. PSR exhibits fluorescence properties, similar to Rhodamine, with excitation at 550nm and emission at 600nm. Z-stacks, obtained using confocal laser-scanning microscopy, were quantified via threshold measurements using ImageJ software, and revealed an approximate 2-fold increase in atrial collagen content in both the RAA and LAA of 12 week aged Ca\textsubscript{v}1.3\textsuperscript{-/-} mice (RAA: 11.67±0.68%, LAA: 11.13±0.93% in Ca\textsubscript{v}1.3\textsuperscript{+/+}; RAA: 23.68±3.42%, LAA: 21.84±1.11%, Ca\textsubscript{v}1.3\textsuperscript{-/-}; P<<0.01) (Fig. 3.5.2). ‘Replacement fibrosis’, where collagen deposition replace functional myocardium secondary to tissue damage and cell death, is commonly observed in models of atrial dilation and congestive heart failure. In a canine mitral regurgitation model, increased AF vulnerability was attributed to structural remodeling of the atria and was associated with leukocyte infiltration and interstitial fibrosis. Macrophages and neutrophils are critical to tissue repair by releasing growth factors such as fibroblast growth factor and TGF\(\beta\), implicating them in fibrogenic remodeling of atria, and are found in the atrium of AF patients. Therefore, I used immunohistochemistry to detect immune cells within Ca\textsubscript{v}1.3 atrial tissue at 12 weeks of age. I failed to observe a significant increase of CD107b (mac-3) positive staining cells or Ly6b.2 (neutrophil) staining cells in Ca\textsubscript{v}1.3\textsuperscript{-/-} atrial myocardium (Fig. 6.6). It is possible that the inflammatory process occurs much earlier in development since atrial fibrosis was already fully developed at 12 weeks of age in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice. Another possibility may involve the stretch-induced activation of p38 MAPK in fibroblasts, which would completely bypass the process of inflammation in fibrotic remodeling.
Figure 3.5.1 Slowed conduction in atria isolated from Ca\textsubscript{v}1.3\textsuperscript{-/-} mice.

Given the prolongation in P-waves observed Ca\textsubscript{v}1.3\textsuperscript{-/-} mice, I next investigated whether conduction slowing of the atria was present. Isolated atria preparations were incubated with the voltage sensitive fast-response dye, di-4-ANEPPS. The LAA was paced at 10Hz (100ms) and retrograde conduction through the appendage was captured with a high-speed camera (934frames/s). A, LAA activation in response to a single pulse delivered at the edge of the appendage. White isochrones represent time elapsed in 2ms increments. Note slower conduction through a Ca\textsubscript{v}1.3\textsuperscript{-/-} LAA (right) compared to a Ca\textsubscript{v}1.3\textsuperscript{+/+} LAA (left), as evident by closer isochrones lines. B, Averaged epicardial CVs reveal significant reductions in CV of the LAA of Ca\textsubscript{v}1.3\textsuperscript{-/-} mice.
Conduction slowing occurs with increased collagen deposition. My observation that conduction velocity was slowed in Ca$_{v}$1.3$^{-/-}$ atria at 12 weeks of age, prompted us to exploit the fluorescent properties of the collagen-specific stain PSR using a spinning disk confocal laser-scanning imaging system. Sections were excited using a 491 nm laser, and Z-stacks were acquired and flattened for quantification. A, Bright yellow regions represent increased PSR staining indicating increased collagen density. Collagen deposition in the interstitial space between myocyte bundles in the LAA (left) and RAA (right) of a Ca$_{v}$1.3$^{+/+}$ mouse (top) compared to a Ca$_{v}$1.3$^{-/-}$ mouse (bottom). Note preserved organization of collagen bundles. B, Quantification, using the threshold method to select and count pixels based on brightness, reveals statistically significant elevations in collagen content (standardized to total tissue) in Ca$_{v}$1.3$^{-/-}$ mice compared to Ca$_{v}$1.3$^{+/+}$ mice.

Figure 3.5.2 Elevated pericapsular and interstitial atrial fibrosis in Ca$_{v}$1.3$^{-/-}$ atria
Chronic hypertension is a strong risk factor for AF, although the exact pathophysiological link between hypertension and AF remains vague. Since Ca\textsubscript{v}1.3\textsuperscript{-/-} mice display diabetic traits, although not overt diabetes, and diabetes is associated with both hypertension\textsuperscript{474} and AF\textsuperscript{475,476}, MAP and left atrial pressure was assessed in anesthetized Ca\textsubscript{v}1.3 mice. Despite the previously described link between Ca\textsubscript{v}1.3\textsuperscript{-/-} mice and diabetes, MAP recordings illustrated an unexpected hypotension in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice when compared to Ca\textsubscript{v}1.3\textsuperscript{+/+} mice (97.5±1.1mmHg in Ca\textsubscript{v}1.3\textsuperscript{+/+}; 82.0±2.0mmHg in Ca\textsubscript{v}1.3\textsuperscript{-/-}; P<<0.01) (Fig. 6.7 D). Furthermore, a significant difference in left atrial pressure (assessed indirectly form left ventricular-end diastolic pressure) was not observed in Ca\textsubscript{v}1.3 mice (Fig. 6.7 C). These results do not support a mechanism of increased vascular resistance due diabetes in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice, which would ultimately pose a hemodynamic stress on the left atrium, and result in activation of renin-angiotensin-aldosterone system.

From clinical and experimental studies, it is well known that chronic hemodynamic stress results in myocardial hypertrophy and fibrosis\textsuperscript{477}. Since conduction slowing, and atrial fibrosis were observed in Ca\textsubscript{v}1.3\textsuperscript{-/-} atria, invasive hemodynamic measurements were used to determine whether these mice display chronic hemodynamic stress, which might explain the presence of fibrosis. Despite severe SA nodal abnormalities, significant changes in left and right developed ventricular pressures were not observed Fig. 6.7 A, B). However, maximum contractility (\(dP/dt_{\text{max}}\)) (measured as the maximum rate of developed pressure) was significantly lower in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice (9791±839.5mmHg/s in Ca\textsubscript{v}1.3\textsuperscript{+/+}; 7056±662.7mmHg/s in Ca\textsubscript{v}1.3\textsuperscript{-/-}; P<0.01) (Fig. 6.7 E). However, once normalizing for after-load differences (dP/dt at 30mmHg), since both genotypes of Ca\textsubscript{v}1.3 mice had significantly different MAPs, the contractility was not significantly changed (Fig. 6.7 E).

Although Ca\textsubscript{v}1.3\textsuperscript{-/-} mice failed to present any evidence of hemodynamic stress under controlled conditions compared to Ca\textsubscript{v}1.3\textsuperscript{+/+} mice, the results above did not take account of potential hemodynamic stress associated with potential AV block, and supraventricular and ventricular extrasystoles. Indeed, Ca\textsubscript{v}1.3 mice have been shown to have extrasysoles\textsuperscript{168}, and a novel ventricular ectopy animal model (using a unique pacing algorithm) by Huizar et al\textsuperscript{478} demonstrated their link with structural remodeling. Accordingly, I then probed Ca\textsubscript{v}1.3\textsuperscript{-/-} mice for ventricular extrasysoles and their hemodynamic consequences by implanting Ca\textsubscript{v}1.3 mice with radio frequency ECG/pressure transmitters. In all three of the Ca\textsubscript{v}1.3\textsuperscript{-/-} implanted with telemetry
units, I identified ventricular ectopy, which was associated with prolonged elevations of left
ventricular pressures (Fig. 3.5.3). Ventricular ectopy was characterized by a unique QRS
waveform, and often presented itself in multiple rapid runs of activation. No signs of any
abnormal ventricular or any other electrical activity was observed in the Ca_{v}1.3^{+/+} mouse
assessed. Consistent runs of ventricular ectopy maintained diastolic pressures well above
30mmHg. These transient elevations in pressure, even during end-diastole (when atrial and
ventricular pressures are equal) may explain the atrial remodeling in Ca_{v}1.3^{-/-} mice. Furthermore,
the hemodynamic consequences observed in the ventricular ectopy model by Huizar et al^{478}
could explain the presence of ventricular hypertrophy in Ca_{v}1.3^{-/-} mice.
Figure 3.5.3 Ventricular extrasystoles in Ca\textsubscript{v}1.3\textsuperscript{−/−} with hemodynamic consequences.

Since premature ventricular contractions produce hemodynamic stress resulting in structural remodeling, and Ca\textsubscript{v}1.3\textsuperscript{−/−} mice have been shown to display ventricular extrasystoles \textit{ex vivo}, I monitored Ca\textsubscript{v}1.3\textsuperscript{−/−} mice \textit{in vivo} to probe for irregular ventricular activity. Radio frequency ECG/pressure transmitters were implanted into Ca\textsubscript{v}1.3 mice, and the pressure-transducing catheter was inserted into the right carotid artery and guided into the LV, while the ECG electrodes were placed in lead II configuration. Representative ECG recordings (top) with the corresponding LV pressures (bottom) are displayed from Ca\textsubscript{v}1.3\textsuperscript{+/+} (left) and Ca\textsubscript{v}1.3\textsuperscript{−/−} mice (right). Note the irregular QRS activation in the Ca\textsubscript{v}1.3\textsuperscript{−/−} ECG recordings, compared to the regular QRS waveform in Ca\textsubscript{v}1.3\textsuperscript{+/+} mice, suggesting ventricular ectopy. Also, note how the ventricular extrasystoles result in sustained elevations in ventricular pressure when the left ventricle should be in diastole. Ventricular extrasystoles were observed in all Ca\textsubscript{v}1.3\textsuperscript{−/−} mice implanted with telemetry units. For Ca\textsubscript{v}1.3\textsuperscript{+/+} n=1; For Ca\textsubscript{v}1.3\textsuperscript{−/−} n=3.
3.6 Electrical versus structural remodeling

In the course of my assessment and characterization of AF events in the atria isolated from Ca\(_{v}1.3^{-/-}\) mice, I noted a greater complexity in the ECG AF signal, especially when compared to a completely structural remodeled atria (when completely denervated), such as our exercise induced AF mouse model (Fig. 3.3.1 A). By performing short-time Fourier transforms analysis on AF events from field ECGs recorded from isolated atria, it was possible to track the \(dfs\) as a function of time throughout the entire length of the AF waveform (Fig. 3.3.1 B). As illustrated in the representative example in Figure 3.3.1 A, there is clear evidence of dynamic variation in \(df\) and multiple sub-harmonic components in the AF waveform recorded from Ca\(_{v}1.3^{-/-}\) atria compared to a structurally remodeled atria (exercise mice). In contrast, the structurally remodeled AF substrate presented a more organized signal and the \(dfs\) often remained stable without secondary components (Fig. 3.3.1 B). Previous studies have demonstrated the usefulness of spectral analysis in gaining insight into the organization of AF\(^{434-437}\). Previous studies have shown distinct OI patterns in structurally or electrically remodeled atria\(^{434}\), and electrically remodeled atria often exhibited AF patterns that were significantly more disorganized than structurally remodeled atria. To quantify the complexities of AF events in Ca\(_{v}1.3\) mice, I applied a similar method to measure OI as in (Fig.****). This analysis revealed AF events with significantly lower OIs in Ca\(_{v}1.3^{-/-}\) when compared to AF in exercised atria (0.57±0.05 in exercised; 0.23±0.03 in Ca\(_{v}1.3^{-/-}\); P<0.01) (Fig. 3.3.1 C). In conclusion, AF events from Ca\(_{v}1.3^{-/-}\) mice are significantly more complex in nature than structurally remodeled atria, and while the fibrosis may contribute to the AF pathology, cardiac alternans are the primary substrate triggering the AF. For example, the Ca\(^{2+}\) alternans are responsible for initiating the concordant APD alternans throughout the tissue, but the conduction discontinuities provided by fibrosis may promote the discordance of the APD alternans.
In a study assessing AF organization, OIs were significantly different in atria with fibrosis compared to electrical remodeling. Accordingly, I calculated the OIs of 3 isolated atria AF ECGs recorded from completely structurally remodeled atria (exercised mice) and Ca\(_{\text{v}}\)1.3\(^{-/-}\) atria. 

**A**, Representative AF ECG traces from exercised (left) and Ca\(_{\text{v}}\)1.3\(^{-/-}\) (right) isolated atria. Note the irregular AF ECG pattern in the Ca\(_{\text{v}}\)1.3\(^{-/-}\) signal. 

**B**, Representative 3D surface plots of short-time FFTs of AF ECGs recorded from isolated atria from exercised (left) and Ca\(_{\text{v}}\)1.3\(^{-/-}\) (right) mice. Note the dynamic \(df\), and multiple sub-harmonic frequency components in Ca\(_{\text{v}}\)1.3\(^{-/-}\) AF. 

**C**, The AF observed in Ca\(_{\text{v}}\)1.3\(^{-/-}\) ex vivo had a significantly lower OI, compared to exercise atria, suggesting a complex electrical AF substrate.
Chapter 4
Discussion
4 Discussion

Initiation of reentry in patients with chronic AF was attributed to an abbreviation in APD and was associated with a reduction in the α1 subunit mRNA, protein and $I_{CaL}^{36, 182-184}$. However, despite increased vulnerability to AF, Ca$_v$1.3$^{-/-}$ mice did not show significant reductions in APD. Recent evident suggests APD alternans, secondary to Ca$^{2+}$ transient alternans, as an important mechanism in the trigger of AF due to dispersion of refractoriness resulting in conduction block (spatially discordant alternans). We are the first to report a novel mechanistic link between loss of Ca$_v$1.3 $I_{CaL}$ and development of an AF substrate. Our results demonstrate that loss of Ca$_v$1.3 exclusively, and not Ca$_v$1.2, produces a dynamic instability in Ca$^{2+}$-cycling when paced at supraphysiological frequencies, and leads to stable Ca$^{2+}$ alternans. These findings support the conclusion that Ca$^{2+}$ alternan development is dependent on reductions in the $I_{CaL}$ trigger for EC coupling$^{330, 398, 403, 404}$, since inotropic agents reduced the sensitivity to Ca$^{2+}$ alternans in Ca$_v$1.3$^{-/-}$ atrial myocytes. In atrial myocytes, large Ca$^{2+}$ transients have been associated with APD prolongation via $I_{NCX}^{408, 409, 410}$, and therefore it wasn’t surprising when translation of Ca$^{2+}$ alternans to electrical alternans displayed positive coupling in Ca$_v$1.3 atrial myocytes. Consistent with the single cell findings, these oscillations in APD translated to the tissue level, and our results suggest spatially discordant alternans was responsible for the perpetuation of AF in Ca$_v$1.3$^{-/-}$ atria.

4.1 Atrial fibrillation in Ca$_v$1.3$^{-/-}$ mice

It has recently been shown that Ca$_v$1.3$^{-/-}$ mice are more susceptible to AF in ex vivo, langendorff perfused hearts$^{167}$ and in vivo, via a midline sternotomy approach to gain access to the cardiac structures$^{136}$. However, the underlying mechanisms for increased vulnerability to AF in Ca$_v$1.3$^{-/-}$ mice have yet to be described. I have further characterized this phenotype ex vivo in isolated atrial preparations from Ca$_v$1.3$^{-/-}$ mice. The advantage to this type of preparation was the ability to qualitatively interpret the complex activation profiles observed in Ca$_v$1.3$^{-/-}$ AF. Not only did I confirm the significantly greater susceptibility to sustained AF in Ca$_v$1.3$^{-/-}$ atria, but I also observed bouts of spontaneous sustained AF which has not been described. The process of “kindling” can initiate reentry through appropriately timed sequences of ectopic foci, which lead to wavefront-wavetail interaction, ultimately leading to abnormal impulse propagation, conduction block, and electrical$^{26-28}$. PV ectopy as a trigger for AF in humans has been
previously described, therefore, the spontaneous ectopic activity present in \( \text{Ca}_V^{1.3}\) isolated atria is likely responsible for initiating the observed spontaneous episodes of AF.

AF induction was preformed in vivo by introducing an octapolar electrophysiology catheter into the right jugular vein and into the RV of anesthetized mice, a more physiological approach than the isolated atria preparations. Contrary to what was expected, I did not observe any increase in susceptibility to inducible AF in \( \text{Ca}_V^{1.3}\) mice. Vagal nerve activity may itself increase vulnerability to AF through APD shortening, heterogeneity and reduced atrial refractoriness. Despite this, administration of the parasympathetic agonist carbachol to reduce atrial refractoriness did not increase the susceptibility of \( \text{Ca}_V^{1.3}\) mice to AF with our rapid stimulation protocols. These discrepancies between the ex vivo and in vivo experiments suggest that perhaps autonomic nerve inputs are interfering/reducing the AF inducibility in \( \text{Ca}_V^{1.3}\). Accordingly, I injected atropine and propranolol to pharmacologically block both branches of autonomic input, but the susceptibility to AF in \( \text{Ca}_V^{1.3}\) mice failed to surface.

The inconsistency in these findings with Zhang et al. may reflect differences in the methodology with respect to the in vivo AF induction protocols. The in vivo protocol employed by Zhang et al., accessed cardiac structures with a midline sternotomy approach while maintaining respiration with an external respirator. The pericardial sac was incised to apply epicardial pacing/recording wires to the exposed epicardium of the atria and bipolar pacing was performed by use of a paired unipolar electrode configuration for stimulation. This approach significantly alters the hemodynamic consequences of respiration on heart function. For example elevations in venus flow-rate with inspiration, and parasympathetic changes associated with respiration (respiratory sinus arrhythmia). In contrast, our protocol involved a much less invasive procedure, since only the availability of the jugular vein was required to gain access to the right atria and ventricle via an electrophysiological catheter. Stimulation of AF by Zhang et al. was carried out with direct epicardial pacing, which involves a much smaller area of myocardial tissue that is depolarized, compared to a larger stimulation site delivered by the electrode rings (0.5mm in diameter) of our octapolar catheter. These differences in methodology may have yielded the observed differences in susceptibility to AF in vivo in the \( \text{Ca}_V^{1.3}\) mice. Of significant note is the discrepancy in AERP which Zhang et al. obtained from intact \( \text{Ca}_V^{1.3}\) mice. I found the AERP to be vastly prolonged in the \( \text{Ca}_V^{1.3}\) intact mice, while Zhang
et al\textsuperscript{136} observed no difference. Electrical remodeling of the atria that shorten the refractoriness predispose to AF\textsuperscript{444}, whereas a significant prolongation of AERP alone is protective, and likely accounts for the reduced susceptibility to \textit{in vivo} AF in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice. Future experiments designed to precisely address the mechanism accounting for the prolonged AERP \textit{in vivo} are in progress.

4.2 Physiological characterization of Ca\textsubscript{v}1.3\textsuperscript{-/-} mice

Studies using the transgenic knockout mouse of Ca\textsubscript{v}1.3 (Ca\textsubscript{v}1.3\textsuperscript{-/-}) revealed the critical role of pacemaker activity in the SA node\textsuperscript{52,167}. Characterization of the electrophysiological parameters of Ca\textsubscript{v}1.3\textsuperscript{-/-} mice has revealed sinus bradycardia, sinus arrest, supraventricular and ventricular extrasystoles, delayed AV conduction, varying degrees of AV node block, a uncoupling of HR and PR interval duration, prolonged PR intervals, and prolonged P-wave durations\textsuperscript{52,160,167-169}. Consistent with these findings, I have observed bradycardia, sinus arrest, AV block and prolonged P-wave and PR intervals in my mice, despite the different strain background. These atrial conduction abnormalities are anticipated since the expression pattern of Ca\textsubscript{v}1.3 in the heart has been described to be limited to the cardiac conduction system (including the SA node, AV node and Purkinje fibers) as well as in the working myocardium of both atria, but not in the ventricles\textsuperscript{159,161,166}. Thus, it is not surprising that we, and others\textsuperscript{160,168}, have uncovered 2\textsuperscript{nd} degree AV block in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice\textsuperscript{160,168}.

Interestingly, Ca\textsubscript{v}1.3\textsuperscript{-/-} mice display ectopic foci located around the region of the atrial myocardium of the PV sleeves, especially during sinus arrest. PV ectopy are typically due abnormal impulse formation (automaticity), triggered activity (EADs or DADs), or microreentry due to the abnormal tissue structure of the PVs. I have found elevated atrial fibrosis in the RAA and LAA of Ca\textsubscript{v}1.3\textsuperscript{-/-} mice, which may facilitate abnormal impulse propagation allowing a smaller input current from the ectopic focus to enable initiation of propagation\textsuperscript{112,113}. In addition, sinus arrest and bradycardia provide an escape from overdrive suppression (a mechanism to maintain a hierarchical pattern of activation in pacemaker cells\textsuperscript{34,35}). The combination of these two processes provides an ideal substrate for the genesis of an ectopic focus in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice.

Another interesting finding in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice was the presence of ventricular hypertrophy. This observation might be explained by the sinus arrest, supraventricular and ventricular extrasystoles, and varying degrees of AV node block observed in Ca\textsubscript{v}1.3\textsuperscript{-/-} hearts\textsuperscript{168}. Chronic AV
block generated in dogs has been associated with the development of myocardial hypertrophy\textsuperscript{442, 456} and genetic knockout mice with a predisposition to heart block, were also associated with ventricular hypertrophy\textsuperscript{481}. Studies on dogs have illustrated the hemodynamic consequences of heart block, wherein severe volume over-load and hemodynamic stress followed AV-block events\textsuperscript{454-456}. Given these findings, it is possible that episodes of heart block in Ca\textsubscript{\textit{v}}1.3 mice may lead to transient rises in LVEDP\textsuperscript{441, 442} that would lead to hypertrophic remodeling of the ventricles\textsuperscript{454, 455}. In addition, premature ventricular contractions in an animal model (using a unique pacing algorithm) demonstrated the link between frequent premature ventricular contractions and hypertrophic remodeling\textsuperscript{478}. The ventricular bigeminy-induced cardiomyopathy was characterized by reduced LVEF, enlarged LV systolic dimensions, and increased severity of mitral valve regurgitation. It is therefore possible that the extrasystoles observed in Ca\textsubscript{\textit{v}}1.3\textsuperscript{−/−} may contribute, in addition to the heart block events, to the observed ventricular hypertrophy.

4.3 Atrial refractoriness in Ca\textsubscript{\textit{v}}1.3\textsuperscript{−/−} mice

Mutations in depolarizing ion channels that affect proper function or that result in reduced expression are often responsible for decreases in atrial refractoriness, consequential of a reduction in APD\textsuperscript{172-177}. Electrophysiological remodeling in the atria, consistent with a shortening in the refractoriness predispose to AF\textsuperscript{444}. In human patients with persistent AF, and rapidly paced atria of dogs, mRNA and protein levels of Ca\textsubscript{\textit{v}}1.2 LTCCs, along with \textit{I}_{\text{CaL}} were reduced\textsuperscript{182-184}. As follows, loss of Ca\textsubscript{\textit{v}}1.3 \textit{I}_{\text{Ca}} could also produce a reduction in APD and consequently atrial refractoriness. However, the AERP measured from intact Ca\textsubscript{\textit{v}}1.3\textsuperscript{−/−} mice were significantly prolonged. These results are consistent with intracellular potentials recorded in isolated atrial tissue. However, erratic pacing and sinus bradycardia likely led to accommodation and restitution mediated changes in APD. Pacing at specific frequencies normalized beating rates between genotypes. It is important to note that sinus bradycardia persisted during the in-tact animal measurements of AERP, so it is possible a similar mechanism accounts for the prolonged AERPs \textit{in vivo}. Consistent with my observations, Zhang \textit{et al}\textsuperscript{52} also revealed a prolongation of APD at 80% repolarization, albeit from SA node cell preparations. These results illustrate that the electrical remodeling characterized by ERP reductions is not present in Ca\textsubscript{\textit{v}}1.3\textsuperscript{−/−}. However, there are other types of electrical remodeling which can predispose to AF, namely cardiac alternans.
4.4 Cardiac Alternans in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice

Cardiac alternans are characterized by the cyclic, beat-to-beat variations in APD (electrical alternans) and contraction (Ca\textsuperscript{2+} transient) amplitude (mechanical or Ca\textsuperscript{2+} alternans)\textsuperscript{235}. Cardiac alternans may induce AF or facilitate the transition from flutter to fibrillation due to conduction block and wavebreak\textsuperscript{244-247}. When we rapidly paced the RAA of Ca\textsubscript{v}1.3\textsuperscript{-/-} mice, we found an increase in the magnitude of APD alternans compared to Ca\textsubscript{v}1.3\textsuperscript{+/+} mice. Consistent with these findings, my simultaneous ECG and AP recordings during AF in isolated atria reveal that when APD alternans stabilize in the LAA, it leads to conduction block. It is also interesting to note that when the atria were paced at rapid frequencies, similar to our stimulation protocols to induce AF, there is a rapid and drastic accommodation of APD in both genotypes. Although completely speculative, the large abbreviation in APD during rapid pacing may act as a trigger to induce AF in both genotypes, but the genesis of APD alternans in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice sustains the AF.

4.5 Electrical restitution in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice

One of the proposed mechanisms to account for increased susceptibility to cardiac alternans is a steep restitution curve. First illustrated using mathematical models to describe the relationship between AP alternation and rate of stimulation\textsuperscript{253}, it was later used to predict the onset and susceptibility to APD alternans leading to fibrillation in cardiac tissue. We speculated that Ca\textsubscript{v}1.3 might be important to the restitution process because APD depends on the recovery from inactivation of LTCCs and cytosolic Ca\textsuperscript{2+} concentrations\textsuperscript{264,445}. Therefore, we generated restitution curves from the LAA and RAA of isolated atria. We failed to observe any difference in the time to rise constant (Tau) between the groups, and more importantly, at the frequencies tested, the slope of the curves never reach >1. These results may be explained by the fact that Ca\textsubscript{v}1.3 may not have as much as a modulatory effect on APD compared to Ca\textsubscript{v}1.2. Ca\textsubscript{v}1.3 channels are activated at more negative potentials compared to Ca\textsubscript{v}1.2\textsuperscript{52,136,160}, suggesting Ca\textsubscript{v}1.2 contributes more preferentially to the plateau phase of the AP\textsuperscript{52,128,129}. Additionally, ionic models\textsuperscript{447,487} and experiments\textsuperscript{259,482} that decrease \(I_{\text{CaL}}\) magnitude suppressed APD alternans.
4.6 Calcium alternans as the cause of APD alternans in Ca\textsubscript{v}1.3 mice

Despite the observation that electrical restitution was not the cause of APD alternans in Ca\textsubscript{v}1.3\textsuperscript{-/-} atria during AF, Ca\textsuperscript{2+} alternans can develop and modulate APD through Ca\textsuperscript{2+} sensitive ion channels and exchangers\textsuperscript{289, 351, 365, 448, 449, 22, 23, 394}. Since loss of Ca\textsubscript{v}1.3 was shown to affect Ca\textsuperscript{2+} homeostasis (reduced intracellular Ca\textsuperscript{2+} transients and delayed times to peak cytosolic Ca\textsuperscript{2+} levels following field stimulation\textsuperscript{167}) we hypothesized loss of Ca\textsubscript{v}1.3 may pose a dynamic instability in Ca\textsuperscript{2+}-cycling. Accordingly, I field paced single LAA myocytes from Ca\textsubscript{v}1.3 mice at supra-physiological frequencies, since it has been show to induce Ca\textsuperscript{2+} alternans in atrial myocytes\textsuperscript{314, 344, 407}. We found that Ca\textsubscript{v}1.3\textsuperscript{-/-} mice had a greater susceptibility to pacing-induced Ca\textsuperscript{2+} alternans, manifested as the development of stable Ca\textsuperscript{2+} alternans at lower frequencies compared to Ca\textsubscript{v}1.3\textsuperscript{+/+} mice. Interestingly, the heterozygous Ca\textsubscript{v}1.3\textsuperscript{+/+} displayed an intermediate phenotype. Reductions in the EC coupling trigger fidelity of \textit{I}\textsubscript{CaL}, via voltage-clamp to reduce LTCC depolarizing currents, has been associated with the development of Ca\textsuperscript{2+} alternans\textsuperscript{327, 376}. In simulation studies of atrial myocytes, reducing the LTCC conductance was associated with Ca\textsuperscript{2+} alternans\textsuperscript{330}. It is likely loss of \textit{I}\textsubscript{CaL}, observed in Ca\textsubscript{v}1.3\textsuperscript{-/-} atrial myocytes\textsuperscript{136, 167}, is producing a similar dynamic instability in Ca\textsuperscript{2+}-cycling and increasing sensitivity to pacing-induced Ca\textsuperscript{2+} alternans.

Not only was there a greater susceptibility to alternans in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice, but the alternans themselves were spatially discordant. When Ca\textsuperscript{2+} alternans develop, they can either be spatially in phase throughout different regions of the myocyte on a given beat, or out of phase, known as spatially discordant Ca\textsuperscript{2+} alternans\textsuperscript{341, 371, 397, 401-403}. Atrial myocytes are inherently more susceptible to discordant alternans\textsuperscript{321, 409} due to their unique EC properties\textsuperscript{313-315, 318}, compared to ventricular myocytes, although disparities in Ca\textsuperscript{2+}-cycling properties (i.e. regional differences in the density of LTCCs, NCX, or SERCA\textsuperscript{341, 401, 440}, reduced RyR open probability\textsuperscript{371}, reduced LTCC open probability\textsuperscript{403, 404}) across the cell will definitely promote heterogeneous Ca\textsuperscript{2+} release and may also promote discordance\textsuperscript{341, 371, 401}. Loss of Ca\textsubscript{v}1.3 across the membrane of atrial myocytes may produce a similar effect due to asynchronous Ca\textsuperscript{2+} release.

When the LTCC trigger was reduced, yielding asynchronous Ca\textsuperscript{2+} release and discordant alternans, a common consequence was observed, Ca\textsuperscript{2+} waves\textsuperscript{327, 376}. We probed for the presence
of Ca$^{2+}$ waves in Ca$_v$1.3$^{-/-}$ in atrial myocytes, and found they were intimately involved in the development and maintenance of the discordant alternans. Ca$^{2+}$ waves activating peripheral CRUs, are facilitated by high recruitment probabilities (high intracellular Ca$^{2+}$ concentration and increased SR sensitivity or load) and can accelerate the development of discordant Ca$^{2+}$ alternans by resetting the phase of SR Ca$^{2+}$ release and refactoriness$^{323,341,371,397,401}$. In Ca$_v$1.3$^{-/-}$ LAA myocytes regions previously activated by a propagated Ca$^{2+}$ wave, were refractory on the next stimulation, promoting enhanced gradients of Ca$^{2+}$ release.

As previously stated, reductions in the EC coupling $I_{\text{CaL}}$ trigger promote Ca$^{2+}$ alternans$^{327,376}$. β-AR stimulation with ISO has been shown to increase $I_{\text{CaL}}$ in atrial myocytes$^{483}$ and ISO was then applied to Ca$_v$1.3$^{-/-}$ atrial myocytes to recover lost $I_{\text{CaL}}$. ISO reduced the sensitivity to pacing-induced Ca$^{2+}$ in both genotypes, but much more significantly in Ca$_v$1.3$^{-/-}$ LAA myocytes. This is not surprisingly since β-AR agonist ISO has been shown to protect against pacing-induced alternans in atrial myocytes$^{360,370,450}$ by increasing Ca$^{2+}$ sequestration via PKA/CaMKII signaling pathways (relief of SERCA inhibition by phosphorylation of phospholamban). Since the effects of ISO are broad, a more discriminatory inotropic agent was used to increase $I_{\text{CaL}}$ conductance. Experimentally, increasing the external Ca$^{2+}$ concentration has been shown to selectively increase $I_{\text{CaL}}$, with negligible effect on SR load, and promote Ca$^{2+}$ release at the peripheral and central regions of field-paced atrial myocytes$^{311}$. We found a protective effect to Ca$^{2+}$ alternans in Ca$_v$1.3$^{-/-}$ LAA myocytes at higher Ca$^{2+}$ concentrations, albeit not as significant as ISO, but this may reflect the additional sequesteration pathways mediated by ISO. This is consistent with observations in atrial myocyte simulations where increased $I_{\text{CaL}}$ conductance reduced Ca$^{2+}$ alternan susceptibility$^{330}$. These findings support the concept of reduced trigger $I_{\text{CaL}}$ promoting Ca$^{2+}$ alternans. The increase in $I_{\text{CaL}}$ in the remaining Ca$_v$1.2 channels (with higher external Ca$^{2+}$) was presumably responsible for the partial reversal of the phenotype in Ca$_v$1.3$^{-/-}$ atrial myocytes.

Experiments using CCBs have demonstrated the asynchronous release of Ca$^{2+}$ throughout the cell$^{399}$, and reductions in LTCC trigger $I_{\text{Ca}}$ influence the development of Ca$^{2+}$ alternans$^{327,376}$. I expected to observe Ca$^{2+}$ alternans in Ca$_v$1.3$^{+/+}$ LAA myocytes treated with the CCB verapamil, although only asynchronous Ca$^{2+}$ release and reduced Ca$^{2+}$ transient magnitudes were observed. These results may reflect a scenario where a large reduction in the primary spark probability is insufficient to trigger recruitment of secondary sparks. Consequently, local heterogeneous Ca$^{2+}$
release failed to synchronize and generate alternans at the whole cell level\textsuperscript{394}. Or this may suggest a novel role of Ca\textsubscript{v}1.3 \( I_{\text{CaL}} \) in the genesis of Ca\textsuperscript{2+} alternans, and is supported by recent evidence where Ca\textsubscript{v}1.3 preferentially localized with RyR2, compared to Ca\textsubscript{v}1.2, in mice SA node myocytes\textsuperscript{170}. If true of all atrial myocytes, then Ca\textsubscript{v}1.3 potentially plays a more significant role in the \( I_{\text{CaL}} \) trigger. Consistent with this idea, Ca\textsubscript{v}1.2 heterozygous mice (Ca\textsubscript{v}1.2\textsuperscript{+/-}), which have been shown in ventricular myocytes to have a 25\% reduction in \( I_{\text{Ca}} \textsuperscript{162} \), displayed an equivalent phenotype to pacing-induced Ca\textsuperscript{2+} alternans as Ca\textsubscript{v}1.2 wild type mice (Ca\textsubscript{v}1.2\textsuperscript{+-}).

### 4.7 Arrhythmogenic consequences of calcium alternans

In order for Ca\textsuperscript{2+} alternans to be a precursor to reentry arrhythmias at this tissue level\textsuperscript{301}, they must first translate to electrical APD alternans. Although there has been no definitive experiment to prove Ca\textsuperscript{2+} alternans are the cause of repolarization alternans, there are is a wide range of evidence to suggest it is highly probable\textsuperscript{258, 289, 337, 339, 342, 340, 341}. In order to determine whether Ca\textsuperscript{2+} alternans were translating to APD and the nature of this interaction (positive vs. negative), we carried out simultaneous current-clamp patch recordings to measure APs, and fluorescence laser scanning confocal microscopy to acquire Ca\textsuperscript{2+} transients. Simultaneous AP and Ca\textsuperscript{2+} transient recordings from both Ca\textsubscript{v}1.3\textsuperscript{+/-} and Ca\textsubscript{v}1.3\textsuperscript{-/-} atrial myocytes exposed that when Ca\textsuperscript{2+} alternans stabilize, APD alternans followed suite. This observation is consistent with the fact that APD alternans can be induced in Ca\textsubscript{v}1.3\textsuperscript{-/-} isolated atria, and are present during AF. Intracellular Ca\textsuperscript{2+} transients can couple to voltage through the Ca\textsuperscript{2+} sensitive ionic currents.

Positive coupling can occur when a large Ca\textsuperscript{2+} transients increases gain through NCX leading to a depolarizing current (\( I_{\text{NCX}} \)) and APD prolongation\textsuperscript{22}. We found that when APD\textsubscript{90} was prolonged it was associated with a large Ca\textsuperscript{2+} transient during alternans in both Ca\textsubscript{v}1.3 genotypes suggesting a normal physiological mechanism for positive coupling. Positive coupling in atrial myocytes can be explained by experimental\textsuperscript{408, 409} and computational\textsuperscript{410} studies in atrial myocytes that illustrate the late phase of the atrial AP (early repolarization) was modulated by a net extrusion of Ca\textsuperscript{2+} through NCX. Since the density of \( I_{\text{K1}} \) is 5- to 10-fold smaller in atrial myocytes compared to ventricular myocytes\textsuperscript{411}, the membrane resistant of atrial myocytes is much greater\textsuperscript{412} and a small depolarizing current by \( I_{\text{NCX}} \) can yield large changes in \( E_{m} \) to prolong APD.
Whole heart mapping studies have demonstrated cardiac alternans to be a precursor to reentry arrhythmias\(^4^{13, 416, 417}\). This is especially true in the case of SDA, since they enhance the gradients and dispersion of repolarization, leading to conduction block. Furthermore, rises in diastolic Ca\(^{2+}\), due to Ca\(^{2+}\) waves, can become arrhythmogenic if they lead to EADs/DADs\(^3^{97, 403, 420}\) or begin to impinge on conduction. Frequency maps from Ca\(_v\)1.3\(^{-/-}\) mice were compared to a completely carbachol electrically remodeled atria. Frequency maps generated from isolated atria optical images of Ca\(_v\)1.3\(^{-/-}\) AF illustrate spatial heterogeneity of activation frequencies. In addition, the OI obtained from frequency maps was significantly more disorganized in Ca\(_v\)1.3\(^{-/-}\) atria compared to Ca\(_v\)1.3\(^{+/+}\) with carbachol. These results support the conclusion that a general reduction in APD is not the substrate in these mice. The spatial heterogeneity of frequencies (represented on the frequency maps) either suggests multiple wavelets oscillating at varying frequencies, or heterogeneity of repolarization/conduction resulting in block. I observed 2:1 and 3:1 coupling between bordering regions of activation frequencies in the maps of Ca\(_v\)1.3\(^{-/-}\) AF. Further characterization of these regions, via the raw optical mapping images of voltage, confirm block, which result in these patterns of coupling between regions in the atria with different activation frequencies. This pattern would be expected to form in AF with SDA as the substrate. Out-of-phase regions are highly susceptible to localized conduction block because the spatial gradient in the APD are the greatest at the nodal line\(^2^{57, 413, 415}\). Further supporting this concept of a meandering wavefront, propagating around regions of block, is the calculated wavelength for the Ca\(_v\)1.3\(^{-/-}\) mice. The product of the APD and CV calculated at 25Hz (data not shown) was approximately 7mm, and the whole length of the atria is around 12mm. Therefore, for stable rotors to exist, or the presence of multiple wavelets, the rotor have to be at least half the size of the atria. However, the frequency maps in the Ca\(_v\)1.3\(^{-/-}\) display df’s encapsulating areas much smaller than the wavelength, which makes the possibly of a rotor and multiple wavelets unlikely. It is also probable that diastolic Ca\(^{2+}\) waves promote raises in \(E_m\), reducing available \(I_{Na}\), and either delaying conduction or completely exhausting it to the point of block.

### 4.8 Structural remodeling In Ca\(_v\)1.3\(^{-/-}\) mice

Atrial fibrosis has been identified as one of the most important risk factors for AF\(^2^{33, 484-487}\). Given the increased vulnerability to AF observed in Ca\(_v\)1.3\(^{-/-}\) mice, and the observed hypertrophic remodeling in the ventricles of these mice, a logical step was to quantify and determine distribution of collagen in atria of Ca\(_v\)1.3\(^{-/-}\). Collagen organization in Ca\(_v\)1.3\(^{-/-}\) mice
was characterized by thicker and more abundant perimysial fibers, which were dispersed randomly and abundantly in the atrium. The organization of these collagen bundles appeared to be lost and instead closely resembled ‘replacement fibrosis’ which is typically seen in response to hemodynamic overload. This replacement of conductive tissue with high resistance collagen bundles invariably leads to conduction slowing and non-uniform anisotropic conduction, which predisposes to AF. Consistent with my histological findings, P-wave durations were significantly prolonged in Ca$_v$1.3$^{-/-}$ mice. In addition, direct measurement of CV via optical mapping studies revealed significant reductions in the CV of the left atria in Ca$_v$1.3$^{-/-}$ mice.

Since the pattern of fibrosis observed in Ca$_v$1.3$^{-/-}$ mice was consistent with replacement fibrosis, the hemodynamic properties of these mice were characterized. To indirectly acquire left atrial pressure, I performed invasive hemodynamics on the left ventricle to obtain end-diastolic pressures. I failed to identify any hemodynamic differences in Ca$_v$1.3 mice. However, these experiments were carried out in anesthetized mice and under these conditions, the typically observed abnormalities (in ambulatory recordings) in conduction (e.g. AV block, sinus arrest, ventricular extrasystoles) were not present. To evaluate the hemodynamic consequences of these abnormal events, which only occurred in ambulatory ECG recordings, careful monitoring of Ca$_v$1.3$^{-/-}$ mice implanted with ECG and pressure radio frequency telemetry units were conducted. These studies in the unanesthetized mouse revealed the presence of ventricle ectopic activity leading to extrasystoles, which lead to sustained pressure elevations of the LV. These findings could explain the development of ventricular hypertrophy, in line with the observations of Huizar et al and their ventricular ectopy model, and can, at least in part, explain the structural remodeling observed in the atria since pressure elevations were maintained above ~40mmHg even in end-diastole, where the atrial and ventricular pressures are equal.

Another significant risk factor associated with AF is hypertension, and it is currently believed that underlying hypertension can result in hemodynamic changes in the left atrium or activation of the renin-angiotensin-aldosterone system, resulting in elevated left atrial pressures and atrial fibrosis, respectively. Ca$_v$1.3 is important to pancreatic beta cell function, and Ca$_v$1.3$^{-/-}$ mice display diabetic traits (although not overt diabetes), and diabetes is associated with both hypertension and AF. However, invasive hemodynamic recordings revealed that Ca$_v$1.3$^{-/-}$ mice were hypotensive. This could be explained by the high expression profile of Ca$_v$1.3 in aortic smooth muscle, which suggests that loss of Ca$_v$1.3 promotes vasodilation.
Inflammation of atrial tissue invariably precedes fibrotic remodeling, especially in models where significant hemodynamic stress is present\textsuperscript{461}. Macrophages and neutrophils are critical to tissue repair in the heart by releasing growth factors such as fibroblast growth factor and TGFβ, implicating them as initiators and perpetuators of fibrotic remodeling in the heart\textsuperscript{468, 469}. In addition, clinical studies on human atrial biopsies have shown elevation in these inflammatory cells in the atria of AF patients\textsuperscript{470}. The pivotal role of inflammatory cells in cardiac fibrosis\textsuperscript{490-492} and AF\textsuperscript{493-495} has led us to characterize inflammation in the atria of Ca\textsubscript{v}1.3\textsuperscript{-/-} mice. Interestingly, I failed to observe any elevations in neutrophils or macrophage in Ca\textsubscript{v}1.3\textsuperscript{-/-} atria using our antibodies (mac-3 and Ly6b.2). It is unlikely that these antibodies have failed to identify a sub-class of leukocyte (as there is considerable heterogeneity in the distribution of these cell markers within leukocyte subtypes) since they stain activated and non-activated monocytes alike (mac-3) or neutrophils, infiltrating monocytes, and activated monocytes (Ly6b.2). It is far more likely that the inflammatory process occurs earlier in development, or that the fibrotic process overrides the traditional inflammatory process, possibly through stretch-induced activation of p38 MAPK in fibroblasts\textsuperscript{471, 472}. Nevertheless, these results suggest that there is structural remodeling consisting with atrial fibrosis in the atria of Ca\textsubscript{v}1.3\textsuperscript{-/-} mice and that this structural remodeling may at the very least contribute to the AF substrate.

4.9 Electrical versus structural remodeling in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice

To further characterize the AF observed in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice, spectral analysis techniques were used in comparison to a known model of structural remodeling. Previous studies have shown that the pattern of atrial activation during AF varies considerably depending on the substrate (electrical, or structural). If structural remodeling were the sole mechanism accounting for the increased vulnerability to AF in Ca\textsubscript{v}1.3\textsuperscript{-/-}, it would be expected that the organization of AF would more closely resemble the structurally remodeled atria (exercised) rather than the electrically remodeled. However, the isolated atria ECGs obtained from Ca\textsubscript{v}1.3\textsuperscript{-/-} AF showed a more complex substrate, with even greater complexity than structurally remodeled atria. In a study assessing AF organization in electrical vs. structural atrial remodeling, significant reductions in the OIs of electrically remodeled atria (by rapid atrial pacing) compared to both control and structurally remodeled atria (pressure overload) were reported\textsuperscript{434}. In atria with fibrosis, AF is driven by what appears to be a stable rapidly activating focus due to microreentry\textsuperscript{233, 496} rather than complex multiple wavelet/reentrant activity. Taken together, these
studies show that the AF of Ca\textsubscript{v}1.3\textsuperscript{+} is not characteristic of primarily structurally remodeled atria, but that other electrical perturbations are present as well.

4.10 Proposed mechanism of atrial fibrillation

The association between arrhythmia risk and cardiac alternans (in the form of T-wave alternans) has been confirmed in human clinical trials\textsuperscript{239, 240}. In fact, instances where predisposing factors to arrhythmias are common (acute myocardial ischemia, genetic channelopathies, and drug and electrolyte disturbances), experimental findings demonstrated the presence of electrical and mechanical alternans\textsuperscript{238}. However, consensus in the field has hypothesized that repolarization alternans are initiated by the beat-to-beat dynamics in SR Ca\textsuperscript{2+} release or Ca\textsuperscript{2+} alternans\textsuperscript{238, 257, 262, 323, 334, 335}. Moreover, convincing evidence in animal models\textsuperscript{241, 242}, numerical simulations\textsuperscript{243}, and human studies\textsuperscript{244-247}, have shown that cardiac alternans may induce AF or facilitate the transition from flutter to fibrillation.

Asynchronous release of Ca\textsuperscript{2+} throughout the cell has been shown to induce alternans in a number of experimental settings\textsuperscript{341, 401}. Restitution of SR Ca\textsuperscript{2+} release or the time-dependence of refractoriness of SR Ca\textsuperscript{2+} release has been suggested as a primary mechanism underlying Ca\textsuperscript{2+} alternans\textsuperscript{335, 370} since it promotes heterogeneous CICR. Experiments impinging on the refractoriness of RyR Ca\textsuperscript{2+} release, by increasing pacing frequency\textsuperscript{386-388}, or decreasing open probability\textsuperscript{371} induced Ca\textsuperscript{2+} alternans. In fact, instabilities in Ca\textsuperscript{2+} release may be due to delayed recovery of CICR or refractoriness of RyR-mediated Ca\textsuperscript{2+} release\textsuperscript{389} and calsequestrin knockout\textsuperscript{375} or overexpression\textsuperscript{391} mice suppress or promote alternans, respectively. Similarly, inhibition of glycolysis in cat atrial and ventricular myocytes increased susceptibility to alternans attributed to the time-dependent recovery of SR Ca\textsuperscript{2+}-release mechanism\textsuperscript{340}. Another mechanism to promote asynchronous Ca\textsuperscript{2+} release, similar to refractoriness of the SR, but further up the CICR hierarchal ladder, is reducing the I\textsubscript{Ca} trigger. Experiments reducing the open probability of LTCCs have been shown to induce asynchronous release resulting in Ca\textsuperscript{2+} alternans\textsuperscript{330, 398, 403, 404}.

I propose that genetic ablation of Ca\textsubscript{v}1.3 produces a similar phenomenon because Ca\textsubscript{v}1.3 has been suggested to be primarily involved in the I\textsubscript{Ca} trigger\textsuperscript{170}. This is supported by the fact that inotropic agents increasing EC coupling fidelity (\(\beta\)-AR stimulation with ISO, or high extracellular Ca\textsuperscript{2+}) reduce susceptibility to pacing-induced Ca\textsuperscript{2+} alternans in Ca\textsubscript{v}1.3\textsuperscript{+} mice.

In order for non-uniform Ca\textsuperscript{2+} sparks, due to reduced primary spark probability, to
generate stable $Ca^{2+}$ alternans, a large enough recruitment probability of couplons must be maintained to facilitate secondary sparks via $Ca^{2+}$ waves. $Ca^{2+}$ waves activating peripheral CRUs can accelerate the development of discordant $Ca^{2+}$ alternans by resetting the phase of SR $Ca^{2+}$ release and refractoriness, which we observed in Ca,1.3<sup>−/−</sup> mice.

Once stable $Ca^{2+}$ alternans has formed, they must first translate to electrical APD alternans before they can lead to reentry arrhythmias at the tissue level. Positive coupling was observed in both Ca,1.3 genotypes in atrial myocytes, which we speculate was due to $I_{NCX}$, and APD alternans were induced experimentally in Ca,1.3<sup>−/−</sup> isolated atria. SDA has essentially provided a mechanistic link of how cardiac alternans develop into VF and AF, since they enhance the gradients and dispersion of repolarization. Optical mapping studies of Ca,1.3<sup>−/−</sup> isolated atria provide indirect evidence that SDA are likely the substrate for AF due to the high degree of regional conduction block. Despite evidence for structural remodeling (atrial fibrosis with delays in conduction), frequency domain analysis suggested a more complex electrically remodeled substrate in Ca,1.3<sup>−/−</sup> atria. Therefore, Ca,1.3<sup>−/−</sup> mice are more susceptibility to AF, primarily due to $Ca^{2+}$ alternans, which translate to electrical alternans and SDA, promoting regional conduction block, a prerequisite for reentry.
Chapter 5

Future Directions
5 Future Directions

5.1 A model of acquired Ca\textsubscript{v}1.3 deficiency

Since ablation of Ca\textsubscript{v}1.3 is a full body knockout of the gene, there is the high probability of indirect effects on the heart via the CNS, pancreas, and vasculature. Classically, Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 could not be distinguished with CCBs. However, a mouse model was created where the high DHP sensitivity of Ca\textsubscript{v}1.2 α1C subunit was eliminated (Ca\textsubscript{v}1.2\textsuperscript{DHP/-}), by replacement of Thr1066 in the helix IIIS5 with a tyrosine residue. This essentially eliminated the contribution of this channel type to DHP effects, and allowed for the specific inhibition of Ca\textsubscript{v}1.3 with DHPs. We are currently in possession of these mice, and are in the process of preforming all of the same pacing-induced Ca\textsuperscript{2+} alternan protocols. Directly blocking Ca\textsubscript{v}1.3 \( I_{\text{Ca}} \) at the time of the experiment will confirm the direct link of Ca\textsubscript{v}1.3 loss in the development of pacing-induced Ca\textsuperscript{2+} alternans. Also, isolated atria AF induction will be preformed in these mice in the presence and absence of DHPs. These experiments will confirm the direct role of Ca\textsubscript{v}1.3 \( I_{\text{Ca}} \) in the development of an AF substrate. Additionally, if Ca\textsubscript{v}1.2\textsuperscript{DHP/-} mice are vulnerable to inducible AF in the presence of DHPs, then fibrosis observed in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice is not the primary substrate for increased arrhythmogenesis in the Ca\textsubscript{v}1.3\textsuperscript{-/-} mice.

5.2 Role of Ca\textsubscript{v}1.3 in excitation-contraction coupling and the \( I_{\text{Ca}} \) trigger

We hypothesized that loss Ca\textsubscript{v}1.3 \( I_{\text{Ca}} \) would reduce the coupling fidelity with the SR release mechanism, and as a result global CICR would become dampened. To directly address the role of Ca\textsubscript{v}1.3 in CICR, experiments measuring the primary spark frequency in response to a stimulus are required. Adequately controlling SR Ca\textsuperscript{2+} loads in these experiments will be critical because SR Ca\textsuperscript{2+} content is a major determinant of EC coupling gain. Enhancement of RyR activity is modulated by luminal sensing sites for Ca\textsuperscript{2+}. To assess EC coupling in Ca\textsubscript{v}1.3 mice, myocytes will be dialyzed using the dual Ca\textsuperscript{2+} buffer dialysis method. The method involves dialyzing myocytes with a low concentration (75µM) of a fluorescent dye (Fluo-3) with EGTA (5mM) and enough Ca\textsuperscript{2+} to clamp the intracellular Ca\textsuperscript{2+} levels at a fixed level (~100nM consistent with other studies). The combination of fluorescent dye, EGTA and Ca\textsuperscript{2+} allows SR Ca\textsuperscript{2+} flux to be determined while clamping SR Ca\textsuperscript{2+} loads to pre-selected levels by fixing the free [Ca\textsuperscript{2+}] in the pipette. With SR Ca\textsuperscript{2+} fixed, myocytes will be stimulated with APs in voltage-clamp...
mode (AP-clamps), and \( \text{Ca}^{2+} \) sparks will be captured with confocal microscopy and a high-speed camera.

Previous experiments in mice SA node myocytes have illustrated the preferential co-localization of \( \text{Ca}_v1.3 \) with RyR2, compared to \( \text{Ca}_v1.2 \). We are currently in contact with Amy Lee at the Department of Molecular Physiology and Biophysics, University of Iowa, USA, who originally produced these results, to perform similar co-localization studies in LAA myocytes. These results would provide an explanation for the potential reduction of EC coupling gain in \( \text{Ca}_v1.3^{-/-} \) atrial myocytes.

### 5.3 Mechanism of spatially discordant electrical alternans and conduction block

Although we suspect \( \text{Ca}_v1.3^{-/-} \) mice are developing SDA at the tissue level due to indirect evidence, we never fully characterized the transition to SDA. We also never ruled out the possibility that diastolic rises in voltage, due to \( \text{Ca}^{2+} \) waves, was responsible for impeding conduction. Similar to APD restitution, CV is also sensitive to the previous DI. Steep CV restitution will predispose to SDA since small changes in DI produce large delays in conduction. The slow propagation of conduction allows the DI at distal sites to become slightly lengthened and as a consequence the APD becomes prolonged. If this process continues, it will self amplify over subsequent beats and will evolve into stable SDA. To determine the role of CV restitution in \( \text{Ca}_v1.3^{-/-} \) mice, similar optical mapping studies to determine CV in the LAA of isolated atria will be performed. Using similar stimulation protocols as the electrical restitution experiments, the LAA will be retrograde paced, and the CV after the DI will be determined from optical images. Simultaneous microelectrode recordings and optical mapping during AF are currently underway. These experiments will conclude whether conduction block on the optical maps is correlated to rise in diastolic voltage in the electrophysiological recordings.
Chapter 6

Appendix
Appendix

Figure 6.1 Significantly prolonged atrial and atrioventricular refractory periods in Ca\(_{\text{v}}\)1.3\(^{-/-}\) mice.

To accommodate the AF induction studies performed in isolated atria, in vivo AF was induced with an octapolar EP recording/stimulation catheter guided through the right jugular vein, and into the right ventricle of anesthetized Ca\(_{\text{v}}\)1.3 mice. A, A representative ECG of a short AF episode in a Ca\(_{\text{v}}\)1.3\(^{+/+}\) mouse, wherein rapid stimulations (20 pulses at 20ms interval) delivered through mid atrial leads (56) induced rapid, chaotic atrial activity (as evident in leads 78). Surface ECG (sECG) shows replacement of distinct p-waves at sinus rhythm with random chaotic atrial activations and AV block following the brief episode of AF. B, To determine the AERP and AVERP, a programmed stimulation was delivered to the mid atria (leads 56) of anesthetized animals using the proximal leads of the EP catheter. Prolongation of both the AERP and AVERP was observed in Ca\(_{\text{v}}\)1.3\(^{-/-}\) mice. C, Unexpectedly, Ca\(_{\text{v}}\)1.3\(^{-/-}\) mice had comparable AF durations compared to the Ca\(_{\text{v}}\)1.3\(^{+/+}\) controls under baseline conditions. In order to increase the likelihood of reentry in Ca\(_{\text{v}}\)1.3\(^{-/-}\), the parasympathetic agonist, carbachol (CCH), was injected into the intraperitoneal space at 50ng/g. Even with a reduction in AERP, Ca\(_{\text{v}}\)1.3\(^{-/-}\) mice failed to present any evidence of sustained AF. Finally, to simulate the conditions of the denervated isolated atrial preparations, the parasympathetic antagonist, atropine, and the sympathetic antagonist, propranolol, were simultaneously injected into the intraperitoneal space at 10mg/kg and 10\(\mu\)g/g, respectively. Similar to carbachol and baseline conditions, Ca\(_{\text{v}}\)1.3\(^{-/-}\) did not express any episodes of sustained AF.
Figure 6.2 Sinus Bradycardia and erratic pacemaker activity in Ca\textsubscript{v}1.3\textsuperscript{-/-} isolated atria.

Spontaneous APs recorded from SA node cells from Ca\textsubscript{v}1.3\textsuperscript{-/-} mice exhibited a decrease in beating frequency, prolonged cycle lengths and reductions in the rate of diastolic depolarization. Therefore, we investigated the nature of SA node firing rate of isolated atria, using ECG field recordings, to determine if pacing would be required to control for beating rates between the two groups. A, Representative ECG recordings from a Ca\textsubscript{v}1.3\textsuperscript{+/+} (left) and a Ca\textsubscript{v}1.3\textsuperscript{-/-} (right) isolated atria. Note the high degree of variance between the peaks of the atrial activation ECG waveforms, which would suggest irregular pacemaker activity. B, The RR interval, or the duration between two consecutive atrial activations (in the case of an isolated atrial preparation) trended to a prolongation in Ca\textsubscript{v}1.3\textsuperscript{-/-} isolated atria. C, An estimation of pacemaker consistency was determined using the variance function of RR intervals. It was found that Ca\textsubscript{v}1.3\textsuperscript{-/-} have a significantly elevated RR variance suggesting erratic pacing of the SA node.
Figure 6.3 Loss of Ca$_{1.3}$ $I_{CaL}$ does not result in a significant change in action potential duration.

Reductions in the APD predispose to AF. To investigate whether there was arrhythmogenic shortening of the APD, APs were recorded using high resistance 3M KCl filled microelectrodes. APDs were acquired from the RAA and LAA of Ca$_{1.3}$ isolated atrial preparations and an epicardial electrode was used to pace atria at 10Hz (100ms) or 25Hz (50ms) from a proximal location to the microelectrode recording. A, Single representative microelectrode APs paced at 10Hz from the RAA of Ca$_{1.3}$ mice. Note the trend towards prolongation in the Ca$_{1.3}^{-/-}$ (dotted line) AP tracing compared to Ca$_{1.3}^{+/+}$ (solid line). B, Membrane potential ($E_{m}$); resting $E_{m}$ (Rest); AP amplitude from resting to peak (Amp.). Microelectrode recordings from Ca$_{1.3}$ atria exposed no significant differences in the resting $E_{m}$ or AP amplitude between the groups. C, Left atrial appendage (LAA); right atrial appendage (RAA); APD repolarization at 30% (APD$_{30}$), 50% (APD$_{50}$), and 90% (APD$_{90}$). Contrary to what was expected, a trend towards a prolongation in the APD of Ca$_{1.3}^{-/-}$ atria was observed was at 10Hz. At pacing frequencies similar to those experienced during AF (25Hz), a general reduction in APD was observed in both groups. However, no significant differences were observed in APD in Ca$_{1.3}$ atria at 25Hz.
Fig 6.4 Similar electrical restitution kinetics between isolated atria from Ca_v 1.3 mice.

Electrical restitution curves are an important determinant in the stability of membrane voltage dynamics, and LTCC I_{Ca} inactivation kinetics plays an important role in the process. Therefore, loss of Ca_v 1.3 could affect activation and recovery of the other ion channels in the restitution process. Restitution values were obtained from a train of 30 pulses at either 10Hz or 25Hz (S_1) followed by a varied delayed after pulse (S_2). APs were acquired with a microelectrode from the LAA and RAA from isolated Ca_v 1.3 atria. Restitution curves were generated from APD_{90} immediately following the S_2 pulse, and plotted against the corresponding S_1-S_2 (DI). A. Representative restitution curves at a CL of 10Hz (left) and 25Hz (right) of Ca_v 1.3+/+ (black) and Ca_v 1.3-/- atria (red). B, the corresponding derivative of the restitution curves from A was plotted. Note how the slope never reached a point >1 in each of the groups. C, the time constant (Tau), obtained using the exponential power equation "y=A1*(1-exp(-x/t1))^n+y0", was averaged from 3 cells from each appendage, and then averaged again between each group. Tau was then used to compare the rise time between each group with respect to the S_1 frequency, and location of recording (appendages). No difference was observed between Ca_v 1.3+/+ and Ca_v 1.3-/- atria at either CL or appendage.
Figure 6.5 A general reduction in calcium transient magnitude, but no increase in susceptibility to pacing-induced calcium alternans in Ca\textsubscript{v}1.3\textsuperscript{+/+} verapamil treated cells.

Experiments using CCBs have demonstrated the asynchronous release of Ca\textsuperscript{2+} during myocyte transients, and have demonstrated their influence in the development of Ca\textsuperscript{2+} alternans. If loss of Ca\textsubscript{v}1.3 \(I_{Ca}\) is responsible for development of Ca\textsuperscript{2+} alternans, then treatment of Ca\textsubscript{v}1.3\textsuperscript{+/+} atrial myocytes with a CCB should increase their vulnerability to pacing-induced Ca\textsuperscript{2+} alternans. We applied the CCB verapamil to field-pacing Ca\textsubscript{v}1.3\textsuperscript{+/+} LAA myocytes paced at only 6Hz and 7Hz (dropping to a baseline of 3Hz between each acquisition) to induce Ca\textsuperscript{2+} alternans. A, Representative raw Ca\textsuperscript{2+} signals from a Ca\textsubscript{v}1.3\textsuperscript{+/+} LAA myocyte paced at 7Hz and in the absence (left) and presence of 50nM verapamil (right). Note the expected reduction in Ca\textsuperscript{2+} transient magnitude upon addition of verapamil (right). B, Application of 50, 100, or 200nM verapamil to Ca\textsubscript{v}1.3\textsuperscript{+/+} LAA myocytes had no effect on pacing-induced Ca\textsuperscript{2+} alternan susceptibility. At least 10 cells were recorded from each condition. Cells were obtained from an n=2 for Ca\textsubscript{v}1.3\textsuperscript{+/+} +50nM verapamil, n=1 for Ca\textsubscript{v}1.3\textsuperscript{+/+} +100nM verapamil, n=1 for Ca\textsubscript{v}1.3\textsuperscript{+/+} +200nM verapamil mice.
Figure 6.6 No presence of acute neutrophil infiltration, or elevated mononuclear phagocyte infiltration in atrial appendages of Ca$_{v}$.1.3$^{-/-}$ mice.

Increased AF vulnerability has been attributed to structural remodeling of the atria, which was associated with leukocyte infiltration and interstitial fibrosis. Macrophages and neutrophils are critical to tissue repair by releasing growth factors such as fibroblast growth factor and TGFβ, implicating them in fibrogenic remodeling of atria. In addition, they are found in the atrium of AF patients. Given the increase in atrial collagen and increased susceptibility to AF induced by ablation of Ca$_{v}$.1.3$, we investigated for the presence of macrophages and neutrophils using immunohistology with CD107b (mac-3) and Ly6b.2 antibodies, respectively. Haematoxylin staining was used to reveal nuclear and cytoplasmic structures. A, Micrographs illustrating representative images of macrophage staining (red arrows showing Mac-3 staining) from the atrial appendages of Ca$_{v}$.1.3$ mice. B, Total macrophage count revealed no difference between groups C, representative images of the neutrophil staining from the atrial appendages of Ca$_{v}$.1.3$ mice (red arrows showing Ly6b.2 staining). Total neutrophil count revealed no difference between groups.
Figure 6.7 Invasive hemodynamics revealed no difference in developed pressure, end diastolic pressure or contractility in the left or right ventricle, but hypotension in vasculature of Ca\textsubscript{v}1.3\textsuperscript{-/-} mice.

Chronic pressure or volume overload of the heart results in myocardial hypertrophy. Underlying hypertension can result in elevated left atrial pressures or elevated atrial fibrosis (via renin-angiotensin-aldosterone system activation). Both characteristics of ventricular hypertrophy and elevated atrial fibrosis were observed in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice. Therefore, to determine whether hemodynamic abnormalities were present in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice, invasive hemodynamics were performed on the right and left ventricle. A 1F pressure-transducing catheter was guided into the left and right ventricle to obtain developed pressure (LVDP and RVDP respectively). Left ventricular end-diastolic pressure (LVEDP) was used as an indirect measure of left atrial pressure. Mean arterial pressure (MAP) was calculated from aortic pressures. A, B, No difference in LVDP or RVDP was observed in of Ca\textsubscript{v}1.3 mice. C, Ca\textsubscript{v}1.3 mice displayed no difference in LVEDP. D, significant elevation of MAP in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice suggests hypotension. E, The maximum contractility (measured as dPdT) was significantly reduced in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice, but when afterload differences were accounted for (by measuring dPdT at 30mmHg), no difference in contractility was present.
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