

The role of the perinexus in Long QT Syndrome Type 3

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ABSTRACT

Gain of function of cardiac voltage-gated sodium channel (Nav1.5) leads to Long QT Syndrome Type 3 (LQT3). LQT3 phenotype can be exacerbated by expanding the perinexus, which is an intercellular nanodomain with high density of Nav1.5 in the intercalated disc. Following this finding, we found that elevating extracellular sodium and widening the perinexus synergistically exacerbated LQT3 phenotype. Importantly, we also found that perinexal expansion increases the susceptibility to cardiac arrest in *aged* LQT3, which demonstrated that perinexal expansion is an arrhythmogenic risk especially in *aged* LQT3 patients. Furthermore, we observed that the perinexus narrows with aging and conceals LQT3 phenotype, which suggests that perinexal narrowing may have a cardio-protective role during aging in LQT3. Surprisingly, following the finding of the synergistic effect of extracellular sodium elevation and perinexal widening on LQT3 phenotype in drug-induced LQT3 guinea pig hearts, we found that this synergistic effect was not observed in genetically-modified LQT3 mouse hearts, which is due to high sodium also increasing transient outward potassium current (I_{to}). In summary, the whole project investigated the role of the perinexus in LQT3 from different conditions including sodium, aging and species. The findings in this project discovered the importance of perinexal expansion in LQT3 and also the involvement of I_{to} in sodium regulating LQT3 phenotype in hearts which functionally express I_{to} channels. Therefore, a LQT3 animal model which has similar electrophysiology close to human may be a great option for translational purpose.

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GENERAL AUDIENCE ABSTRACT

Long QT Syndrome Type 3 (LQT3) is an inherited heart disease with the phenotype of long QT interval in ECG. It has been found that LQT3 phenotype gets worse when a very tiny space in the heart, termed as the perinexus, is wide due to cardiac edema. Following this finding, we also found that increasing sodium concentration together with wide perinexus can further exacerbate LQT3 phenotype in guinea pig hearts. Furthermore, we found that widening the perinexus in *aged* LQT3 hearts causes cardiac death but not in *adult*, which suggests that perinexal widening worsens LQT3 phenotype and even leads to cardiac death in aged hearts. Besides, we found that the perinexus narrows with aging and there is no difference in LQT3 phenotype between adult and aged hearts, which suggests that the narrow perinexus during aging may protect the hearts from cardiac death in LQT3. Surprisingly, we discovered that increasing sodium and widening the perinexus together fails to exacerbate LQT3 phenotype when compared with widening the perinexus alone in LQT3 mouse hearts, which is due to high sodium increasing transient outward potassium current (I_{to}). Notably, I_{to} channels are not functionally expressed in guinea pig hearts. In summary, the whole project investigated the role of the perinexus in LQT3 from different conditions including sodium, aging and species. The findings in this project discovered the importance of perinexal expansion in LQT3 and also the involvement of I_{to} in sodium regulating LQT3 phenotype in hearts. Therefore, a LQT3 animal model which has similar electrophysiology close to human may be a great option for translational purpose.

“A butterfly flapping its wings in Brazil could set off a tornado in Texas.”

--Edward Norton Lorenz

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GLOSSARY OF ABBREVIATIONS

LQT3	Long QT Syndrome Type 3
APD	Action Potential Duration
ATXII	Anemone Toxin Type II
βadp1	Sodium Channel β 1 -Subunit Adhesion Domain Antagonist
GOF	Gain-of-function
Nav1.5	Cardiac Voltage-Gated Sodium Channel A Subunit
I_{NaL}	Late Sodium Current
ECG	Electrocardiogram
ID	Intercalated Disc
W_p	Perinexal Width
E_{Na}	Sodium Reversal Potential
CL	Cycle Length
TEM	Transmission Electron Microscopy
EAD	Early Afterdepolarization
BrS	Brugada Syndrome
NCX	Sodium-Calcium Exchanger
w	Cleft Width
I_{to}	Transient Outward Potassium Current
WT	Wild-Type
4-AP	4-Aminopyridine
CaMKII	Calcium/Calmodulin-Dependent Protein Kinase
QTc	Corrected QT Interval
CV	Conduction Velocity
CVL	Longitudinal Conduction Velocity
CVT	Transverse Conduction Velocity
TDR	Transmural Dispersion of Repolarization
TpTe	$T_{peak} - T_{end}$ Interval

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Chapter 1: Introduction

LQT3 overview

Cardiovascular disease remains the leading cause of death in the United States.¹ Sudden cardiac death accounts for over 40% in total death associated with cardiovascular disease.² Numerous studies have demonstrated that sudden cardiac death is associated with prolonged QT interval measured in electrocardiogram (ECG).³⁻⁷ The etiology of prolonged QT interval has been extensively described in a group of cardiac diseases named as Long QT Syndromes (LQTS).⁸

As an exemplar of LQTS, Long QT Syndrome Type 3 (LQT3) is caused by gain of function (GOF) of cardiac voltage-gated sodium channels ($\text{Na}_v1.5$),⁹ which are predominant in ventricles and crucial for electrical initiation and propagation in the heart.¹⁰ $\text{Na}_v1.5$ GOF due to disruption of $\text{Na}_v1.5$ inactivation leads to late sodium current (I_{NaL}), which is associated with prolonged action potential duration (APD) and increased risk of arrhythmias.¹¹ This GOF could occur in acquired disorders such as heart failure, coronary artery disease, diabetes mellitus and atrial fibrillation,¹²⁻¹⁵ and congenital condition⁹ basically due to the mutation in the gene *SCN5A* encoding $\text{Na}_v1.5$. So far, more than 150 mutational sites have been identified in LQT3 patients.^{9, 16} Although the incidence of cardiac arrhythmias in inherited LQT3 (7,000 to 10,000 patients in the US) is less frequent than other types of LQTS,^{17, 18} LQT3-associated life-threatening arrhythmias (35%) are very high when compared with LQT1 (14%) and LQT2 (24%) especially in patients over age 40,^{19, 20} which suggests that the risk of sudden cardiac death increases with age in LQT3. These lethal arrhythmias commonly occur during sleep or rest (65%) than other

conditions.⁷ Importantly, the first ever cardiac arrhythmia occurred in LQT3 patients is very fatal,^{19, 21} which highlights the importance of genetic test for LQT3 families before the symptom is present. The current therapeutic strategies for LQT3 include drugs such as mexiletine,²² lidocaine,²³ flecainide,²⁴ ranolazine²⁵ and β -blocker,²⁶ and placement of implanted cardioverter defibrillator (ICD),²⁷ and left cardiac sympathetic denervation.²⁸ Interestingly, LQT3 patients are not always symptomatic. Multiple clinical studies have found that LQT3 patients could have normal QT interval.^{21, 29} In a clinical study including three main LQTS population of LQT1, LQT2 and LQT3,²¹ 16% of LQTS patients with normal QT interval is LQT3. Importantly, in only LQT3 cases, over 60% LQT3-associated missense mutations don't produce prolonged QT interval, which is associated with low risk of life-threatening arrhythmias compared with symptomatic patients. However, the mechanism of this concealed LQT3 phenotype is clinically unclear.

Ventricular arrhythmias

The mechanisms responsible for ventricular arrhythmias can be generally divided into 2 major categories: 1) abnormal impulse formation and 2) reentry.³⁰ Each category includes several different types of abnormal activities leading to arrhythmias. Current LQT3 studies³¹ have suggested that two main mechanisms may be responsible for LQT3-related ventricular arrhythmias: early afterdepolarization and phase 2 reentry.

Early afterdepolarization

Early afterdepolarization (EAD) is a secondary depolarization during action potential repolarization phase 2 and/or 3, which retards the repolarization to cause APD prolongation.³² The occurrence of EAD requires an increase in net inward current, a

condition called reduced repolarization reserve.³³ In LQT3, the increase in I_{NaL} is the prerequisite of EAD formation. The common mechanism of EAD formation in the presence of $Na_v1.5$ GOF is the re-activation of L-type calcium channels which increases the intracellular calcium to elevate action potential again.³² Frequent EADs in one action potential can induce triggered activity which is a cause of ventricular arrhythmias.^{34, 35}

Prolonged APD is a substrate of EAD formation. However, APD prolongation doesn't always cause EAD formation. Studies have investigated LQT3-associated APD prolongation and EAD formation in isolated cardiomyocytes using ATXII with different doses and found that EAD or triggered activity occurs with high dose of ATXII,³⁶⁻³⁸ which suggests that EAD formation requires a sufficient APD prolongation. Importantly, the life-threatening cardiac arrhythmias in LQT3 patients mainly occur during extreme bradycardias which are associated with very long APD.⁷ Therefore, EAD formation is a mechanism responsible for LQT3-associated cardiac arrhythmias.

Phase 2 reentry

By contrast to EAD formation, phase 2 reentry is caused by conduction disturbance due to action potential heterogeneity.³⁹ Action potential heterogeneity could be found on the epicardium primarily due to heterogenous distribution of transient outward potassium channels.^{40, 41} Phase 2 reentry occurs when the electrical current propagates from a spike-and-dome action potential region to re-excite a loss-of-dome action potential region.^{41, 42} Phase 2 reentry can be manifested by R-on-T phenomenon in ECG^{43, 44} and spiral waves on the epicardium by using optical mapping techniques.^{45, 46}

Action potential heterogeneity could also be found across the ventricular wall.⁴⁷ Three layers of the ventricle from inside to outside are endocardium, myocardium and

epicardium, each of which produces different APDs with longest APD from myocardium due to M cells and shortest APD from epicardium. The difference of the longest and shortest APDs is called transmural dispersion of repolarization (TDR).⁴⁸ Studies have found that $\text{Na}_v1.5$ GOF is associated with increased TDR by preferentially prolonging APD in myocardium and thereby exacerbate transmural APD heterogeneity.^{38, 49} Importantly, this incidence of cardiac arrhythmias occurs when the increased TDR reaches a threshold.⁴⁸⁻⁵⁰ The relationship between increased TDR and arrhythmias can be interpreted by phase 2 reentry as the electrical current from myocardium during action potential plateau re-excite the epicardium which just completes the repolarization.^{41, 49, 51}

The perinexus

Although the mechanism of the concealed LQT3 phenotype is unclear, the recent findings from our group suggest that an intercellular nanodomain, named as the perinexus, may contribute to the normal QT interval in LQT3.^{37, 52} The perinexus is a 200-nm long and 20-30nm wide $\text{Na}_v1.5$ -rich space next to gap junctions in the intercalated disc.^{53, 54} Although the perinexus is extremely narrow relative to the size of a cardiomyocyte, but it could produce 'butterfly effect' on LQT3 phenotype. Our group found that widening the perinexus could exacerbate LQT3 phenotype by further prolonging APD^{37, 52} and inducing EAD formation and R-on-T phenomenon,⁵² which suggests that the concealed LQT3 phenotype may be associated with the narrow perinexus. Furthermore, the other side effect of widening the perinexus is conduction slowing.^{55, 56} We found that widening the perinexus in heart model without $\text{Na}_v1.5$ gain of function leads to the reduction in conduction velocity (CV), which is associated with cardiac arrhythmias.⁵⁷⁻⁵⁹ However, the mechanisms of perinexal widening leading to APD prolongation and EAD formation in

LQT3 and reducing CV cannot be interpreted by providing direct evidence, the reason being no feasible technique can directly measure dynamics in the perinexus. Fortunately, the well-established cardiac computational model can be used to explore the mechanism with the theories of self-attenuation and ephaptic coupling.

Self-attenuation

As we know $\text{Na}_v1.5$ activation causes rapid sodium influx which leads to a large peak sodium current. Computational model found that the dense $\text{Na}_v1.5$ in the narrow perinexus could deplete local sodium ions and thereby limit EAD formation, which is called self-attenuation.^{52, 60} By contrast, this self-attenuation decreases when the perinexus is wider, which couldn't completely deplete sodium ions and may increase I_{NaL} to cause EAD formation.^{52, 61} In the simulation, the mechanism of EAD formation is explored by measuring transmembrane potential, cleft I_{NaL} and sodium reversal potential. When the perinexus is narrow, LQT3 mutant indeed increases I_{NaL} relative to wild type (WT). But the sodium reversal potential greatly decreases due to self-attenuation. Although sodium ions refill the perinexus to increase reversal potential, the increased sodium driving force leads to sodium ions depletion again. This protective negative feedback limits I_{NaL} increase and thereby decrease EAD formation. However, when the perinexus is wide, sodium ions refill the perinexus very rapidly after $\text{Na}_v1.5$ activation so that the self-attenuation is reduced to increase I_{NaL} and thereby lead to EAD formation. Therefore, the reduced self-attenuation is responsible for perinexal expansion leading to APD prolongation and EAD formation in LQT3.

Theoretically, the reduced self-attenuation can also be achieved by increasing sodium concentration without widening the perinexus. In order to demonstrate this hypothesis,

we investigated the effect of high sodium on APD prolongation in LQT3 hearts.³⁷ The experiments and simulations show that high sodium prolonged APD with the reduced self-attenuation. Importantly, high sodium with wide perinexus, which could further reduce self-attenuation, produced a synergistic effect on APD prolongation.

According to the computational model, an important prerequisite for perinexal expansion regulating LQT3 phenotype is that at least 70% sodium channels must be located in the intercalated disc.⁶¹ Considering sodium channels distribution changes over age, it is important to know how sodium channel distribution and cell size affect the regulation of the perinexus during aging. The simulation of neonatal and adult LQT3 cardiac tissues showed that APD prolongation and EAD formation are minimally associated with perinexal expansion in neonatal cells primarily due to diffusive distribution of Nav1.5 in neonatal cells. By contrast, APD prolongation and EAD formation are highly associated with perinexal expansion in adult cells due to Nav1.5 preferentially locating in the ID.⁶² However, the effect of perinexal expansion on LQT3 phenotype has not been investigated in aged cells. The most recent investigation of atrial perinexus in human tissue suggests that atrial perinexus widens in aged hearts.⁶³ Still, the mediation of ventricular perinexus on LQT3 phenotype in aged hearts is unknown.

Ephaptic coupling

It is widely accepted that conduction in cardiac tissue is mediated by gap junctions, which are low-resistance intercalated-disc pathways between adjacent cells. ^{64,}

⁶⁵Electrophysiologists believe that the conduction can also be regulated by the extracellular electrical field, also known as ephaptic coupling (EpC).⁶⁶ By definition, EpC occurs when a stimulated excitable cell activates the adjacent cell through the

extracellular electrical field produced by conductive ions. The concept of EpC at the first time is proposed in the late 19th century by observing the firing of a primary nerve induces the firing of an adjacent inactive nerve.⁶⁷ Later on, EpC in cardiac tissue was investigated by Sperelakis and Mann in 1977⁶⁸ and further elaborated by Seprelakis and McConnell in 2002.⁶⁹

EpC was confirmed by cardiac studies that reduction in gap junction conductance doesn't always decrease conduction velocity due to a concept of conduction reserve^{70, 71}. It was suggested that this conduction reserve is caused by increased electrical field in the intercalated disc, specifically located in the sodium channel-rich perinexus. The results showed that widening the perinexus increases CV sensitivity to 50% reduction in gap junctions while narrowing the perinexus leads to CV insensitivity to reduced gap junctions, which suggest that the perinexus could regulate CV through EpC.

However, the relationship between the perinexal width and CV is not linear. Instead, CV has a biphasic change with perinexal width when gap junction conductance is small.⁷² The computation model suggests that the relationship between CV and perinexal width is biphasic when gap junction conductance is less than 0.2534 μS . CV increases when the perinexus gets mildly wider due to enhanced electrical field. However, if the perinexus further gets wider, CV decreases due to attenuated electrical field. This relationship is also gap junction conduction dependent. If gap junction conductance is over 0.2534 μS , CV always increases when the perinexus widens.

The effect of perinexus expansion on CV has been investigated in animal models with different agents including different calcium concentrations,⁶⁰ hyperosmotic agent mannitol⁵⁶ and cell-cell de-adhesion peptide βadp1 .⁵⁵ These different agents produce the same

results that widening the perinexus decrease CV. Importantly, the investigation of perinexal expansion vs CV in multiple diseased heart models is required to explore the CV-related mechanisms.⁷³ However, it remains unclear if $\text{Na}_v1.5$ GOF affects the relationship between perinexal expansion and CV.

Research Objective

The investigation of the role of the perinexus during $\text{Na}_v1.5$ GOF is very limited. So far, only two animal studies demonstrated that widening the perinexus exacerbates $\text{Na}_v1.5$ GOF due to reduced self-attenuation, which are followed by a couple of $\text{Na}_v1.5$ GOF computational studies. Importantly, the relationship between perinexal expansion and CV during $\text{Na}_v1.5$ GOF is unknown. Therefore, the overall objective of the whole project is to investigate the role of the perinexus in LQT3 under different conditions including hypernatremia, species and aging.

First, we investigated the individual and combined effects of hypernatremia and perinexal expansion on LQT3 phenotype in drug-induced LQT3 guinea pig hearts. The results suggest that combination of hypernatremia and perinexal expansion greatly decreased self-attenuation and played a synergistic effect on APD prolongation. The details of this study are described in Chapter 2.

Second, in order to rule out the off-target effect of ATXII which was used to induce LQT3 phenotype in guinea pig hearts, the combined effect of hypernatremia and perinexal expansion was investigated in a genetically-modified LQT3 mouse heart model. Interestingly, hypernatremia produced the shortening effect on APD, which is different from the findings in a drug-induced guinea pig model of LQT3. Patch clamping in isolated mouse cardiomyocytes demonstrated that this shortening effect of hypernatremia on APD

is due to increased transient outward potassium current (I_{to}). Importantly, hypernatremia and perinexal expansion have no synergistic effect on APD due to increased I_{to} in mouse ventricular myocardium. The details of this study are described in Chapter 3.

Third, the main purpose of this study is to investigate if perinexal expansion contributes to age-dependent risk of sudden cardiac death in LQT3. Surprisingly, we found that the perinexus narrows with aging and may conceal the age-related cellular I_{NaL} and thereby didn't show APD difference between adult and aged guinea pig heart groups. As we expected, perinexal expansion in aged LQT3 hearts is an arrhythmic risk factor according to the arrhythmia susceptibility test. Very interestingly, we found that perinexal expansion in the presence of ATXII leads to an increase in CV in both age groups opposite to previous findings, but the mechanism remains unclear. Furthermore, we found that the increased risk of arrhythmias due to perinexal expansion in aged LQT3 hearts may be associated with the increased TDR which leads to the development of reentry. The details of this study are described in Chapter 4.

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Chapter 2: Hypernatremia and Intercalated Disc Edema Synergistically Exacerbate Long QT Syndrome Type 3 Phenotype

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This chapter has been published in Am J Physiol Heart Circ Physiol (<https://doi.org/10.1152/ajpheart.00366.2021>). Author contributions: XW and SP designed research question and project. XW and GAB collected experimental data. XW analyzed data, XW drafted text and figures. SHW designed computational model and analyzed simulation data. All authors contributed to text and figure revisions.

Abstract

Background: Cardiac voltage-gated sodium channel gain-of-function prolongs repolarization in the Long-QT Syndrome Type 3 (LQT3). Previous studies suggest that narrowing the perinexus within the intercalated disc, leading to rapid sodium depletion, attenuates LQT3-associated action potential duration (APD) prolongation. However, it remains unknown whether extracellular sodium concentration modulates APD prolongation during sodium channel gain-of-function. We hypothesized that elevated extracellular sodium concentration and widened perinexus synergistically prolong APD in LQT3.

Methods and Results: LQT3 was induced with anemone toxin type II (ATXII) in Langendorff-perfused guinea pig hearts (n=20). Sodium concentration was increased from 145 to 160 mM. Perinexal expansion was induced with mannitol or the sodium channel β 1-subunit adhesion domain antagonist (β adp1). Epicardial ventricular action potentials were optically mapped. Individual and combined effects of varying clefts and

sodium concentrations were simulated in a computational model. With ATXII, both mannitol and β adp1 significantly widened the perinexus and prolonged APD, respectively. The elevated sodium concentration alone significantly prolonged APD as well. Importantly, the combination of elevated sodium concentration and perinexal widening synergistically prolonged APD. Computational modeling results were consistent with animal experiments.

Conclusions: Concurrently elevating extracellular sodium and increasing intercalated disc edema prolongs repolarization more than the individual interventions alone in the LQT3. This synergistic effect suggests an important clinical implication that hypernatremia in the presence of cardiac edema can markedly increase LQT3-associated APD prolongation. Therefore, this is the first study to provide evidence of a tractable and effective strategy to mitigate LQT3 phenotype by managing patient sodium levels and preventing cardiac edema.

Introduction

Congenital Long-QT Syndrome Type 3 (LQT3) is associated with gain-of-function (GOF) mutations in the SCN5A gene that encodes the cardiac voltage-gated sodium channel α subunit ($\text{Na}_v1.5$).¹ These mutations result in incomplete inactivation of $\text{Na}_v1.5$, leading to increased late sodium current (I_{NaL}) and prolonged ventricular repolarization, together forming an arrhythmogenic substrate.^{2, 3} Prolonged repolarization can manifest on the surface electrocardiogram (ECG) as a prolonged QT interval.¹ However, some patients with confirmed LQT3-associated variants present with normal corrected QT intervals, which is referred to as a concealed LQT3 phenotype.^{4, 5} A clinical study investigating the incidence and prevalence of Long-QT Syndromes suggests that 28% of all LQT3 patients have a concealed phenotype.⁶ Although the mechanism of the concealed LQT3 phenotype has not been clinically confirmed, our group has determined that this phenotype can be masked experimentally through changes in ephaptic coupling that are mediated by narrowing an intercalated disc (ID) nanodomain, named the perinexus.⁷⁻¹⁰ A fundamental prerequisite for this mechanism is that $\text{Na}_v1.5$ is densely expressed within the ID,^{11, 12} specifically within the perinexus,¹³ and thereby the associated integrated current through $\text{Na}_v1.5$ is limited by local extracellular sodium ion availability. Thus, the perinexus may serve as a key extracellular nanodomain modulating I_{NaL} in diseases such as LQT3.⁷

We previously proposed that changes in perinexal width (W_p) can modulate action potential repolarization in LQT3 through an ionic self-attenuation mechanism.^{7, 14, 15} This putative mechanism suggests that narrowing the perinexus shortens action potential duration (APD) via rapid depletion of local sodium ions within this $\text{Na}_v1.5$ -rich nanodomain.

Briefly, activation of Nav1.5 causes a large and fast inward sodium current (I_{Na}), rapidly depleting sodium ions in the narrow extracellular perinexus. This depletion of local sodium ions within the perinexal nanodomain subsequently reduces the sodium reversal potential (E_{Na}) via a negative feedback mechanism, thereby attenuating I_{NaL} , and ultimately resulting in normal APD. Conversely, expanding the perinexus would blunt the ability of I_{Na} to deplete local sodium ions and the associated reduction of E_{Na} , thereby permitting increased I_{NaL} and prolonged APD in LQT3. However, under normal conditions with physiological Nav1.5 inactivation, perinexal widening has no effect on APD in *ex vivo* hearts,^{7, 16} which suggests that APD prolongation due to widened perinexi preferentially occurs under conditions of incomplete Nav1.5 inactivation. Therefore, perinexal expansion could play a role in APD prolongation in any condition associated with increased I_{NaL} , including heart failure, coronary artery disease, diabetes mellitus and atrial fibrillation.¹⁷⁻²⁰

Currently, perinexal expansion in isolated hearts can be achieved by two approaches with different mechanisms. The first approach utilizes mannitol, a hyperosmotic agent inducing acute interstitial edema, which has been demonstrated to expand the perinexus.²¹ The second one utilizes β adp1, a novel inhibitor of homophilic adhesion by voltage-gated sodium channel β 1 subunit, which is required for regulating perinexal intermembrane distance.¹⁶ Importantly, while previous studies and computational models suggest local sodium ion depletion may conceal LQT3, it remains unknown whether modulating extracellular sodium alone or in conjunction with perinexal expansion exacerbates APD prolongation secondary to sodium channel gain of function. This gap in knowledge has

important clinical implications if extracellular sodium concentrations can conceal Nav1.5 GOF.

We reason that, for a fixed extracellular sodium concentration, widening the perinexus increases the number of sodium ions within this nanodomain, just as increasing extracellular sodium concentration should increase the number of ions for a given perinexal volume. Therefore, we hypothesize that increasing extracellular sodium concentration prolongs APD and, when perinexi are widened, exacerbates APD prolongation under conditions of sodium channel GOF. In this study, we investigate the effect of elevated extracellular sodium concentration on APD with and without perinexal expansion in both a drug-induced animal model and a computational model of LQT3. We determine that perinexal widening and elevated sodium concentration each prolong APD, and combined, they have a synergistic effect on APD prolongation. Lastly, the results of this study imply that high levels of serum sodium could be a risk factor for APD prolongation in cardiac diseases associated with enhanced I_{NaL} .

Methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). All animal study protocols were approved by the Institutional Animal Care and Use Committee at the Virginia Polytechnic Institute and State University.

Optical Mapping

Optical mapping of Langendorff-perfused guinea pig hearts has been extensively described by our group.^{7, 22, 23} Briefly, male, 3-month old, albino, 500-gram Hartley guinea

pigs (n=34, Hilltop Lab Animals, Scottsdale, PA, USA) were anesthetized with isoflurane inhalation. Hearts were rapidly excised, cannulated (<5 minutes) and retrogradely perfused with a crystalloid perfusion solution (in mM: CaCl₂ 1.25, NaCl 139.5, KCl 4.56, dextrose 5.5, MgCl₂ 0.7, HEPES 10 and NaOH 5.5; pH=7.4). A 145-mM sodium concentration (145Na) is a result of 139.5-mM NaCl and 5.5-mM NaOH, and a high sodium concentration of 160 mM (160Na) is a result of 154.5-mM NaCl and 5.5-mM NaOH. Importantly, 160Na was chosen to model clinically-relevant hypernatremia.²⁴ Temperature maintained at 36°C in a 3D printed PLA bath.²² Atria were removed and ventricles electrically stimulated by a bipolar plunge electrode placed in the right ventricular septum near the atrioventricular node with current set at 1.5X the minimum stimulation threshold.

The voltage-sensitive fluorophore, di-4-ANEPPS (15 µM, Biotium, CA, USA), was perfused into the heart, and the heart position was visually adjusted to move the left anterior descending artery in the center of the field of view so that the anterior epicardial surfaces of both ventricles were mapped during external pacing to record optical action potentials with a MiCAM HS02-CMOS camera (SciMedia: 92x80 pixels, field of view = 14.02x12.19 mm). The electromechanical uncoupler, blebbistatin (10 µM, ApexBio Tech LLC, TX, USA), was added to the perfusate to reduce cardiac motion. An I_{NaL} agonist, sea anemone venom ATXII (7 nM, alomone labs, Jerusalem, Israel),⁷ was perfused into the heart to simulate the phenotype of Long QT Syndrome Type 3 (LQT3). Mannitol (26.1 g/L, Sigma-Aldrich, MO, USA), which induces cardiac edema,^{7, 21} or βadp1 (1 µM, LifeTein LLC, NJ, USA), which is a sodium channel β1-subunit adhesion domain antagonist,¹⁶ were perfused to induce perinexal expansion.

Time control experiments with ^{145}Na (baseline, $n=6$) or ATXII ($n=6$) over 60 minutes were performed to determine APD stability over time. $^{145}\text{Na}+\text{ATXII}$ significantly prolonged APD relative to ^{145}Na at either 30 or 60-minute perfusion respectively and there is no significant change in APD between 30 and 60 minutes with ^{145}Na or $^{145}\text{Na}+\text{ATXII}$ (Supplemental Material 1, Figure S1-A and C). Therefore, for experiments involving ATXII and mannitol, a subset of hearts ($n=6$) was perfused with ATXII for 60 minutes, while another subset of hearts ($n=5$) was perfused with ATXII and mannitol for 30 minutes following 30 minutes of perfusion with ATXII.

Time control experiments with βadp1 were also performed to determine APD stability over 40 minutes. There is no significant change in APD between 20 and 40 minutes with $^{145}\text{Na}+\beta\text{adp1}$ ($n=4$) or $^{145}\text{Na}+\text{ATXII}+\beta\text{adp1}$ ($n=4$), respectively. As expected, ATXII still significantly prolonged APD in the presence of βadp1 (Supplemental Material 1, Figure S1-B and D). Therefore, for βadp1 experiments, the hearts were perfused with βadp1 for 20 minutes after 20-minute perfusion of ATXII ($n=5$). Notably, the sodium concentration used in the experiments above was 145 mM. For high sodium concentration experiments, the hearts were perfused with ^{160}Na for 20 minutes after 20-minute perfusion of ^{145}Na in the absence and presence of βadp1 ($n=8$). Half of the experiments were performed in a blinded manner by a second experimentalist for the purpose of rigor and reproducibility. Since the interventions cannot be washed out over the time course of experiments, the solution order is not randomized.

Optical signals were recorded during steady-state pacing at progressively increasing cycle lengths (CL) of 200, 300, 400, 500, 600, 700 and 800 ms until intrinsic activity prevented 1:1 capture. Action potential alternans were not observed under any

experimental condition (Supplemental Material 1, Figure S2); therefore, action potential duration (APD) of only one optical action potential in each of 1840 pixels was calculated after 2x2 spatial binning. The average APD across the array was considered a single data point for a heart at one pacing CL. APD was measured as the difference between the time of the maximum upstroke velocity and 90% or 70% repolarization (APD90 and APD70, respectively) of the optical action potential. APD90 and APD70 were only reported when the heart was successfully paced at the indicated CL. The data for APD70 can be found in the Supplemental Materials.

Arrhythmia Induction protocol

A S1-S2 stimulation protocol^{25, 26} was performed as follows. S1 and S2 were stimulated with a 2ms pulse width through the same bipolar plunge electrode inserted from the right ventricular septum in order to evaluate the susceptibility to cardiac arrhythmia perfused in the following order: 145Na+ATXII, 145Na+ATXII+ β adp1, and 160Na+ATXII+ β adp1. The heart was paced with a train of 10 regular (S1) pulses followed with a premature extra stimulus (S2). All hearts were stimulated with an S1 of 700ms, and the S2 was progressively reduced by as low as 1ms until loss of 1:1 capture or arrhythmia induction.

Transmission Electron Microscopy (TEM)

Left ventricular tissue was collected at the end of the optical mapping experiment and fixed with glutaraldehyde as previously described for TEM.²⁷ A minimum of 20 images of gap junctions and the associated perinexus were obtained at 150,000X magnification for each heart. For each image, i.e. each perinexus, the mean perinexal width (W_p) was calculated over the distance of 30-105 nm from the edge of the gap junction plaque by a

MATLAB algorithm developed in our lab.²⁸ The average W_p for each heart was obtained by averaging the measured W_p from each image for a given heart. W_p from $^{145}\text{Na}+\text{ATXII}$, $^{145}\text{Na}+\text{ATXII}+\text{mannitol}$, and ^{145}Na or $^{160}\text{Na}+\text{ATXII}+\beta\text{adp1}$ is reported.

Simulations

We simulated a 50-cell cable (one-dimensional tissue) comprised of individual myocytes coupled via gap junctions and ephaptic coupling, as in prior work by us and others.^{7-9, 29-31} We accounted for non-uniform sodium channel subcellular localization by spatially discretizing each cell into multiple (10) axial membrane patches along the length of the cell and two intercalated disc (ID) membrane patches at the ends of the cell. Unless otherwise stated, 90% of sodium channels were localized in the ID patches. A T-shaped network of cleft axial resistances and a radial resistance, proportional and inversely proportional, respectively, to the intercellular cleft width (w , proportional to experimentally measured W_p), governed extracellular potentials at the ID and cleft. ID sodium currents and diffusion with the bulk extracellular space governed the extracellular cleft sodium ion concentration ($[\text{Na}^+]_e^{\text{cleft}}$) dynamics, which also depended on the cleft volume.

We utilized an established ventricular guinea pig myocyte ionic model³² representing individual ion channel dynamics, and incorporated a Markov model of a LQT3-associated $\text{Na}_v1.5$ GOF mutant³³ which reproduces a pronounced I_{NaL} . Specifically, the mutant channel model exhibited two modes of gating - a “background mode” and a “burst mode,” the latter of which represented a population of channels that transiently fail to inactivate. Full model equations, parameters, and details of the numerical integration are reported in our prior work.⁷

The cable was paced at one end with a specified CL. We performed a computational parameter study, varying values for CL, w , and bulk extracellular sodium concentration (Na_o). For each simulation, APD was calculated by taking the difference between the action potential repolarization time (defined as the time V_m crossed below -60 mV) and activation time (above -60 mV) for each cell and averaging the values for cells 15 to 35. The simulation codes can be found in the Supplemental Material 2.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). The results are presented as mean $\pm$ standard deviation. The unpaired Student's t-test was performed for statistical analysis of W_p . Comparisons of APD90, APD70 and QT interval at a single pacing CL were analyzed with the unpaired Student's t-test or one-way ANOVA followed by Bonferroni correction for multiple comparisons. Statistical comparisons of APD90, APD70, Δ APD90 and Δ QT interval as a function of CL were made using a two-way ANOVA followed by Bonferroni correction for multiple comparisons. Specific statistical methods are described in the figure legends. A post-hoc power analysis reveals a power of greater than 80% for $\alpha=0.05$, and an $n=4$ based on mean and standard deviations for all experimental interventions. Therefore, a $p<0.05$ was considered statistically significant.

Results

We previously demonstrated that widening the perinexus in the intercalated disc (ID) can prolong action potential duration (APD) in this drug-induced Long-QT Syndrome Type 3 (LQT3) model in aged guinea pig hearts (15 months old).⁷ As a positive control, previous

findings were replicated in the present study with adult animals (3 months old). Representative perinexal images (Figure 1A) acquired by transmission electron microscopy (TEM) and summary data for perinexal width (W_p) (Figure 1B) show that mannitol in the presence of $^{145}\text{Na}+\text{ATXII}$ significantly widens the perinexus relative to $^{145}\text{Na}+\text{ATXII}$, consistent with our previous study using older guinea pigs.⁷ Optical mapping experiments reveal that at 30 minutes of perfusion with $^{145}\text{Na}+\text{ATXII}$, APD is significantly increased (Figure 1C) and the intrinsic heart rate is also significantly decreased (data not shown) relative to ^{145}Na (baseline). Importantly, APD at 90% repolarization (APD₉₀) is significantly prolonged with $^{145}\text{Na}+\text{ATXII}+\text{mannitol}$ relative to $^{145}\text{Na}+\text{ATXII}$ (Figure 1C). The representative and summary data for APD reveal that $\text{ATXII}+\text{mannitol}$ significantly increases APD relative to ATXII at a cycle length of 600 ms (bradycardia for a guinea pig, Figure 1D). The findings for APD₉₀ are similar to those found for APD at 70% repolarization (Figure S3). Furthermore, early afterdepolarization (EAD) or R-on-T events in ECG were not observed in any experimental hearts.

With ATXII , APD prolongation is reflected in the volume-conducted ECG obtained during pacing. $^{145}\text{Na}+\text{ATXII}$ significantly prolongs QT interval at 30 and 60 minutes of perfusion relative to ^{145}Na (Figure 1E, CL = 500 ms), with no difference between 30 and 60 minutes. $^{145}\text{Na}+\text{ATXII}+\text{mannitol}$ significantly increases QT interval when compared with ^{145}Na and $^{145}\text{Na}+\text{ATXII}$ (Figure 1F, CL = 500 ms). Overall, these results, together with the data from our previous work,^{7, 8} demonstrate that the widened intercellular cleft correlates with prolonged APD in the drug-induced LQT3 model in both adult and aged guinea pig hearts. Interestingly, Li et al demonstrated that mannitol alone can increase APD in rabbit ventricular myocytes,³⁴ presumably by inhibiting outward potassium currents,^{35, 36} and we

recently confirmed that mannitol prolongs APD in isolated guinea pig myocytes.⁸ In contrast, our previous optical mapping studies in the intact guinea pig whole-heart preparation did not observe changes in APD in response to mannitol.^{7, 8, 37}

In order to determine whether perinexal expansion is a generalizable mechanism of APD prolongation with increased I_{NaL} , the perinexus was expanded with the sodium channel β 1-subunit adhesion domain antagonist (β adp1) instead of mannitol in subsequent experiments. We have previously shown that, under normal conditions without ATXII, β adp1 significantly increases W_p without prolonging APD in either isolated cardiomyocytes or intact guinea pig heart preparations.¹⁶ In this drug-induced LQT3 model, representative perinexal images (Figure 2A) and summary data for W_p (Figure 2B) reveal that, in the presence of ATXII, β adp1 widens W_p relative to ATXII alone. With regards to effects on repolarization, perfusion of $145Na+ATXII+\beta$ adp1 significantly prolongs APD relative to $145Na+ATXII$ (Figure 2C). In order to confirm that β adp1 preferentially prolongs APD in the presence of ATXII and has little effect on APD in the absence of ATXII reported previously,¹⁶ the effect of β adp1 on APD in the presence and absence of ATXII is evaluated in Figure S4 in Supplemental Material 1. In contrast with the minimal effect of β adp1 alone on APD, β adp1 in the presence of ATXII causes a significantly rate-dependent APD prolongation. Again, APD70 responds similarly to APD90 for all interventions in this experimental group (Supplemental Material 1 Figure S5-A and B). Overall, these results demonstrate that β adp1 widens the perinexus and prolongs APD in the drug-induced LQT3 model, consistent with the effects observed with mannitol.⁷

Computational models suggest that one consequence of narrow intercellular cleft separation is rapid depletion of sodium ions during depolarization of Nav1.5-rich ID nanodomains; this phenomenon is termed ‘self-attenuation’.⁷ For a fixed extracellular sodium concentration, widening the intercellular cleft (increased volume) would theoretically increase the total number of sodium ions in this nanodomain. With a wider cleft, the capacity for incomplete Nav1.5 inactivation to substantively deplete sodium levels in this nanodomain should be reduced, thereby preventing negative feedback (self-attenuation) and permitting enhanced I_{NaL} . However, it is unknown if an increased sodium ion concentration in the widened perinexus would further reduce self-attenuation in the drug-induced LQT3 model.

Since Nav1.5 is densely expressed in the ID,^{11, 12} and in particular within the perinexus,¹³ it is important to determine if an elevated extracellular sodium concentration can alter perinexal width and prolong APD in the drug-induced LQT3 model. Neither representative TEM images of perinexi (Figure 3A) nor summary data (Figure 3B) reveal significantly different perinexal widths in ATXII+ β adp1-treated hearts perfused with either 145 mM sodium (145Na) or 160mM sodium (160Na) concentrations. This suggests that the sodium concentration has little effect on W_p under these conditions. Despite a lack of difference in W_p , elevating sodium concentration to 160Na did significantly prolong APD relative to 145Na during treatment with β adp1 (Figure 3C and Supplemental Material 1, Figure S5-C). In order to demonstrate whether elevated sodium concentration alone prolongs APD independent of perinexal expansion (i.e. in the absence of β adp1), APD as a function of CL is compared between 145Na and 160Na in the presence of ATXII. Figure 3D and Figure S5-D in Supplemental Material 1 show that even without perinexal

expansion, elevated sodium concentration alone significantly prolongs APD₉₀. Furthermore, no EAD or R-on-T events were observed in hearts perfused with β adp1, even with an elevated sodium concentration.

In order to investigate whether region-dependent APD heterogeneity exists across the ventricular epicardium, or if the interventions affect APD heterogeneity, quartile APDs in the base and apex of left and right ventricles are presented in Figure S6 in Supplemental Material 1. In short, neither mannitol, β adp1 nor elevated sodium in the presence of ATXII significantly modulate APD heterogeneity.

To investigate whether the combination of 160Na and β adp1 prolongs APD synergistically or additively relative to either 160Na or β adp1 separately, the change in APD with ATXII is plotted in Figure 3E. The black dashed line represents the change in APD caused by switching from 145Na to 160Na (160Na+ATXII vs 145Na+ATXII; effect of sodium alone). The red solid line represents the change in APD caused by the addition of β adp1 to 145Na (145Na+ATXII+ β adp1 vs 145Na+ATXII; effect of perinexal widening alone). Summing the individual effects on APD caused by elevating sodium alone and adding β adp1 alone produces the thick orange solid line (sum of black dashed line and red solid line), revealing the APD prolongation predicted by the calculated additive effect.

The red dotted line indicates the measured change in APD in the presence of ATXII caused by perfusion of 160Na and β adp1 (160Na+ATXII+ β adp1) relative to 145Na+ATXII. The comparison of these four lines reveals that perfusion with β adp1 and elevated sodium prolongs APD to a greater extent than the summation of the individual effects of increasing sodium from 145Na to 160Na or adding β adp1, i.e. the red dotted line (measured) is well above the thick orange line (predicted). Finally, the change in QT

interval as a result of increasing sodium and perinexal width concurrently is greater than the individual effects, consistent with the changes observed with APD (Figure 3F). Taken together, these results demonstrate that in this LQT3 model, elevating sodium concentration alone prolongs APD and when combined with widened intercellular clefts, the effect on APD prolongation is synergistic.

We also investigated the susceptibility to cardiac arrhythmia with S1S2 stimulation protocol. Cardiac arrhythmias are rare and not significantly different with the different perfusates (Supplemental Material 1, Figure S7). Specifically, one polymorphic and self-terminating arrhythmia is observed in one of four hearts perfused with 145Na+ATXII. Additionally, no arrhythmias are induced in any heart perfused with either 145Na+ATXII+ β adp1 or 160Na+ATXII+ β adp1. Therefore, widened perinexi and elevated sodium prolong APD, but do not induce cardiac arrhythmias in this model of LQT3.

In our prior work,⁷ simulations predicted that preferential localization of Na_v1.5 at the ID was necessary for perinexal cleft width regulation of Na_v1.5 GOF and self-attenuation. Here, we performed new simulations of cardiac tissue with an LQT3-associated Na_v1.5 GOF mutation³³ in the setting of variable extracellular sodium concentrations and Na_v1.5 ID localization (Supplemental Material 1, Figure S8). Simulations predict that both cleft expansion and elevated sodium concentration prolong APD, in agreement with the above experimental results, only for conditions of preferential Na_v1.5 localization at ID, consistent with our prior work.^{7, 8} Next, we broadly investigated the complex relationship between APD, cleft width, sodium concentration, and pacing rate in simulated cardiac tissue with the LQT3-associated Na_v1.5 GOF mutation (Figure 4). For all conditions, APD gradually increases with increasing CL, consistent with expected APD restitution. For a

given sodium concentration, APD prolongs as cleft width increases (Figure 4A), and similarly, for a given cleft width, APD prolongs as the sodium concentration increases (Figure 4B). Further, these effects are more pronounced for longer CLs (i.e., slower pacing rates). Thus, simulations predict that separately, both cleft expansion and elevated sodium concentration prolong APD, as demonstrated in the experiments presented above. We next quantified the summative nature of these predictions in mutant cardiac tissue.

Performing parallel analyses to experiments in Figure 3 (Panel C, D and E), we plot APD as a function of CL for ^{145}Na (solid lines) and ^{160}Na (dashed lines) for nominal (20 nm, black lines, Figure 5A) and expanded (50 nm, red lines, Figure 5B) cleft widths. As in Figure 4, we find that elevated sodium concentration prolongs APD for both normal and wide cleft widths. Importantly, simulations predict that the observed combined effect (dashed red line) is greater than the calculated sum of the individual contributions to APD prolongation (Figure 5C, solid orange line) of cleft expansion (solid red line) and elevated sodium (dashed black line), denoting a synergistic effect consistent with the experiments presented in Figure 3E.

Expanding on the above experiments and simulations, we next investigated whether these synergistic effects are similarly predicted for the case in which sodium concentration is reduced. In Figure S9 of Supplemental Material 1, we plot APD as a function of CL for ^{145}Na (solid lines) and ^{130}Na (dashed lines) for nominal (20 nm, Supplemental Material 1, Figure S9-A) and expanded (50 nm, Supplemental Material 1, Figure S9-B) cleft widths. As in Figure 4, the reduced sodium concentration shortens APD for both cleft widths. In this case, simulations predict that the individual contributions of APD prolongation via cleft expansion (Supplemental Material 1, Figure S9-C, solid red

line) and APD shortening via reduced sodium concentration (dashed black line) together sum (solid orange line) to shorten APD overall. However, the simulated combined effect (dashed red line) exhibits an even greater degree of APD shortening. That is, cleft expansion-mediated APD prolongation is dampened in a synergistic manner by reduced sodium concentration that is more than the individual contributions.

To quantify the comparison of the combined effects with the summed individual contributions, we define the “synergy ratio” as the ratio of the combination of cleft expansion and altered sodium concentration (dashed red lines in Figures 5C and S9-C) divided by the summed individual contributions (solid orange lines in Figures 5C and S9-C), with the ratio presented as positive for APD prolongation and negative for APD shortening. In Figure 5D, we plot the synergy ratio measured for CL of 750 ms as a function of the sodium concentration. Consistent with Figures 5C and S9-C, the synergy ratio in the model is greater than 1 for sodium concentrations above 145 mM, while the ratio is less than -1 for sodium concentration below 140 mM. These measures thus predict that elevated sodium concentrations synergistically enhance the influence of cleft expansion, while reduced sodium concentrations synergistically mitigate the influence of cleft expansion.

Finally, we investigated the mechanism of the synergistic effects of elevated sodium concentration and cleft expansion in the LQT3-associated $\text{Na}_v1.5$ GOF cardiac tissue. Simulated cardiac tissue was paced at one end of the strand at a CL of 750 ms. In Figure 6, we plot the time series for the transmembrane potential (V_m , Figure 6A), $\text{Na}_v1.5$ open probability (Figure 6B), intercellular cleft sodium concentration (Figure 6C), I_{Na} at the ID (Figure 6D), and ID E_{Na} (Figure 6E) for different combinations of nominal (20 nm) and

expanded cleft width (50 nm) and normal (^{145}Na) and elevated (^{160}Na) extracellular sodium. Note that for clarity, traces are only shown for a single ID membrane or cleft (cell 25 out of 50). For nominal cleft width and normal sodium concentration (^{145}Na , $w=20$ nm; black lines), mutant $\text{Na}_v1.5$ promotes an increased open probability during the cardiac action potential and a persistent I_{NaL} . However, both the initial sodium influx during the action potential upstroke and subsequent late current deplete the local cleft sodium concentration (Figure 6C) and reduce the ID E_{Na} (Figure 6E). This reduced E_{Na} in turn reduces the magnitude of I_{NaL} .

As discussed in our prior work,⁷ ID cleft sodium depletion represents a negative feedback mechanism that suppresses APD prolongation. However, in the setting of cleft expansion (^{145}Na , $w=50$ nm; red lines), cleft sodium depletion is mitigated, resulting in an enhanced I_{NaL} and prolonged APD. In the setting of elevated sodium concentration (^{160}Na , $w=20$ nm; gray lines), local cleft sodium is depleted to a greater extent than the setting of cleft expansion alone (red line). However, the larger extracellular sodium concentration results in rapid cleft refilling during the plateau, which in turn increases I_{NaL} and thus prolongs APD so much so that the red and grey lines in Figure 6A are nearly indistinguishable. That is, cleft expansion and elevated sodium individually prolong APD to a similar extent. Finally, for the combination of both cleft expansion and elevated sodium (^{160}Na , $w=50$ nm; magenta lines), both of individual effects are enhanced: cleft sodium level after rapid depletion is maintained at the level greater than that in the setting of either cleft expansion or elevated sodium concentration alone (Figure 6C), which in turn promotes I_{NaL} and prolongs APD.

In our prior work,⁷ we demonstrated that the altered I_{NaL} driving force could also promote EADs, in particular under bradycardic conditions. In the Supplemental Material 1 (Figures S10-S13), we demonstrate that for longer CLs the synergy between cleft expansion and altered sodium concentration is more pronounced, such that small individual contributions to APD prolongation combine to promote pronounced EADs (Supplemental Material 1, Figure S10). Plots of APD restitution for longer CLs under the above explored conditions further demonstrate the synergistic effects on EAD promotion (Supplemental Material 1, Figures S11-S13). Further, we find that the synergy ratio magnitude (as described above) is generally greater than 1 for longer CLs, illustrating both synergistic APD prolongation and shortening for elevated and reduced extracellular sodium concentration, respectively (Supplemental Material 1, Figure S14).

Discussion

Previous experimental and simulation studies revealed that widening intercellular separation in the gap junction adjacent and $Na_v1.5$ dense perinexus can unmask a LQT3 phenotype.^{7, 8} However, to our knowledge, this is the first study to elucidate how altering extracellular sodium concentration modulates action potential duration (APD) prolongation secondary to $Na_v1.5$ gain-of-function (GOF). Specifically, we demonstrate the synergistic effects of increased perinexal width and elevated sodium concentration on APD prolongation. Both *ex vivo* and *in silico* experiments consistently show that both perinexal widening and elevated sodium concentration alone prolong APD. Importantly, by simultaneously widening the perinexus and elevating sodium concentration, the singular effects are amplified in a synergistic manner. The results suggest that a high

concentration of sodium in widened intercellular clefts greatly increases I_{NaL} and subsequently prolongs ventricular repolarization in response to $Na_v1.5$ GOF.

Despite the synergistic effect of widened perinexus and high sodium concentration on APD, neither spontaneous arrhythmia nor induced arrhythmia was observed in the same condition. In contrast with spontaneous arrhythmias observed in a previous aged animal study,⁷ the current study using younger animals suggests an age-related difference in the incidence of spontaneous arrhythmia. S1S2 is a well-established protocol to induce arrhythmias, but the premature stimulus is usually delivered on the epicardium.^{25, 26} In this study, S1S2 was stimulated from the septum. However, we do not know if stimulation site could affect the arrhythmia induction. Additionally, we do not know the three-dimensional distribution of APD gradients in the whole heart with these interventions. Therefore, future studies are required to address these specific and important questions.

It is well established that incomplete inactivation of $Na_v1.5$ causes APD prolongation in a number of diseases including LQT3.^{1, 17-20} Previous work suggested that decreasing the extracellular volume in nanodomains between cardiomyocytes such as the perinexus leads to extracellular sodium depletion and attenuation of APD prolongation despite incompletely inactivated and therefore conductive $Na_v1.5$.⁷⁻⁹ In effect, our previous studies explored how changing the total number of sodium ions available (fixed sodium concentration and altered volume) modulates the rate of extracellular sodium depletion. In contrast, this study explored how altering both the perinexal volume and sodium concentration individually and concurrently modulate APD prolongation during $Na_v1.5$ GOF.

A proposed mechanism for increased sodium concentration modulating APD during $\text{Nav}1.5$ GOF is as follows. According to ephaptic coupling theory, rapid transactivation of $\text{Nav}1.5$ withdraws sodium ions from a shared restricted space and decreases extracellular sodium in that space with two electrophysiological consequences.^{7, 8} First, sodium charge withdrawal will reduce extracellular potentials and thereby increase transmembrane potential of both cells. Second, rapid extracellular sodium depletion will reduce E_{Na} along those shared membrane boundaries. The net effect of reduced extracellular potential and E_{Na} reduces the driving force for sodium through the sodium channel due to incomplete inactivation, so I_{NaL} is markedly decreased. However, the elevated extracellular sodium increases the number of ions available in the extracellular space, thereby increasing E_{Na} during the diastolic interval. As a result, I_{Na} induced sodium depletion of shared nanodomains such as within the perinexus is now insufficient to reduce E_{Na} to the level required for I_{Na} induced self-attenuation. As a result, $\text{Nav}1.5$ can continue to conduct, thereby sustaining I_{NaL} , which prolongs the APD.

As demonstrated previously and explored in greater detail here, these effects are highly dependent on $\text{Nav}1.5$ localization within the sarcolemma and whether those channels localize to relatively narrow extracellular domains that can be self-regulated by the same currents. This suggests that enhancing I_{NaL} without self-attenuation would more frequently cause dramatic and potentially life threatening APD prolongation during $\text{Nav}1.5$ GOF. This is important because mannitol is commonly used in the priming solution for patients with cardiac surgery and traumatic brain injury due to its osmotic diuretic effect.^{38, 39} However, it has been reported that mannitol is associated with prolonged QT interval which correlates with increased cardiac arrhythmia in brain injury patients.⁴⁰ QT prolongation

could be either due to mannitol induced hERG inhibition^{35, 36} or age-associated, increased Nav1.5 late-current.⁴¹ Therefore, the mechanism of QT prolongation with mannitol requires additional research in order to separate the effects of hERG inhibition and unmasking Nav1.5 gain-of-function.

Although it was shown that mannitol prolongs APD in the current LQT3 heart study due to widening the perinexus,^{7, 8} mannitol is also associated with hERG inhibition to prolong APD in the wild-type isolated cardiomyocytes^{8, 34-36} However, mannitol did not prolong APD in the isolated wild-type whole-heart preparations.^{7, 8, 37} Despite the unclear mechanism, the consistency of the mannitol data with β adp1 data in the current LQT3 model raises our confidence that APD prolongation in LQT3 hearts is not an effect of mannitol inhibiting hERG channels, but rather an effect of both agents expanding the perinexus.

Sodium channel subunit beta-1 (SCN1B) facilitates cell-cell adhesive interaction, and *Scn1b* loss of function is associated with increased cardiac arrhythmias in *Scn1b* null mouse hearts⁴² and hearts perfused with 10 μ M β adp1.¹⁶ In the current study, β adp1 widened the perinexus, but we did not observe spontaneous or inducible cardiac arrhythmias even in the presence of ATXII. Previously, 10 μ M β adp1 was shown to increase arrhythmias in the isolated guinea pig heart,¹⁶ but this concentration is an order of magnitude greater than the 1 μ M used in this study. Therefore, the degree of perinexal disruption in this manuscript is difficult to compare to genetically reduced *Scn1b* in mouse models or studies using larger β adp1 concentrations. Future studies are necessary to determine the degree of sodium channel beta-1 subunit loss-of-function that will be associated with APD prolongation and increased arrhythmia risk.

Synergy, as proposed in this study, suggests that the rate of change of sodium concentration in $\text{Na}_v1.5$ -rich nanodomains is modulated by both cleft volume and the bulk extracellular ionic concentration (through diffusive coupling between the cleft and bulk extracellular spaces). Importantly, the synergy ratio reveals that both a widened cleft and elevated sodium will dramatically prolong APD in this model, and in a manner that is greater than the sum of the individual effects. Equally important is the predicted negative synergy ratio. When clefts are small and extracellular sodium reduced, the study suggests that APD can be shortened as a result of a $\text{Na}_v1.5$ GOF. Therefore, our study indicates that the electrophysiological phenotype of $\text{Na}_v1.5$ GOF in LQT3 may be fully concealed by reducing extracellular sodium concentrations synergistically with narrowing perinexal width. While these findings are highly provocative and tantalizing due to the translational potential of managing LQT3 by modulating plasma sodium concentration, the negative synergy ratio requires additional experimental evidence and careful consideration.

High sodium intake may increase the risk of cardiac events in diseases associated with increased I_{NaL} . High serum sodium is associated with prolonged QT interval in patients⁴³⁻⁴⁶ suggesting that elevated plasma sodium may exacerbate QT interval prolongation in diseases associated with increased I_{NaL} .¹⁷⁻²⁰ Importantly, prolonged QT interval and structural disruption of the intercalated disc (ID) are each associated with aging^{41, 47} suggesting that elevated plasma sodium may greatly increase the incidence of cardiac events in aged patients. Interestingly, our recent simulation study of the age progression in LQT3 predicted that ID structural properties have minimal influence on arrhythmias for conditions associated with neonatal and early developmental stages, while cellular properties, specifically cell size and sodium channel density, are more critical.⁹ In contrast,

for adult cardiac tissue, we found that ID structure is a key determinant of arrhythmias, consistent with our current study. Here, we have further identified elevated sodium concentration as an additional regulatory factor for LQT3 patients. Importantly, the synergistic effect observed in this study suggests that elevated sodium concentrations within the structurally disrupted intercalated disc may increase the propensity for cardiac events in aged LQT3 patients.

However, reducing serum sodium levels must be carefully considered in LQT3 patients. Current pharmacologic therapies aimed to treat LQT3 may also cause a LQT3 overlap syndrome with Brugada Syndrome (BrS) as a consequence of the therapy reducing both late and peak I_{Na} . Some $Nav1.5$ variants, including insD1795⁴⁸ and E1784K⁴⁹, cause increased I_{NaL} and reduced peak I_{Na} , which are associated with the LQT3 and BrS overlap syndrome. Importantly, the R1193Q⁵⁰ and V2016M⁵¹ variants are not associated with a change in peak current, but I_{NaL} is increased, and both variants are associated with the LQT3 and BrS overlap syndrome. The negative synergy ratio predicted by the model supports a hypothesis that even in the absence of reduced peak I_{Na} measured in isolated cells, $Nav1.5$ GOF may contribute to BrS because of enhanced I_{Na} self-attenuation that will ultimately reduce the peak current in tissue. Therefore, hyponatremia may increase arrhythmogenic risk in some BrS patients. In fact, several case reports have shown that BrS ECG patterns were observed during hyponatremic episodes and became normal again after serum sodium concentrations were restored.⁵²⁻⁵⁴

Limitations

The LQT3 model in the present study was established by perfusing isolated hearts with ATXII based on previous studies establishing that the compound prevents complete

inactivation of Nav1.5. Like all pharmacologic interventions, ATXII may have off-target effects. For example, ATXII may increase GOF of other sodium channels beyond the cardiac Nav1.5 isoform, and it may also enhance the inward rectifier potassium current.⁵⁵

⁵⁶ Even if all sodium channels in the lateral membrane, t-tubules, or intercalated disc⁵⁷ respond similarly to changes in the extracellular ionic concentration, or to the β adp1 peptide, the model predicts only ID-localized Na channels underlie APD prolongation under the conditions explored in this study. Additionally, we have previously shown that the mechanism of perinexal expansion induced APD prolongation is independent of at least three different LQT3 models including one experimental drug-induced LQT3 guinea pig model and two simulated Nav1.5 LQT3 variants.⁷ Still, future studies are needed to demonstrate broader applicability based on other sodium channel specific dynamics, different voltage-gated sodium channel isoforms and the development of a computational model of sodium channel interactions with pharmacologic agents.

Experiments were conducted in pharmacologically arrested hearts with the agent blebbistatin. All electromechanical uncouplers have effects that may limit interpretation of the present results. For example, blebbistatin could prolong APD in the isolated hearts⁵⁸

⁵⁹ by a proposed mechanism of altering intracellular calcium handling.⁶⁰

The effects of elevated sodium concentration on intracellular calcium homeostasis are not investigated in the current study. Nav1.5 GOF should increase intracellular sodium during the action potential plateau followed by increased intracellular calcium via reduced sodium-calcium exchanger (NCX) forward mode.^{61, 62} Altered calcium homeostasis is associated with the occurrence of EADs.⁶³ However, elevating extracellular sodium should reduce intracellular calcium accumulation,⁶⁴ by a mechanism thought to occur later

in the action potential and diastolic intervals. Future studies that manipulate NCX and pacing rate are needed to understand the effect of increased extracellular sodium on intracellular calcium homeostasis. With self-attenuation though, sodium entry into myocytes during prolonged action potential repolarization will be reduced, and coupled with long diastolic intervals, should result in further intracellular calcium depletion. Thus, one might assume elevating extracellular sodium would be anti-arrhythmic by a mechanism of reducing the likelihood of spontaneous calcium release events or delayed after-depolarizations. Our data and models suggest that LQT3 is associated with EADs, and the prolonged APs are more likely associated with re-activation of calcium channels as the arrhythmic substrate rather than intracellular calcium overload. Importantly, the models in this study and our previous work incorporate NCX and demonstrate significant APD prolongation and EADs in the presence of widened clefts and increased extracellular sodium concentration.

We note that simulations predict smaller changes in APD due to cleft width expansion and altered extracellular sodium, compared with experiments, for shorter cycle lengths, while simulations predict larger APD changes for longer cycle lengths. We speculate that these quantitative discrepancies arise due to model sensitivity in the positive feedback mechanism in which I_{NaL} promotes EADs. Although our model incorporates significant structural details, specifically non-uniform $Na_v1.5$ distribution and restricted intercellular cleft spaces, this formulation is nonetheless a simplified representation of complex cardiac tissue structure. Structural details not incorporated in our model may dampen this positive feedback and thus result in quantitative measurements closer to experiments. While this limitation results in quantitative differences, the simulations qualitatively predict

trends consistent with experiments, and importantly identify the mechanism underlying the synergistic effect of cleft expansion and altered extracellular sodium.

All experiments were performed with adult male guinea pigs and the findings will need to be confirmed in female animals, particularly given the well-established differences in the expression of a variety of potassium channels between the sexes.^{65, 66}

Lastly, caution should be exercised before directly translating these results into humans since Langendorff-perfused isolated hearts operate outside *in vivo* ionic control mechanisms that are provided by the renal, gastrointestinal, and pulmonary systems. It is therefore unclear if serum sodium concentrations are a mechanism of prolonged QT interval in LQT3 patients with normal electrolyte homeostasis. Clinical studies have shown that hypernatremia is an important risk factor for QT interval prolongation.⁴³⁻⁴⁶ However, *in vivo* studies need to be conducted to evaluate the safety and efficacy of modulating serum sodium in conditions associated with Nav1.5 GOF.

Conclusions

In conclusion, Nav1.5 GOF can be exacerbated by either expanding cellularly regulated shared extracellular nanodomains densely expressing highly conductive sodium channels or elevating extracellular sodium. Importantly, the combination of widened extracellular nanodomains like the perinexus with elevated sodium may constitute a putative mechanism that synergistically exacerbates APD prolongation during Nav1.5 GOF.

Although several drugs have been used as relatively specific I_{NaL} inhibitors to treat LQT3 in patients,⁶⁷ little progress has been made in recent years to treat enhanced I_{NaL} .

Therefore, our findings potentially provide a new and cost-effective strategy to mitigate sodium channel GOF related diseases like LQT3 by means of managing sodium levels and preventing cardiac edema in patients.

Supplemental data

Supplemental Material 1 (Supplemental Figs. S1–S14):

<https://doi.org/10.6084/m9.figshare.14825058>.

Supplemental Material 2 (simulation code):

<https://doi.org/10.6084/m9.figshare.16624240>.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Author contributions

S.P. conceived and designed research; X.W., G.B., and S.H.W. performed experiments; X.W., G.B., and S.H.W. analyzed data; X.W., G.B., S.H.W., and S.P. interpreted results of experiments; X.W., G.B., S.H.W., and S.P. prepared figures; X.W. and S.P. drafted manuscript; G.S.H., G.B., D.R.K., R.G.G., S.H.W., and S.P. edited and revised manuscript; X.W., G.S.H., G.B., D.R.K., R.G.G., S.H.W., and S.P. approved final version of manuscript.

Figures

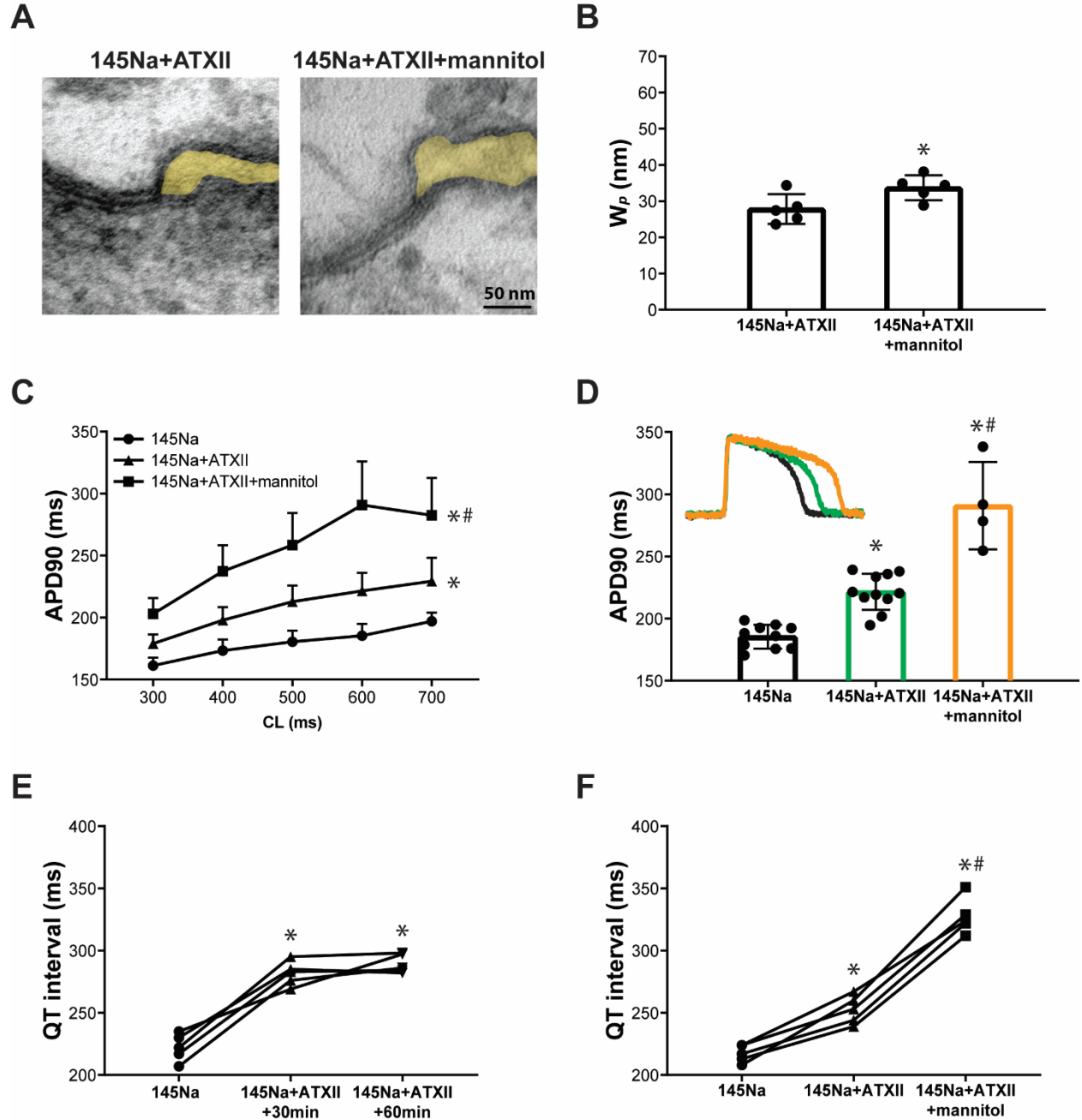


Figure 1. Perinexal widening induced by mannitol correlates with prolonged APD in LQT3. A. Representative TEM images of the perinexus (shaded yellow) after perfusion of ATXII alone (145Na+ATXII) for 60 minutes or ATXII and mannitol (145Na+ATXII+mannitol) for 30 minutes. **B.** Summary data for perinexal width (W_p) with perfusion of 145Na+ATXII or 145Na+ATXII+mannitol (145Na+ATXII: n=5;

145Na+ATXII+mannitol: n=5; * p <0.05 by unpaired t-test). **C.** Action potential duration at 90% repolarization (APD90) as a function of cycle length (CL) for 145Na, 145Na+ATXII, or 145Na+ATXII+mannitol (145Na: n=10; 145Na+ATXII: n=11; 145Na+ATXII+mannitol: n=5; * p <0.05 relative to 145Na; # p <0.05 relative to 145Na+ATXII; two-way ANOVA with mixed-effects model). Perfusion duration of each solution is 30 minutes. **D.** Representative action potentials superimposed to demonstrate differences in repolarization (inset) and summary data for APD90 at CL=600 ms with 145Na, 145Na+ATXII and 145Na+ATXII+mannitol (* p <0.05 relative to 145Na; # p <0.05 relative to 145Na+ATXII; one-way ANOVA with mixed-effects model). **E.** QT interval during pacing at 500 ms in paired experiments (n=5) with 145Na, 145Na+ATXII at 30 and 60 mins (* p <0.05 relative to 145Na; one-way ANOVA with repeated measures). **F.** QT interval during pacing at 500 ms in paired experiments (n=5) with 145Na, 145Na+ATXII and 145Na+ATXII+mannitol. (* p <0.05 relative to 145Na; # p <0.05 relative to 145Na+ATXII; one-way ANOVA with repeated measures). Perfusion duration of each solution is 30 minutes

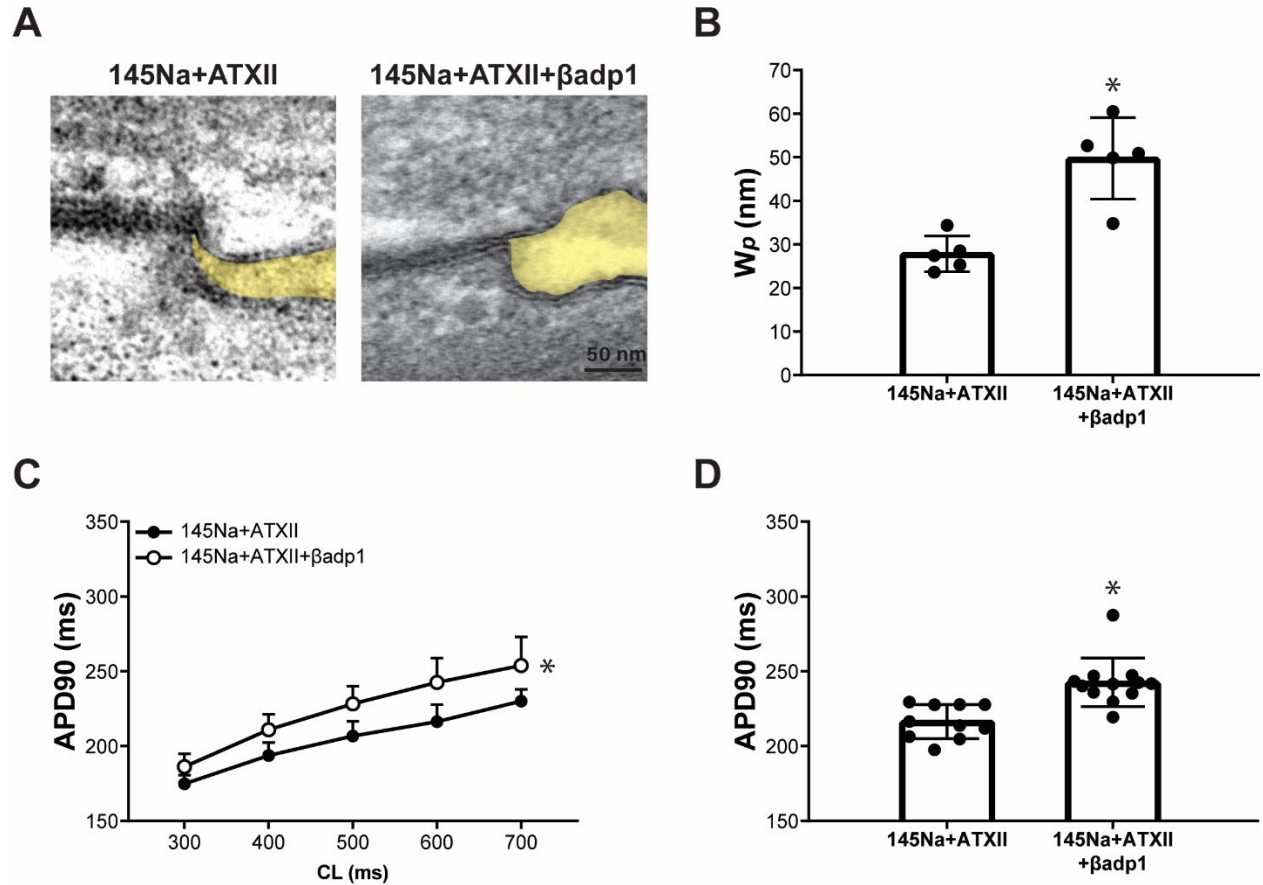


Figure 2: Perinexal widening induced by β adp1 is associated with prolonged APD in LQT3. **A.** Representative TEM images of the perinexus (shaded yellow) after perfusion of ATXII (145Na+ATXII) or ATXII and β adp1 (145Na+ATXII+ β adp1). **B.** Summary data for perinexal width (W_p) with perfusion of 145Na+ATXII or 145Na+ATXII+ β adp1 (145Na+ATXII: $n=5$; 145Na+ATXII+ β adp1: $n=5$; * $p<0.05$ by unpaired t-test). **C.** Action potential duration at 90% repolarization (APD90) as a function of cycle length (CL) for 145Na+ATXII and 145Na+ATXII+ β adp1 (145Na+ATXII: $n=11$; 145Na+ATXII+ β adp1: $n=13$; * $p<0.05$ by two-way ANOVA with mixed-effects model). Perfusion duration of each solution is 20 minutes. **D.** Summary data for APD90 at CL=600 ms with 145Na+ATXII and 145Na+ATXII+ β adp1 (* $p<0.05$ by unpaired t-test).

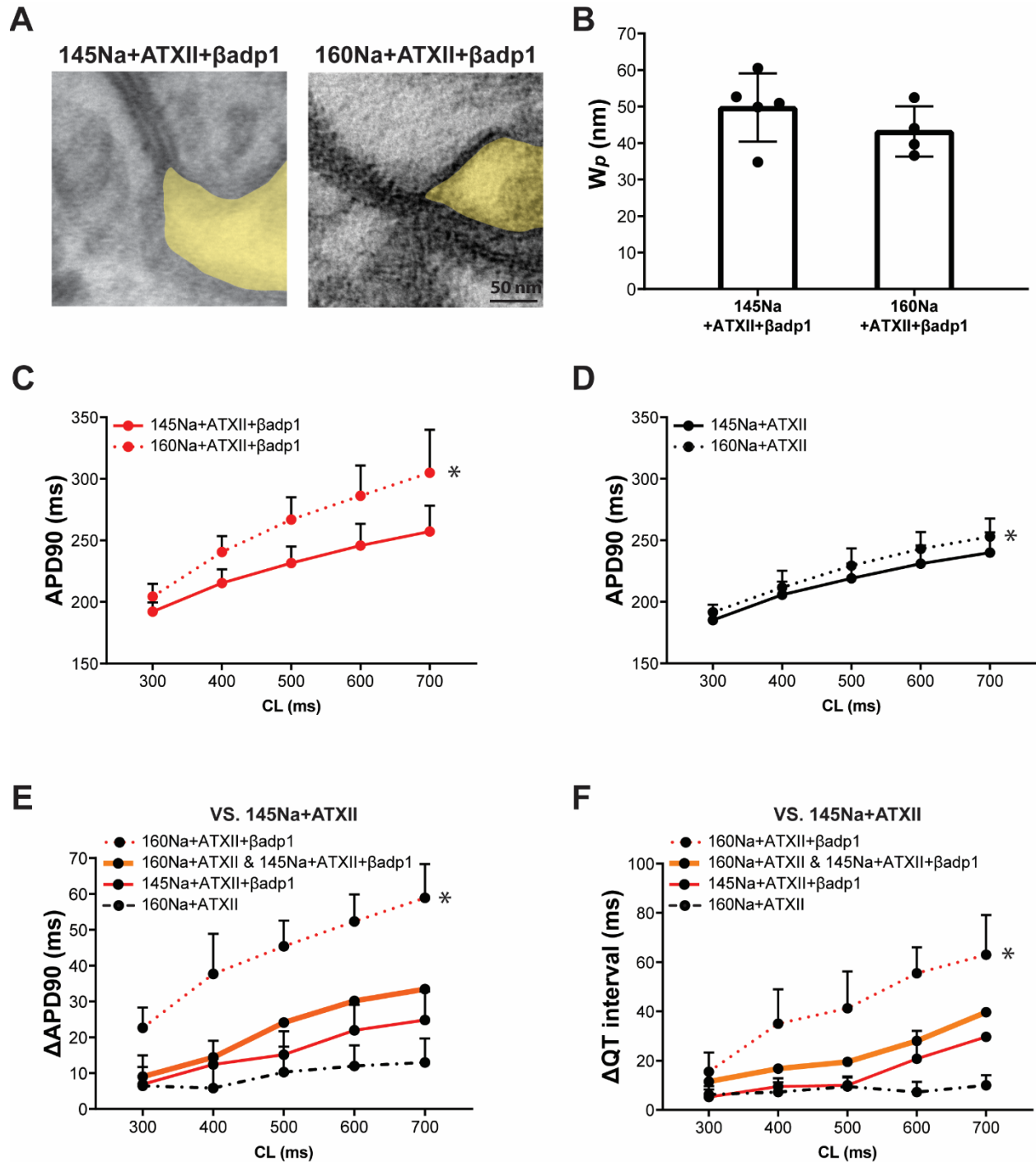


Figure 3: Perinexal widening and high sodium concentration synergistically prolong APD in LQT3. **A.** Representative TEM images of the perinexus (shaded yellow) after perfusion of 145-mM sodium concentration, ATXII and βadp1 (145Na+ATXII+βadp1), and 160-mM sodium concentration, ATXII and βadp1

(160Na+ATXII+ β adp1). **B.** Summary data for perinexal width (W_p) with perfusion of 145Na+ATXII+ β adp1 (n=5) and 160Na+ATXII+ β adp1 (n=4). **C.** Action potential duration at 90% repolarization (APD90) as a function of cycle length (CL) for 145Na+ATXII+ β adp1 and 160Na+ATXII+ β adp1 (145Na+ATXII+ β adp1: n=9; 160Na+ATXII+ β adp1: n=8; * p <0.05 by two-way ANOVA with mixed-effects model). Perfusion duration of each solution is 20 minutes. **D.** APD90 as a function of CL for 145Na+ATXII and 160Na+ATXII (145Na+ATXII: n=4; 160Na+ATXII: n=4; * p <0.05 by two-way ANOVA with mixed-effects model). Perfusion duration of each solution is 20 minutes. **E.** The change in APD (relative to 145Na+ATXII) due to perfusion with 160Na+ATXII (n=4; black dashed line), 145Na+ATXII+ β adp1 (n=7; red solid line), the summation of the individual effects of 160Na and β adp1 perfusion (160Na+ATXII & 145Na+ATXII+ β adp1, thick orange solid line), and the effect of combined perfusion with 160Na and β adp1 (n=6; 160Na+ATXII+ β adp1, red dotted line) (* p <0.05 relative to 160Na+ATXII and 145Na+ATXII+ β adp1; two-way ANOVA with repeated measures). **F.** The change in QT interval (relative to 145Na+ATXII) due to perfusion with 160Na+ATXII, 145Na+ATXII+ β adp1, the summation of the individual effects of 160Na and β adp1 perfusion (160Na+ATXII & 145Na+ATXII+ β adp1), and the effect of combined perfusion with 160Na and β adp1 (160Na+ATXII+ β adp1) (* p <0.05 relative to 160Na+ATXII and 145Na+ATXII+ β adp1; two-way ANOVA with repeated measures).

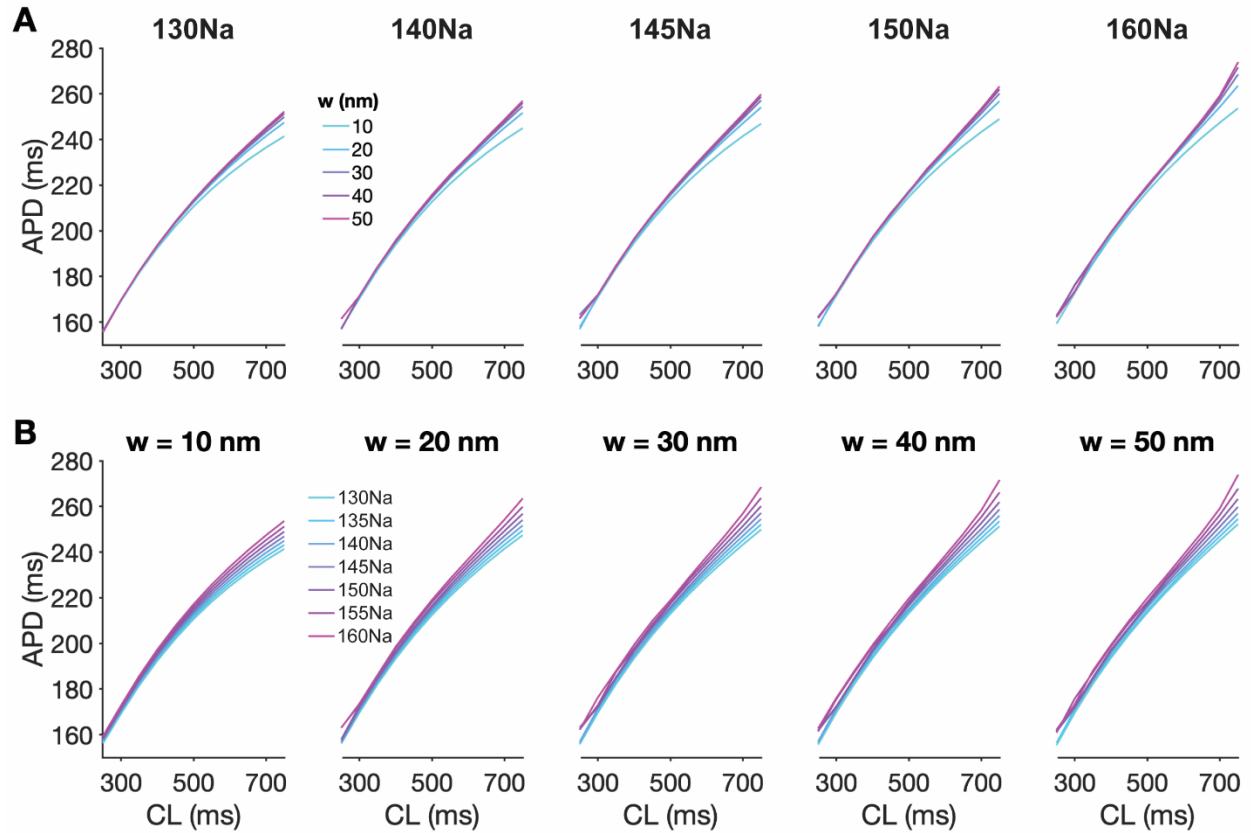


Figure 4: Cleft expansion and elevated sodium concentration separately prolong APD in cardiac tissue with a LQT3-associated sodium channel gain-of-function mutation. Action potential duration (APD) is shown as a function of cycle length (CL) for different sodium concentrations and cleft widths (w) in simulations of tissue with LQT3-associated sodium channel gain of function. **A.** APD prolongation for increasing cleft width (w) and variable sodium concentration. **B.** APD prolongation for elevated sodium concentration and variable cleft widths (w).

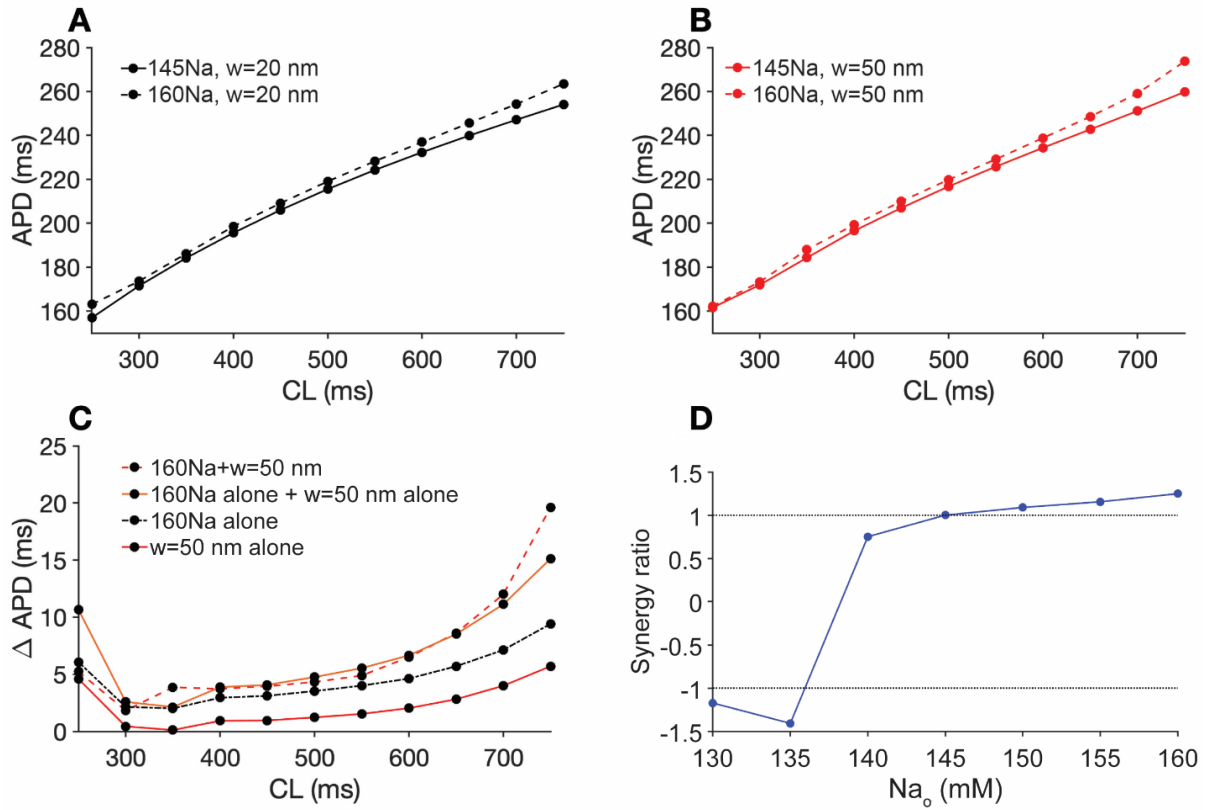


Figure 5: Simulation predicts the synergistic effect of expanded intercellular clefts and elevated sodium concentration on APD prolongation in cardiac tissue with a LQT3-associated sodium channel gain-of-function mutation. A. Action potential duration (APD) as a function of cycle length (CL) for two different sodium concentrations (145Na and 160Na) and nominal cleft width (w) of 20 nm. **B.** APD as a function of CL for two different sodium concentrations and a widened cleft width (w) of 50 nm. **C.** The change in APD due to expanded cleft width 50 nm ($w=50$ nm alone), elevated sodium concentration 160 mM (160Na alone), summation of expanded cleft width and elevated sodium concentration (160Na alone + $w=50$ nm alone) and combination of expanded cleft width and elevated sodium concentration (160Na+ $w=50$ nm) relative to cleft width of 20 nm and sodium concentration of 145 mM. **D.** The synergy ratio with different sodium concentrations. For sodium concentration above 145 mM, a synergy ratio greater than 1

indicates that APD is prolonged synergistically, and for sodium concentration below 140 mM, the synergy ratio less than 1 indicates that APD is shortened synergistically.

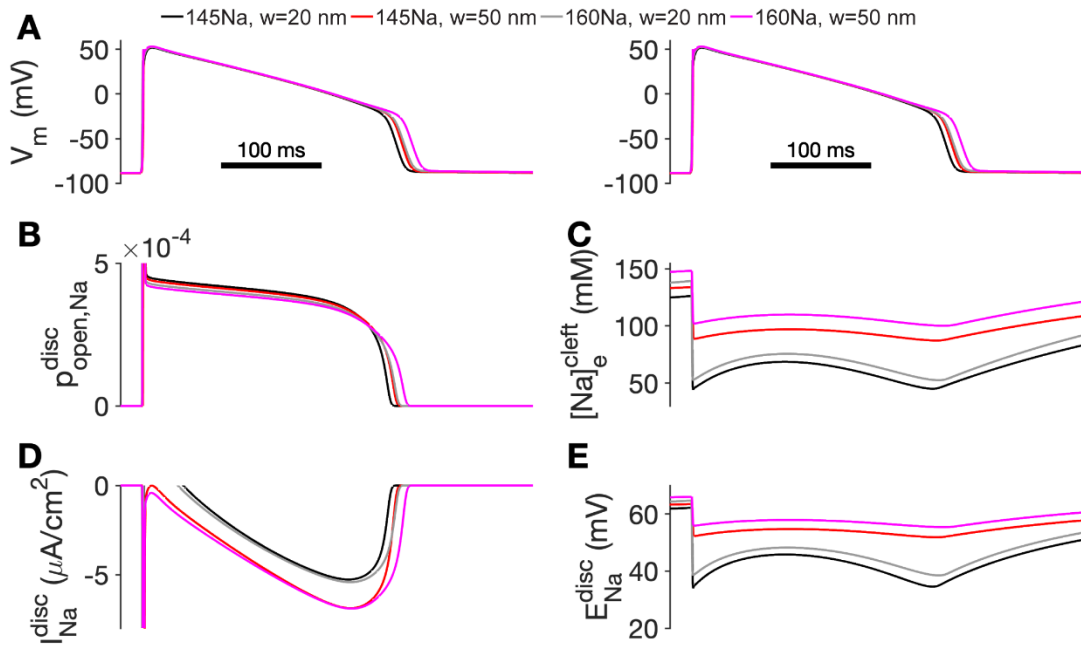


Figure 6: Combination of expanded intercellular clefts and elevated sodium concentration prolongs APD by reducing cleft sodium depletion and maintaining I_{Na} driving force. **A.** Transmembrane potential (V_m), **B.** Nav1.5 open probability, **C.** Intercellular cleft sodium concentration, **D.** Sodium current at the intercalated disc (ID), and **E.** ID sodium reversal potential (E_{Na}) are shown as a function of time for different cleft widths and sodium concentrations for an action potential elicited during pacing at cycle length (CL) = 750 ms in cardiac tissue with a LQT3-associated sodium channel gain-of-function mutation.

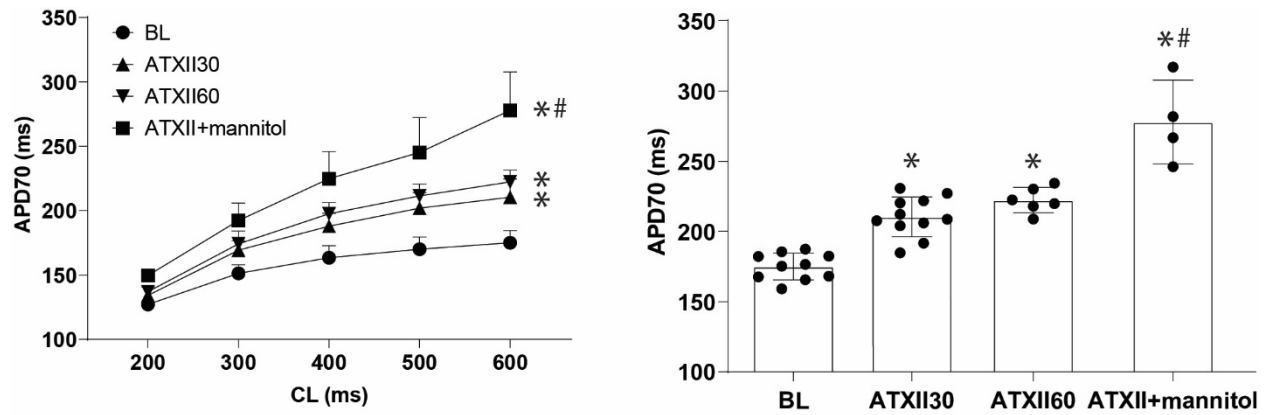


Figure S1: Mannitol prolongs APD in the drug-induced LQT3 model. A. Action potential duration at 70% repolarization (APD70) as a function of cycle length (CL) for baseline (BL), ATXII for 30 minutes (ATXII30), ATXII60 or ATXII+mannitol (BL: n=10; ATXII30: n=11; ATXII60: n=6; ATXII+mannitol: n=5; *p<0.05 relative to BL; #p<0.05 relative to ATXII30 and ATXII60; two-way ANOVA with mixed-effects model). **B.** Summary data for APD70 at CL=600 ms (*p<0.05 relative to BL; #p<0.05 relative to ATXII30 and ATXII60; one-way ANOVA with mixed-effects model).

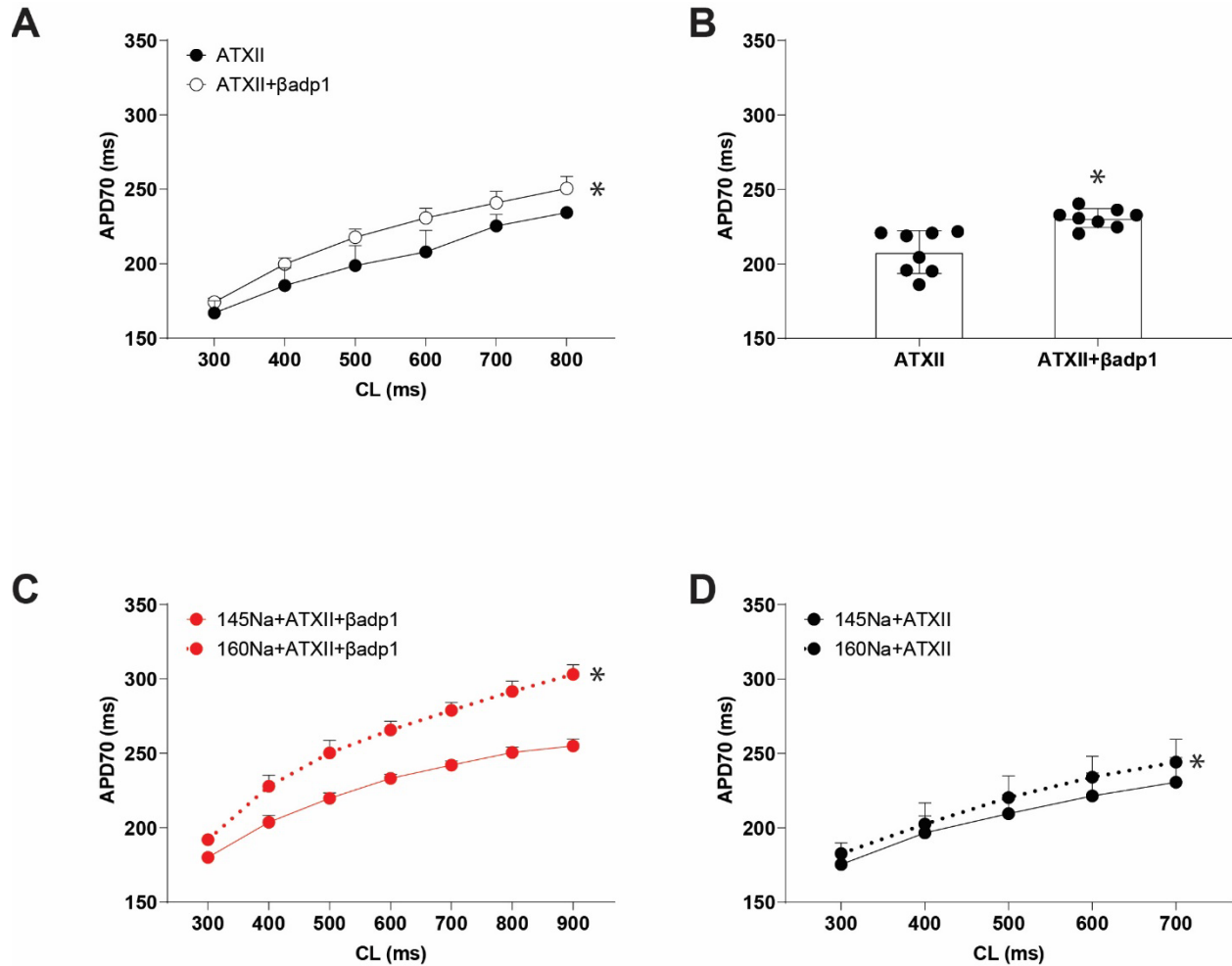


Figure S2: βadp1 and high sodium concentration individually or together prolong APD in the drug-induced LQT3 model. **A.** Action potential duration at 70% repolarization (APD70) as a function of cycle length (CL) for ATXII and ATXII+βadp1 (ATXII: n=8; ATXII+βadp1: n=9; * $p<0.05$ by two-way ANOVA with mixed-effects model). **B.** Summary data for APD70 at CL=600 ms (* $p<0.05$ by unpaired t-test). **C.** APD70 as a function of CL for 145Na+ATXII+βadp1 and 160Na+ATXII+βadp1 (145Na+ATXII+βadp1: n=5; 160Na+ATXII+βadp1: n=4; * $p<0.05$ by two-way ANOVA with mixed-effects model). **D.** APD70 as a function of CL for 145Na+ATXII and 160Na+ATXII (145Na+ATXII: n=4; 160Na+ATXII: n=4; * $p<0.05$ by two-way ANOVA with mixed-effects model).

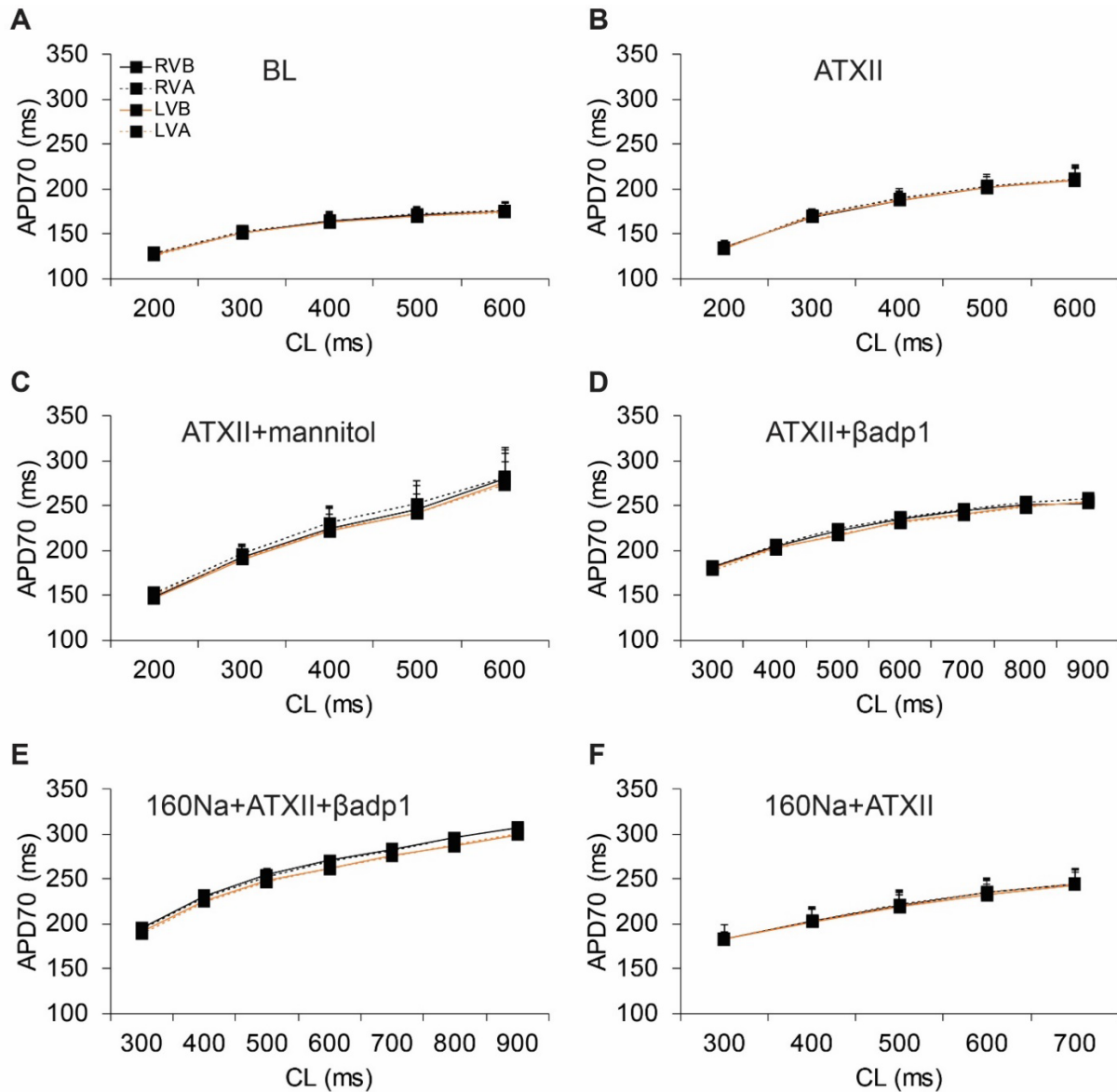


Figure S3: No region-dependent APD heterogeneity was observed in any conditions in the drug-induced LQT3 model. A-F: Action potential duration at 70% repolarization (APD70) in RVB, RVA, LVB and LVA as a function of cycle length (CL) at baseline (BL), ATXII, ATXII+mannitol, ATXII+ β adp1, 160Na+ATXII+ β adp1 and 160Na+ATXII, respectively. RVB, right ventricular base; RVA, right ventricular apex; LVB, left ventricular base; LVA, left ventricular apex. Two-way ANOVA with repeated measures.

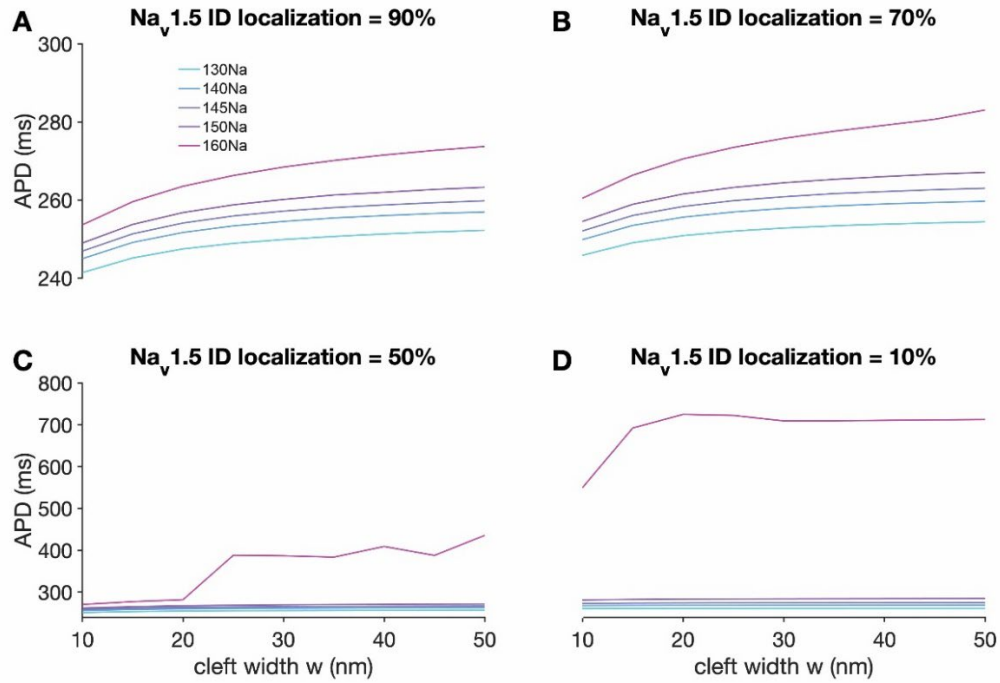


Figure S4: Preferential $\text{Na}_v1.5$ ID localization is critical for LQT3-associated APD prolongation. Action potential duration (APD) is shown as a function of intercellular cleft width (w) for different sodium concentrations and **A.** 90%, **B.** 70%, **C.** 50%, and **D.** 10% (uniform) $\text{Na}_v1.5$ localization at the intercalated disc (ID) in cardiac tissue with a LQT3-associated sodium channel gain-of-function mutation. Parameter: cycle length (CL) = 750 ms.

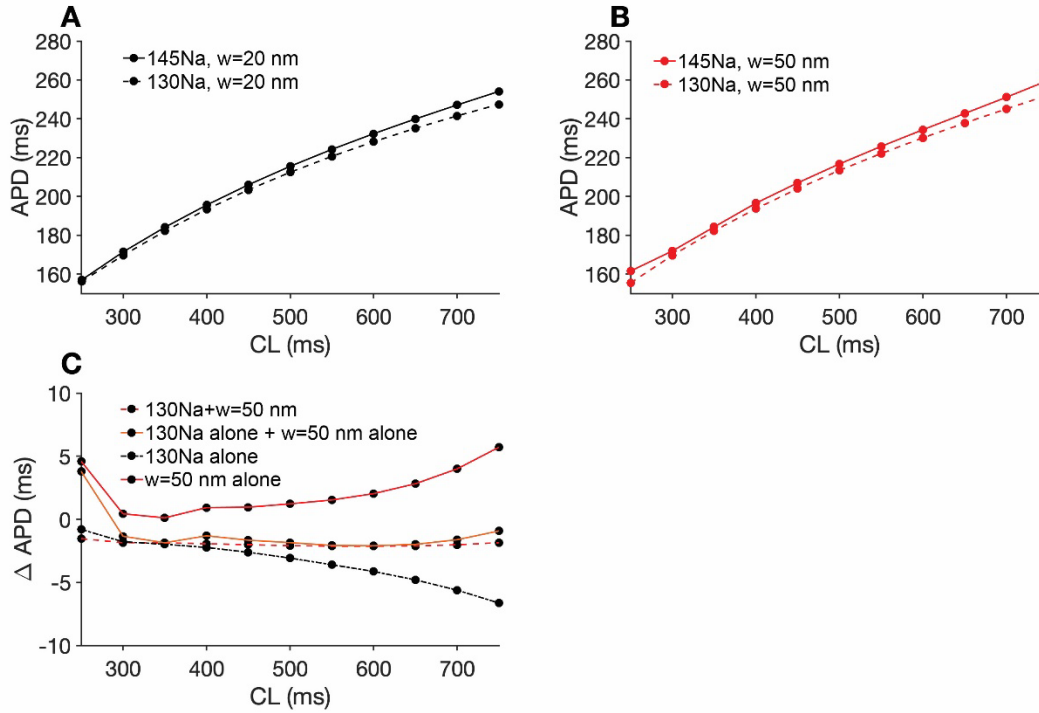


Figure S5. Simulations predict the synergistic effect of expanded intercellular cleft and reduced sodium concentration on APD shortening for long CL. **A.** APD as a function of CL for two different sodium concentrations (145 mM and 130 mM) and cleft width of 20 nm. **B.** APD as a function of CL for two different sodium concentration and cleft width of 50 nm. **C.** The change in APD due to expanded cleft width 50 nm (w=50 nm alone), reduced sodium concentration 130 mM (130Na alone), the summation of expanded cleft width and reduced sodium concentration (130Na alone + w=50 nm alone) and combination of expanded cleft width and reduced sodium concentration (130Na+w=50 nm) relative to cleft width of 20 nm and sodium concentration of 145 mM.

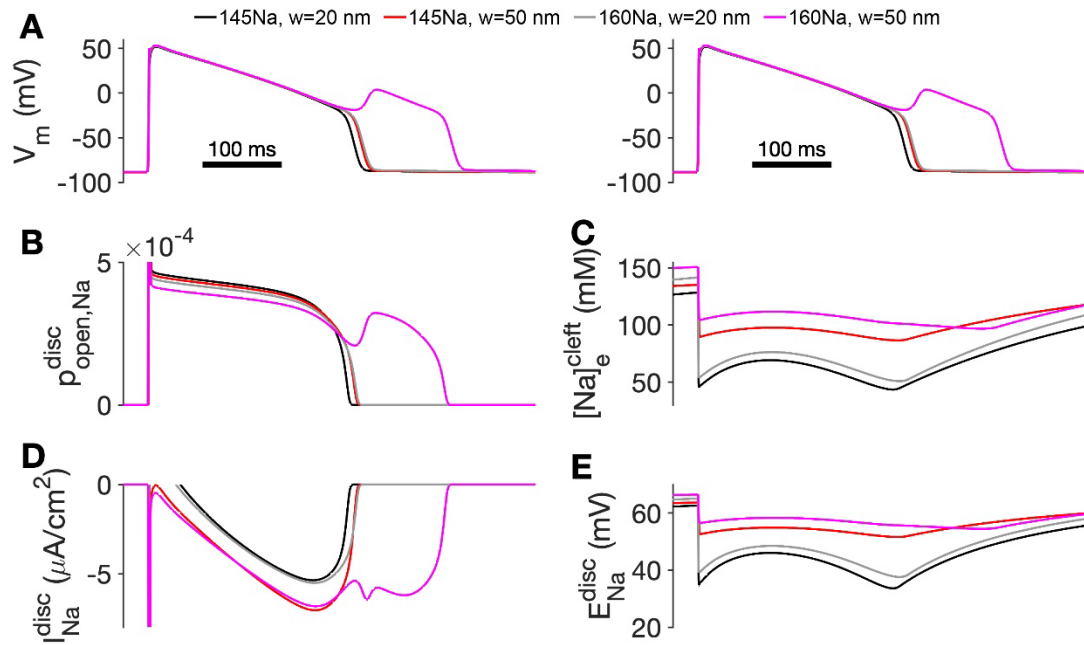


Figure S6. Combination of expanded intercellular cleft and elevated sodium concentration promote early afterdepolarizations by reducing cleft sodium depletion and maintaining I_{Na} driving force. **A.** Transmembrane potential (V_m), **B.** Sodium channel open probability, **C.** Intercellular cleft sodium concentration, **D.** Sodium current at the ID. **E.** ID sodium reversal potentials are shown as a function of time for different cleft widths (w) and sodium concentrations for an action potential elicited during pacing at $CL = 800$ ms.

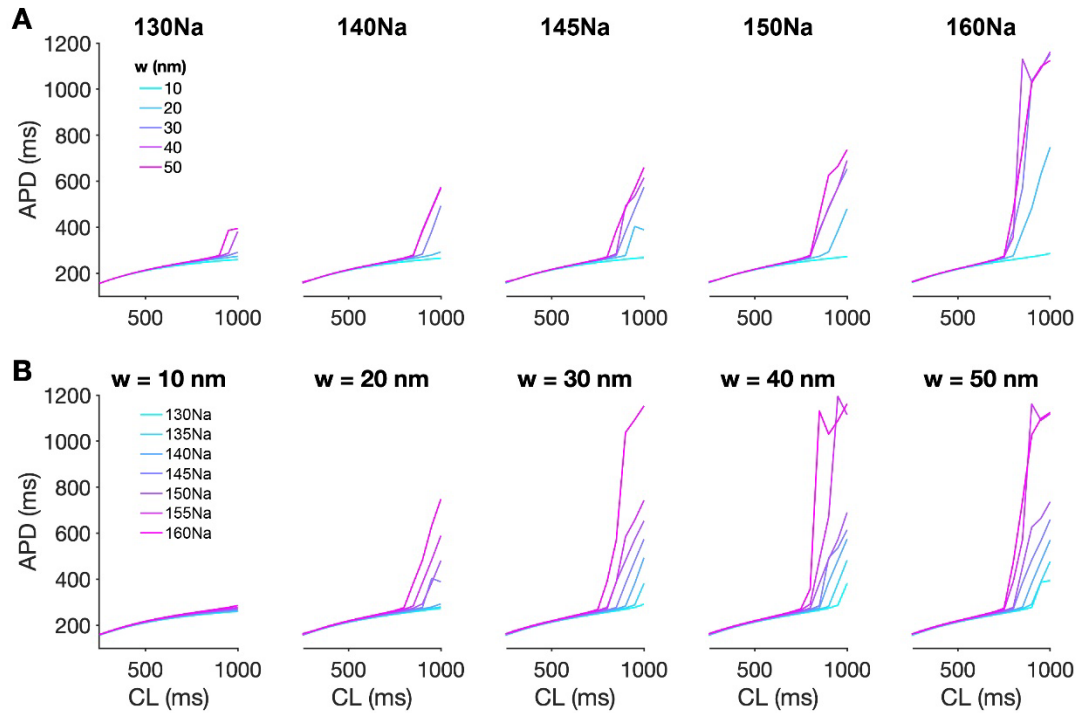


Figure S7. Intercellular cleft expansion and elevated sodium concentration separately prolong APD and promote early afterdepolarization (EADs) for long cycle lengths (CLs). APD is shown as a function of CL for different sodium concentrations and cleft widths (w) in simulations of tissue with LQT3-associated sodium channel gain-of-function. **A.** APD prolongation for increasing cleft width and variable sodium concentration. **B.** APD prolongation for elevated sodium concentration and variable cleft widths.

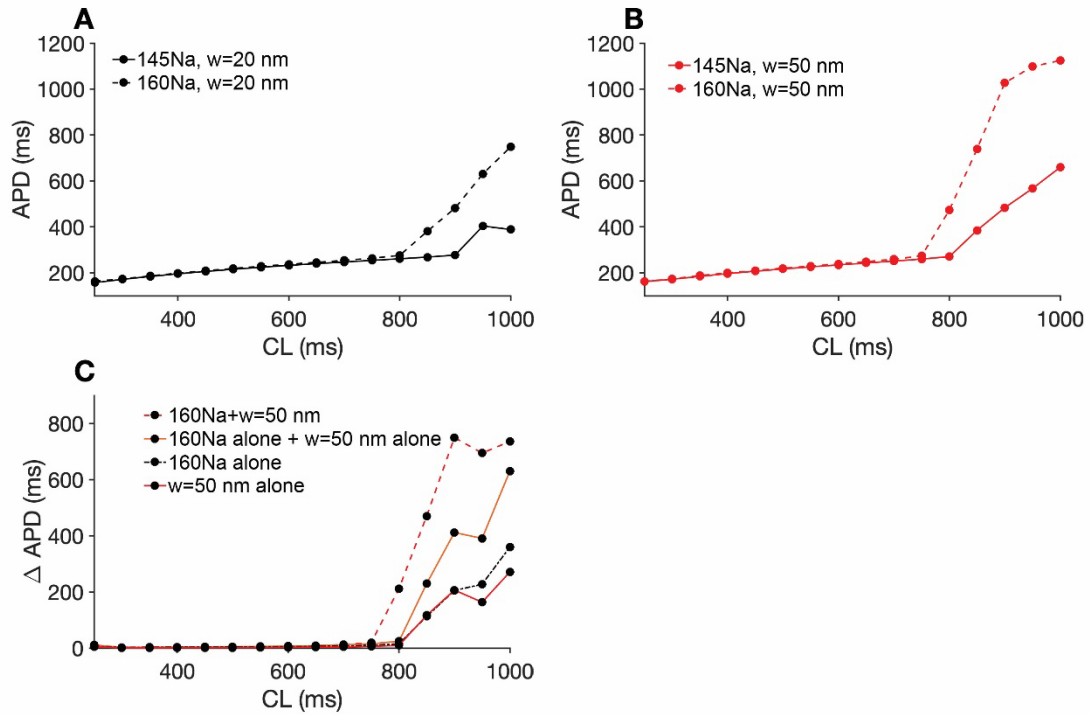


Figure S8. Simulations predict the synergistic effect of expanded intercellular cleft and elevated sodium concentration on early afterdepolarization (EAD) formation for long CL. **A.** APD as a function of CL for two different sodium concentrations (145 mM and 160 mM) and nominal cleft width of 20 nm. **B.** APD as a function of CL for two different sodium concentrations and a widened cleft width of 50 nm. **C.** The change in APD due to expanded cleft width 50 nm ($w=50$ nm alone), elevated sodium concentration 160 mM (160N alone), summation of expanded cleft width and elevated sodium concentration (160N alone + $w=50$ nm alone) and combination of expanded cleft width and elevated sodium concentration (160N+ $w=50$ nm) relative to cleft width of 20 nm and sodium concentration of 145 mM.

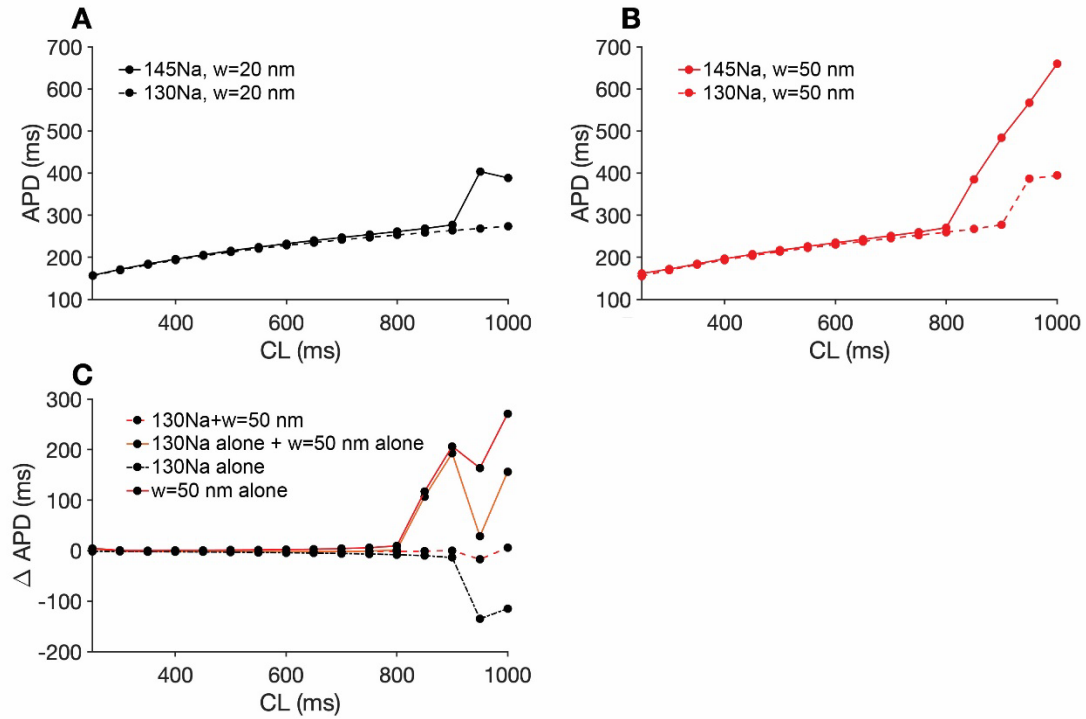


Figure S9. Simulations predict the synergistic effect of expanded intercellular cleft and reduced sodium concentration on APD shortening and early afterdepolarization (EAD) suppression for long CL. A. APD as a function of CL for two different sodium concentrations (145 mM and 130 mM) and nominal cleft width of 20 nm. **B.** APD as a function of CL for two different sodium concentrations and a widened cleft width of 50 nm. **C.** The change in APD due to expanded cleft width 50 nm (w=50 nm alone), reduced sodium concentration 130 mM (130N alone), summation of expanded cleft width and reduced sodium concentration (130N alone + w=50 nm alone) and combination of expanded cleft width and reduced sodium concentration (130N+w=50 nm) relative to cleft width of 20 nm and sodium concentration of 145 mM.

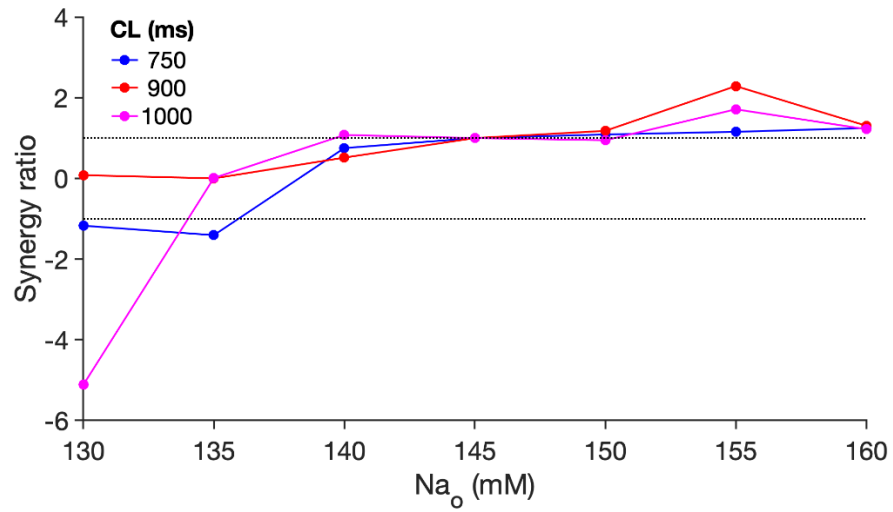


Figure S10. Simulations predict that the synergistic effect on APD prolongation and shortening depends on sodium concentration and cycle length (CL). For sodium concentrations above 145 mM, the synergy ratio greater than 1 indicates that APD is prolonged synergistically, and for sodium concentration below 140 mM, the synergy ratio less than -1 indicates that APD is shortened synergistically. These effects tend to be enhanced for longer CLs.

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Chapter 3: Extracellular sodium regulates transient outward potassium and late sodium currents in a mouse model of Long QT Syndrome Type 3

Running title: High sodium fails to exacerbate LQT3 phenotype in mice hearts

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Abstract

Background: Long QT Syndrome Type 3 (LQT3) is caused by cardiac voltage-gated sodium channel (Nav1.5) gain of function and characterized by action potential duration (APD) prolongation due to increased late sodium current (I_{NaL}). A guinea pig model of drug-induced LQT3 demonstrated that elevating sodium and widening the perinexus, an intercalated disc cleft adjacent to gap junctions, individually and synergistically exacerbate APD prolongation. However, previous studies have suggested that high sodium may increase transient outward potassium current (I_{to}) to shorten APD. Therefore, it remains unclear if this synergistic effect occurs in a genetically-modified LQT3 mouse heart model that functionally expresses I_{to} channels.

Purpose: To investigate individual and combined effects of high extracellular sodium and perinexal expansion in a mouse model of LQT3.

Methods: Ventricular action potential duration at 30 (APD30) and 90 (APD90) percent of repolarization was measured from optically mapped, Langendorff-perfused wild type (WT) and genetically-modified LQT3 (Δ KPQ) mice hearts. Hearts were perfused with sodium concentrations of 145 (145Na) or 160 mM (160Na), in the absence or presence of an intercalated disc adhesion inhibitor (β adp1) inducing perinexal widening. I_{to} from isolated mouse ventricular myocytes was measured through whole-cell patch clamping with 145Na and 160Na.

Results: High sodium (160Na) significantly decreased both APD30 and APD90 in WT but only APD30 in Δ KPQ hearts. Interestingly, 160Na did not change APD90 relative to 145Na suggesting that high sodium may also produce the shortening effect on APD in

mice hearts. β adp1 had no effect on APD30 and APD90 in WT hearts while decreased APD30 and increased APD90 in Δ KPQ hearts. Combination of 160Na and β adp1 did not produce the synergistic effect on APD in Δ KPQ hearts likely due to the shortening effect caused by 160Na alone. Importantly, the whole-cell patch clamping demonstrated that high sodium increased I_{to} in isolated mouse ventricular myocytes, which suggests that the shortening effect of high sodium on APD is caused by increased I_{to} .

Conclusions: elevated extracellular sodium doesn't prolong APD in LQT3 mice hearts due to increasing both I_{to} and I_{NaL} . Although perinexal widening still prolongs APD in LQT3 mouse hearts, combination with high sodium fails to synergistically exacerbate APD due to increased I_{to} .

Introduction

Cardiac voltage-gated sodium channels are very critical for maintaining normal electrical initiation and propagation in hearts.¹ Sodium channel protein type 5 subunit alpha, as known as Nav1.5, which is predominantly expressed in ventricles,² is involved in several congenital heart diseases including Sick Sinus Syndrome,³ Brugada Syndrome⁴ and Long QT Syndrome Type 3 (LQT3).⁵ As an exemplar, congenital LQT3 is caused by gain of function mutation of Nav1.5-associated gene, which leads to disruption of sodium channel inactivation and thereby increases late sodium current (I_{NaL}) to prolong action potential duration and QT interval. We have previously demonstrated in drug-induced LQT3 guinea pig models that APD could be exacerbated by widening the perinexus, which is adjacent to gap junctions in the intercalated disc.⁶⁻⁸ When the perinexus widens, sodium ion availability in it increases and the high density of Nav1.5⁹ in the perinexus allows the increase in sodium influx (I_{NaL}) during disrupted Nav1.5 inactivation and thereby to prolong APD. We also demonstrated that increased sodium concentration without perinexal widening also prolongs APD.⁸ Importantly, with perinexal widening, elevating sodium concentration could further greatly increase I_{NaL} and produce a synergistic effect on APD prolongation.⁸ Therefore, managing the sodium intake and preventing cardiac edema should benefit LQT3 patients.

However, high sodium may increase transient outward potassium current (I_{to}) to shorten APD. Voltage-gated potassium channels such as Kv4.2 and Kv4.3¹⁰ are responsible for fast early repolarization after Nav1.5 activation by generating I_{to} . Previous studies have suggested that high sodium may increase I_{to} with intracellular sodium ions leaving the

cells through I_{to} channels.¹¹⁻¹⁴ Since increased I_{to} shortens APD, it becomes unclear if high sodium prolongs APD in a LQT3 heart which functionally expresses I_{to} .

In the current study, we aim to investigate the individual and combined effects of extracellular sodium and perinexal widening on APD in a genetically-modified LQT3 (Δ KPQ) mouse model. We found that high sodium produced a shortening effect on APD in both WT and Δ KPQ hearts, which is inconsistent with the findings from the drug-induced LQT3 guinea pig hearts.⁸ Consistent with previous findings, widening the perinexus with sodium channel $\beta 1$ subunit de-adhesion peptide (β adp1) prolongs APD in Δ KPQ hearts. Furthermore, combination of high sodium and β adp1 did not produce synergistic effect on APD likely due to the shortening effect on APD caused by high sodium. Importantly, the whole-cell patch clamping showed that this shortening effect of high sodium on APD is caused by increased I_{to} . Furthermore, the association of APD with high sodium and perinexal widening will be investigated in a computational LQT3 mouse model and the results should also show the shortening effect of increased I_{to} on APD in the presence of increased sodium and perinexal widening. Therefore, we concluded that high sodium produced an opposite effect on APD by increasing I_{to} and I_{NaL} in Δ KPQ hearts and the combined effect of high sodium and perinexal widening on APD is mediated by increased I_{to} .

Methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). All animal study protocols were approved by the Institutional Animal Care and Use Committee at the Virginia Polytechnic Institute and State University.

Establishment of Langendorff-perfused heart model

Male and female wild-type (WT, SvEv) and Δ KPQ mice (23-28 weeks) were involved. All animals were anesthetized with isoflurane inhalation. After cervical dislocation, hearts were rapidly excised, cannulated (<5 minutes) and retrogradely perfused with a crystalloid perfusion solution, consisting of (in mM) 139.5 NaCl, 1.0 MgCl₂, 1.2 NaH₂PO₄, 4.0 KCl, 1.8 CaCl₂, 10 HEPES; 5.6 Glucose, and 5.5 NaOH (pH=7.4 measured at 36°C). A 145 mM sodium concentration (145Na) is a result of 139.5 mM NaCl and 5.5 mM NaOH, and a high-sodium concentration of 160 mM (160Na) is a result of 154.5 mM NaCl and 5.5 mM NaOH. Importantly, 160Na was chosen to model clinically relevant hypernatremia.¹⁵ Heart temperature was maintained at 36°C in a three-dimensional printed polylactic acid bath. Atria were removed in order to induce ventricular bradycardia and thereby pace the heart successfully and stably. The perfusion pressure was maintained at 60-80 mmHg by adjusting the flow rate of perfusion solution. Each heart was stabilized for 15 mins before the experiment started.

Optical mapping

The voltage-sensitive fluorophore, di-4-ANEPPS (15 μ M, Biotium, CA, USA), was perfused into the heart, and the heart position was visually adjusted to move the left anterior descending artery (LAD) in the center of the field of view so that the anterior epicardial surfaces of both ventricles were mapped during external pacing to record optical action potentials with a MiCAM HS02-CMOS camera (SciMedia: 92 x 80 pixels, field of view = 14.02 x 12.19 mm). The electromechanical uncoupler, blebbistatin (10 μ M, ApexBio Tech LLC, TX, USA), was added to the perfusate to reduce cardiac motion. A

sodium channel β 1-subunit de-adhesion peptide, β adp1^{8, 16}(1 μ M, LifeTein LLC, NJ, USA), was perfused to induce perinexal expansion.

In order to investigate the epicardial action potential duration (APD) under same conditions in WT and Δ KPQ hearts, each heart was paced with a unipolar electrode that was placed in the septum near the atrioventricular node to stimulate the electrical propagation through the conduction system and the reference electrode was placed in the bath. The final stimulation current was set at 1.5X the minimum stimulation threshold which was tested with a cycle length (CL) of 150 ms (pulse width = 2ms). Each heart was perfused with different solutions in order. To investigate the effect of high sodium concentration on APD, each heart was perfused with 145Na and then 160Na. To investigate the effect of high sodium and β adp1 on APD, each heart was perfused with 145Na, 145Na+ β adp1 and then 160Na+ β adp1. The perfusion duration of each test solution was 30 mins. Optical signals were recorded during steady-state pacing at CL of 150 ms. For analysis, APD of one optical action potential in each of 1840 pixels was calculated after 2x2 spatial binning by using a custom MatLab program. The average APD across the array was considered a single data point for a heart. APD was measured as the difference between the time of the maximum upstroke velocity (i.e. activation time, AT) and 30% repolarization time (APD30) or 90% repolarization time (APD90) of the optical action potential (Figure 1A). APD30 and APD90 were only reported when both adult and aged hearts were successfully paced.

Patch clamp

Single cell isolation

Myocytes were enzymatically isolated from the left ventricles of adult mouse hearts in Langendorff-perfusion at 37 °C with collagenase II. The perfusion buffer for Langendorff-perfused adult mouse hearts consists of (in mM): 120.3 NaCl, 14.75 KCl, 0.6 KH₂PO₄, 0.6 Na₂HPO₄, 1.2MgSO₄, 10 HEPES, 4.64 NaHCO₃, 30 Taurine, 9.9 2,3-Butanedione 2-monoxime (BDM). The solution pH was 7.4 adjusted with NaOH. The digestion buffer was made with 135-mg collagenase II (Worthington-biochem, USA) in 60-mL perfusion buffer. Stop buffer was made with 6-mL fetal bovine serum in 54-mL perfusion buffer. A 100-mM CaCl₂ stock solution was prepared and then 10- μ L, 40- μ L and 90- μ L CaCl₂ were added into three 10-mL stop buffers (CaCl₂ 100, 400 and 900 μ M). Setup of Langendorff-perfused mouse heart has been described in last section. The digestion buffer perfused the heart for 8 mins after cannulation. Then 3- μ L CaCl₂ stock solution was added in the digestion buffer and then continue for another 8 mins. The digested heart was then placed in a plate and gently teared into about 20 pieces. A wide mouth transfer pipette was used to mechanically dissolve the pieces into cells for 2 mins. After 2 mins, add 2-mL stop buffer without CaCl₂ and mechanically dissolve the pieces for 2 more mins. Transfer the cell suspension to a 50-mL conical tube and then use 5-mL stop buffer to wash the plate and add to the conical tube. Spin the conical tube for 3 mins with a centrifuge with 30g at room temperature. Remove supernatant and add stop buffer with 100- μ M CaCl₂, and then spin the tube for 3 mins. Repeat last step with stop buffer with 400- μ M and then 900- μ M CaCl₂. After removing supernatant, then resuspend cells in 7.5 mL cardiomyocyte media. Change the media in two hours. Then cells are ready for experiments.

Patch clamp protocol

External solution consists of (in mM): 1.0 CaCl₂, 5.5 Glucose, 10 HEPES, 5.4 KCl, 1.0 MgCl₂, 145 or 160 NaCl, and 0.33 Na₂HPO₄. The pH was 7.4 adjusted with NaOH. Internal solution consists of (in mM): 1.0 CaCl₂, 10 EGTA, 5 HEPES, 110 K-Aspartate, 20 KCl, 1.0 MgCl₂, 8 NaCl and 5 MgATP. The pH was 7.2 adjusted with KOH. Myocytes were patch-clamped using the whole-cell configuration in either current-clamp (for AP recording) or voltage (or AP)-clamp mode (for I_{to} recording). For action potential recording, 1-3 ms stimulus current (0.4-0.7 nA) was delivered. For I_{to} recording, 3-mM 4-aminopyridine (4-AP) was used to confirm I_{to} current. To measure I_{to} , hold the potential at -80mV for 10 seconds before a 15-ms prepulse to -45 mV in order to activate and inactivate sodium channels to minimize the contamination of I_{to} .¹⁷ After 15-ms with -45 mV, step the potentials between -80 mV and +60 mV with an increment of 10 mV for 500ms.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA). The results are presented as mean $\pm$ standard deviation. Comparisons of APD30 and APD90 between WT and Δ KPQ (Figure 1) was analyzed with unpaired Student's *t*-test. The individual effects of high sodium and β adp1 on APD in same genotypes were investigated with paired Student's *t*-test (Figures 2, 3 and 5). The individual and combined effects of high sodium and β adp1 were compared with one-way ANOVA followed by Bonferroni correction for multiple comparisons (Figure 4). One-sample *t*-test was used to determine the individual and combined effects relative to 0 (Figure 4). A $p < 0.05$ was considered statistically significant.

Results

Epicardial action potentials from anterior left and right ventricles were optically mapped in Langendorff-perfused WT and Δ KPQ mice hearts. Representative action potential tracings and APD90 contours from WT and Δ KPQ hearts were shown in Figure 1A and 1B, respectively. Overall, comparison of APD30 and APD90 from all WT and Δ KPQ hearts at baseline (sodium concentration is 145 mM) demonstrated that Δ KPQ hearts manifest with the phenotype of prolonged APD (Figure 1C).

Increased extracellular sodium prolongs APD in drug-induced LQT3 guinea pig hearts due to enhancing I_{NaL} .⁸ In the current study, we replicated the effect of high sodium on APD in mice hearts. Surprisingly, 160-mM sodium (160Na) decreased APD30 in both WT and Δ KPQ hearts relative to 145-mM sodium (145Na). 160Na also significantly decreased APD90 in WT hearts suggesting that high sodium shortened APD in WT hearts. By contrast, high sodium did not significantly change APD90 in Δ KPQ hearts. Overall, the different effects of high sodium on APD between drug-induced LQT3 guinea pig and Δ KPQ mouse hearts suggest that the related mechanism in both animal models may be different.

We have previously demonstrated that widening the perinexus prolongs APD in the drug-induced LQT3 model.^{6, 8} The putative mechanism states that widening the perinexus accommodates the amount of sodium ions and thereby increases I_{NaL} to prolong APD. Importantly, the synergistic effect on APD suggests that the amount of sodium ions greatly increases in the widened perinexus when the sodium concentration increases, therefore I_{NaL} is greatly enhanced to exacerbate APD prolongation. Therefore, the amount of sodium ions can be increased either by increasing sodium concentration or widening the

perinexus or both. In order to investigate if widening the perinexus could produce similar effect on APD in Δ KPQ hearts, β adp1 was used to widen the perinexi in both WT and Δ KPQ hearts. We found that β adp1 did not change APD30 and APD90 in WT hearts, which is consistent with previous findings.^{8, 16} By contrast, β adp1 decreased APD30 while increased APD90 in Δ KPQ hearts. The unexpected effect of β adp1 on APD30 need to be further investigated. Overall, β adp1 prolonged APD in Δ KPQ hearts as we expected.

We have demonstrated that high sodium and β adp1 could individually and synergistically prolong APD the drug-induced LQT3 model.⁸ However, the current study showed that high sodium has no effect on APD90 and β adp1 prolonged APD in Δ KPQ hearts. Therefore, the synergistic effect of high sodium and β adp1 on APD is uncertain. In order to investigate if the synergistic effect still exists, WT and Δ KPQ hearts were perfused with solution of $^{160}\text{Na}+\beta$ adp1 and then changes in APD30 (Δ APD30) and APD90 (Δ APD90) due to ^{160}Na alone (Δ Na), β adp1 alone ($\Delta\beta$ adp1) and $^{160}\text{Na}+\beta$ adp1 ($\Delta(\text{Na}+\beta$ adp1)) relative to ^{145}Na were compared (Figure 4). $^{160}\text{Na}+\beta$ adp1 did not significantly change Δ APD30 and Δ APD90 relative to ^{160}Na alone or β adp1 alone in both WT and Δ KPQ hearts suggesting the combination of high sodium and β adp1 has no synergistic effect on APD. Furthermore, $^{160}\text{Na}+\beta$ adp1 marginally ($p=0.056$) increased APD90 in Δ KPQ hearts probably due to decreased APD30.

The different effects of high sodium and β adp1 on APD observed in the current study suggests that high sodium may affect APD in another way in addition to enhancing I_{NaL} . Considering that transient outward potassium channels are functionally expressed in mice hearts, not guinea pig hearts, we hypothesized that high sodium increases transient outward potassium current (I_{to}) to shorten APD. I_{to} channels are responsible for the early

repolarization right after sodium channel activation. Increasing I_{to} shortens APD. Importantly, previous studies have suggested that high sodium may increase I_{to} .¹¹⁻¹⁴ Therefore, in order to investigate if high sodium increases I_{to} , isolated cardiomyocytes from a ΔKPQ heart was used to measure I_{to} under sodium concentrations of 145mM and 160mM. I-V curves of I_{to} under 145Na and 160Na was shown in Figure 5 (left). 160Na moved the I-V curve upward suggesting that high sodium increases I_{to} . Furthermore, the I_{to} at 60mV was significantly higher with 160Na than 145Na (Figure 5 – right). Overall, we demonstrated that high sodium increased I_{to} in the isolated mouse cardiomyocytes.

We proposed a mechanism to interpret the effects of high sodium and $\beta adp1$ on APD in WT and ΔKPQ hearts (Figure 6). In WT hearts, high sodium increases I_{to} to decrease APD30 and APD90 and thereby shortens APD. Since $\beta adp1$ has no effect on APD30 and APD90, high sodium with $\beta adp1$ should not further change APD. By contrast, in ΔKPQ hearts, high sodium increases both I_{to} and I_{NaL} . But the increase in I_{to} and I_{NaL} produces different effects on APD. The increase in I_{to} shortens APD while the increase in I_{NaL} prolongs APD. Therefore, the overall effect of high sodium on APD depends on the counterbalance of I_{to} and I_{NaL} . Since $\beta adp1$ increases I_{NaL} , addition of $\beta adp1$ in the presence of high sodium weights I_{NaL} and thereby may increase APD90. Notably, the $\beta adp1$ dose (1 μM) is very small in the current study, so the overall effect of high sodium and $\beta adp1$ on APD prolongation is not obvious.

Discussion

We have previously demonstrated that high extracellular sodium with and without perinexal widening prolongs action potential duration (APD) in drug-induced LQT3 guinea pig hearts⁸ which don't functionally express transient outward potassium channels.¹⁸ By

contrast, in the current study, we found that high extracellular sodium produced the shortening effect on action potential duration (APD) in both wild-type (WT) and Δ KPQ mice hearts due to increased transient outward potassium current (I_{to}). The synergistic effect of high sodium and β adp1 found in LQT3 guinea pig hearts doesn't exist in Δ KPQ mice hearts, which is likely caused by increased I_{to} due to high sodium. This opposite effect caused by high sodium observed in guinea pig and mouse LQT3 hearts suggests that the effect of extracellular sodium on LQT3 phenotype can be mediated by I_{to} .

To our knowledge this was the first time to provide direct evidence that high sodium increases I_{to} in isolated mouse cardiomyocytes. However, it remains unclear in which pathway that sodium impacts I_{to} . Previous studies involving other animal models have suggested that intracellular sodium ions may go through I_{to} channels to contribute to I_{to} .¹¹⁻¹⁴ Notably, high extracellular sodium can lead to the increase in the intracellular sodium by enhancing I_{NaL} and sodium-calcium exchanger (NCX) forward mode.¹⁹ More importantly, it has been shown that increased intracellular sodium leads to APD shortening in canine cardiomyocytes,²⁰ which supports that high sodium increases I_{to} . Furthermore, another possible mechanism we need to discuss is the interaction between calcium/calmodulin-dependent protein kinase II (CaMKII) and I_{to} channels. High extracellular sodium could indirectly decrease CaMKII level through reducing intracellular calcium due to enhanced NCX forward mode. However, studies have demonstrated that inhibition of CaMKII decreases I_{to} peak current and facilitates I_{to} inactivation.^{21, 22} Therefore, even if high sodium negatively affects I_{to} through CaMKII-associated pathway, the main effect is still the increase in I_{to} .

We also demonstrated that widening the perinexus with β adp1 didn't change APD in WT while increased APD90 in Δ KPQ hearts, which are consistent with the findings in previous studies.^{8, 16} Interestingly, we found that β adp1 decreased APD30 in Δ KPQ hearts, which is probably caused by increased I_{to} due to β adp1 increasing I_{NaL} . Although APD30 decreases with β adp1, the main effect of β adp1 on APD remains prolongation in LQT3. However, this prolonging effect cannot be exacerbated by high sodium even if high sodium increases I_{NaL} , suggesting the increasing effect of high sodium on I_{to} .

Previously reported synergistic effect of high sodium and β adp1 on LQT3 phenotype suggests that LQT3 patients should carefully manage sodium intake while the finding in the current study seems not to support this. In order to address this paradox, we have to understand the electrophysiological differences between mouse and other animals. First, mouse cardiomyocytes have overexpressed I_{to} channels relative to other animals.²³ Therefore, I_{to} is primarily responsible for shaping action potential in the mouse cardiomyocyte. Second, there is no action potential plateau in mouse cardiomyocytes relative to other animals such as guinea pig, rabbit and human. Action potential plateau is very critical for maintaining I_{NaL} during repolarization to prolong APD. Normally, Nav1.5 activation could rapidly deplete sodium ions in narrow perinexi, which is called as self-attenuation.⁶ This self-attenuation could limit I_{NaL} and thereby to mediate APD. However, longer action potential plateau could allow sodium ions continuously refill the perinexus to increase sodium ion availability to attenuate this self-attenuation and then increase I_{NaL} to prolong APD. Therefore, the unique differences in cardiac electrophysiology between mouse and other animal hearts suggest that selection of animal LQT3 model is very

critical for translational purpose especially in studies associated with different sodium concentrations.

Limitations

There are several limitations in the current study. First, measurement of perinexal width was not involved. However, the findings that β adp1 has no effect on APD in WT while prolonged APD in Δ KPQ mice hearts suggest that β adp1 should widen the perinexus. Second, we did not investigate the mechanism that high sodium increases I_{to} in mice hearts. Third, we did not further investigate if other ionic channels such as L-type calcium channels and potassium channels are involved in the regulation of high sodium on APD. Last, we did not address if the unique electrophysiological properties in mouse cardiomyocytes is responsible for the shortening effect of high sodium on LQT3 phenotype. Therefore, a different animal model which functionally expresses I_{to} channel and has action potential plateau such as the rabbit should be used.

Conclusions

High sodium produced the shortening effect on APD in both WT and KPQ mice hearts. This shortening effect is caused by increased I_{to} due to high sodium. Widening the perinexus still prolongs APD in LQT3 mice hearts, which is consistent with previous findings. However, combination of high sodium and perinexal widening fails to exacerbate APD prolongation in LQT3 mice hearts due to increased I_{to} . Therefore, the effect of high sodium on I_{to} must be considered in LQT3 studies involving different sodium concentrations.

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Disclosures

None.

Figures

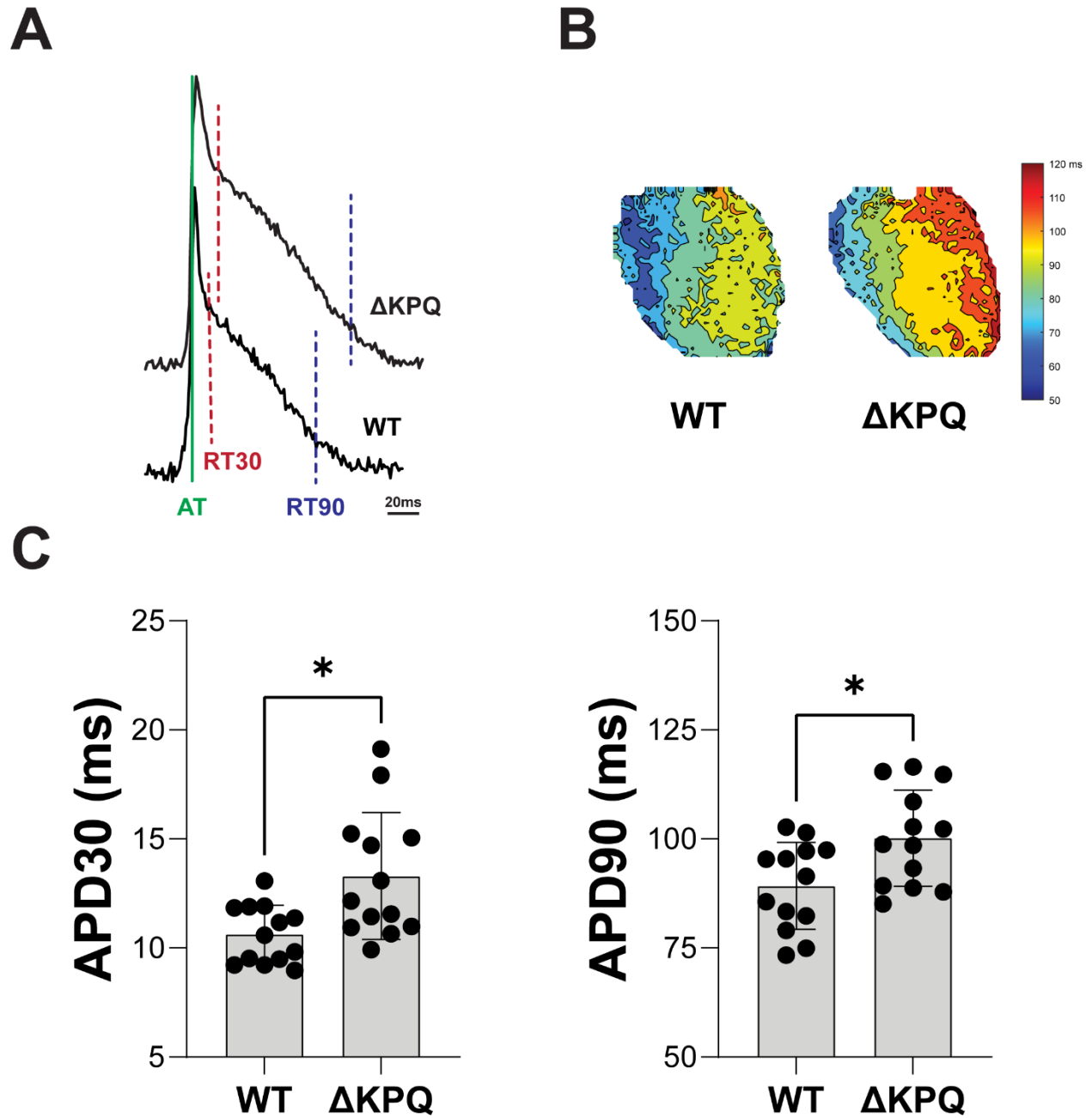


Figure 1. The phenotype of prolonged APD is manifested in ΔKPQ mice hearts. A.

Ventricular action potential duration (APD) was quantified with APD30 (the time difference between AT and 30% repolarization time, i.e. RT30) and APD90 (the time difference between AT and 90% repolarization time, i.e. RT90) in WT and ΔKPQ hearts. **B.**

Representative ventricular APD90 contours from a WT and a Δ KPQ heart, respectively.

C. APD30 and APD90 were significantly greater in Δ KPQ (n=13) than WT hearts (n=13).

*p<0.05 (unpaired Student's *t* test). AT, activation time; RT30 and RT90, 30% and 90% repolarization time.

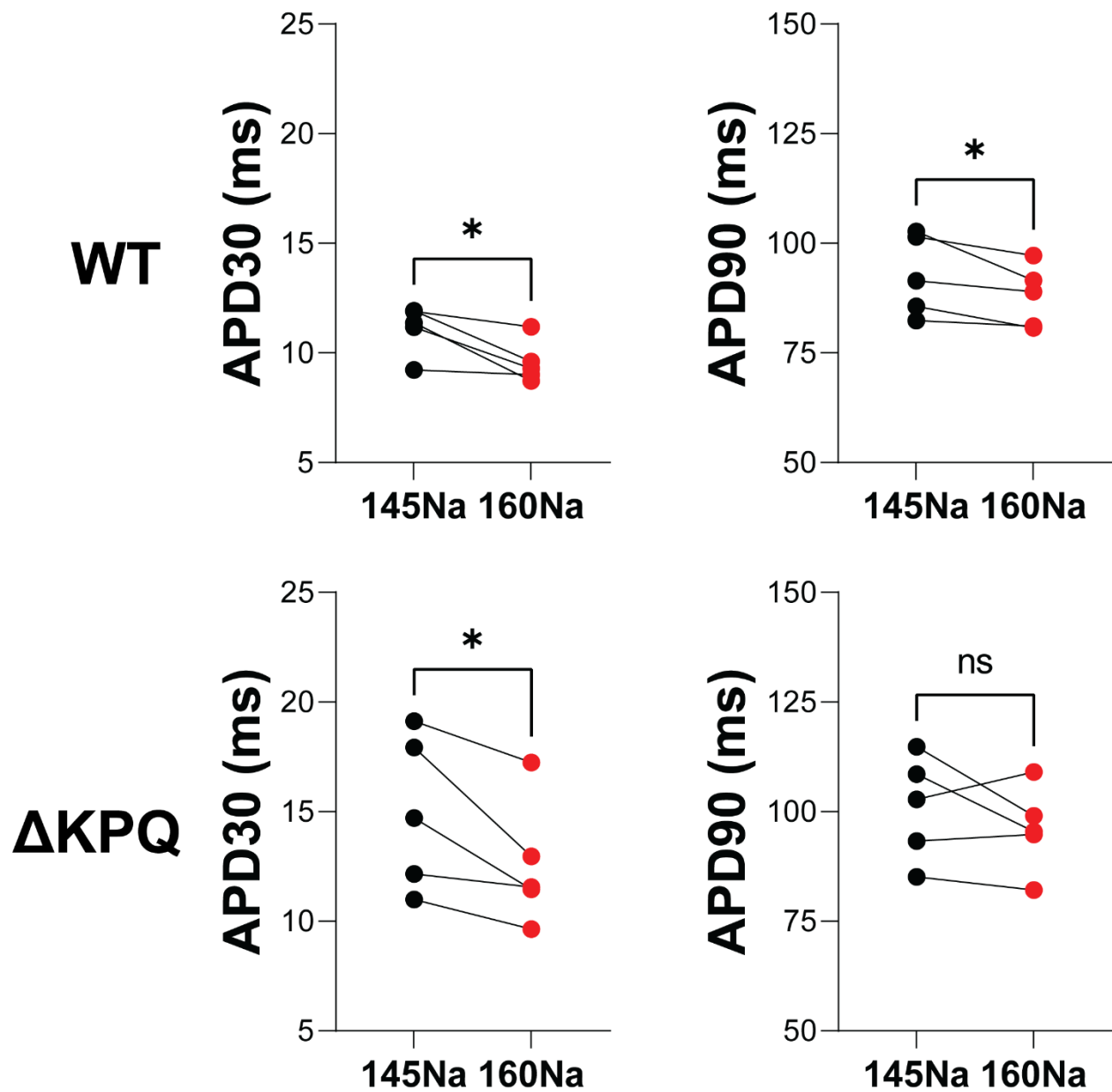
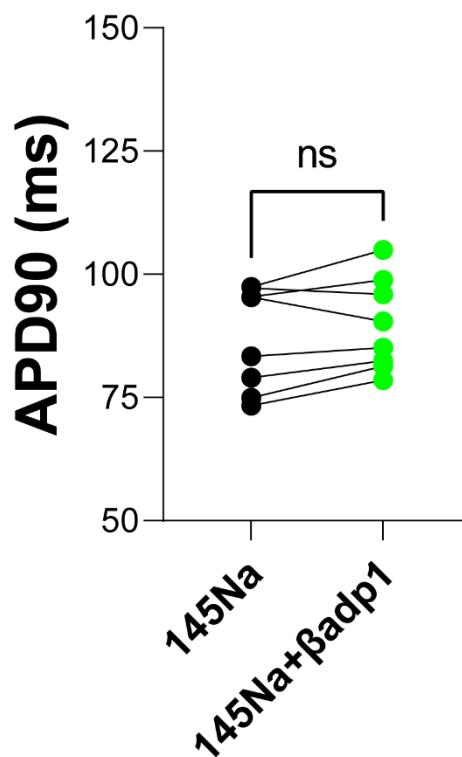
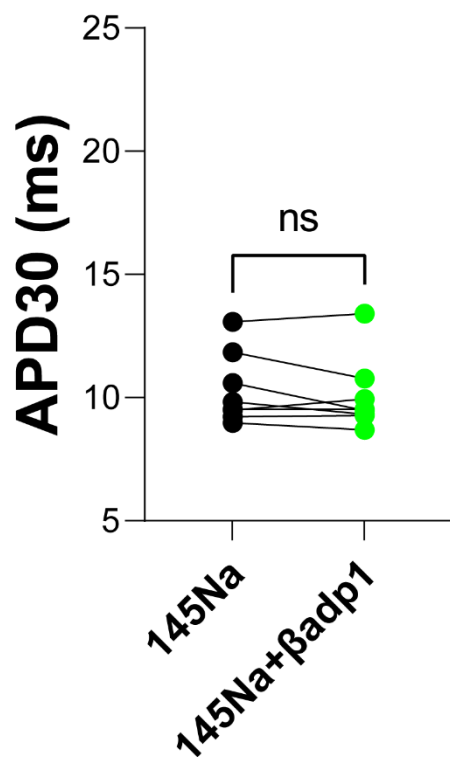


Figure 2. Increased extracellular sodium shortens APD differently in WT and Δ KPQ mice hearts. **Top:** High sodium significantly decreased APD30 and APD90 in WT hearts (n=5). **Bottom:** High sodium significantly decreased APD30 but not APD90 in Δ KPQ hearts (n=5). 145Na and 160Na, 145-mM and 160-mM sodium concentrations respectively. * $p < 0.05$ (paired Student's *t* test). ns, not significant.

WT



ΔKPQ

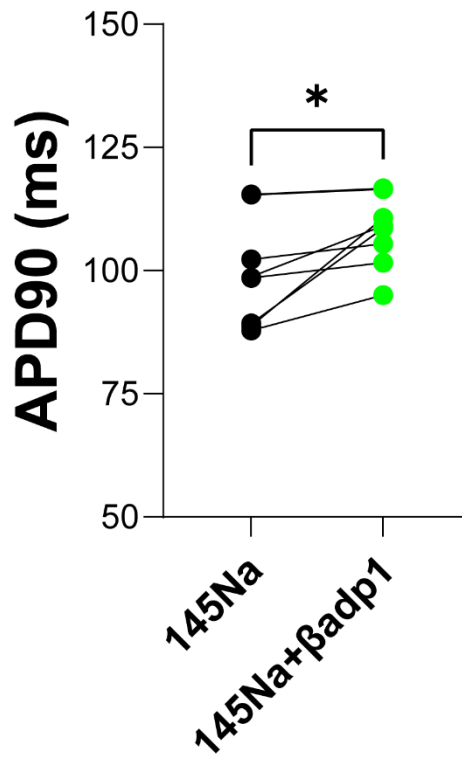
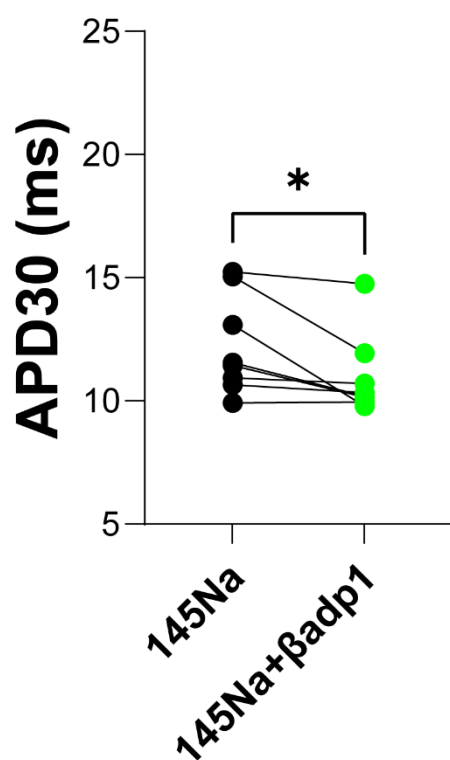
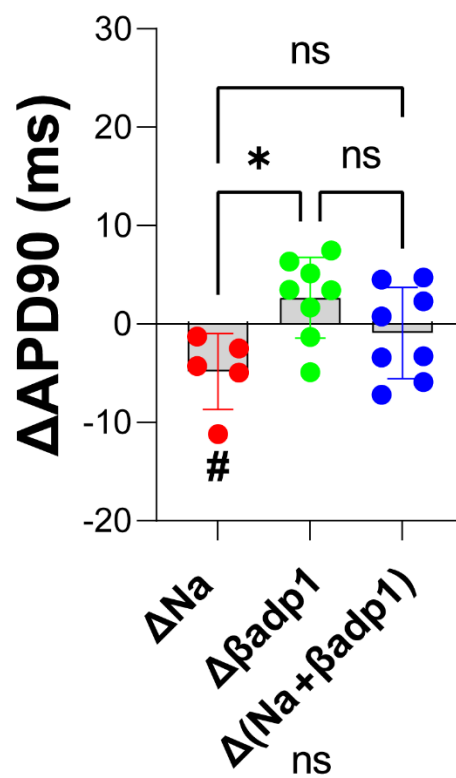
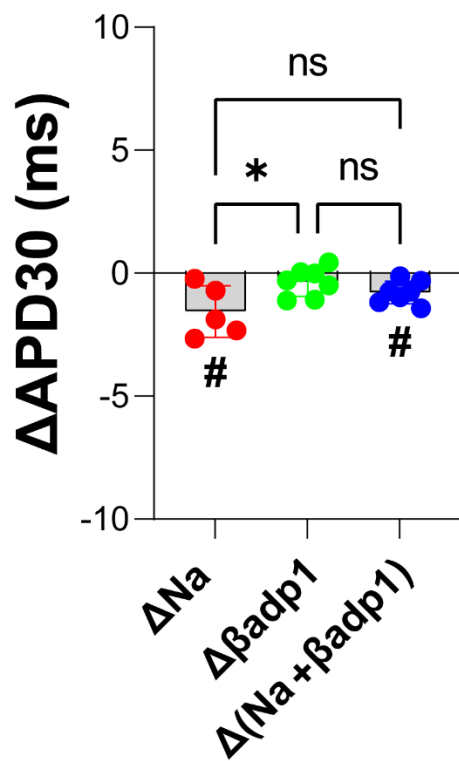


Figure 3. Application of β adp1 changes APD in Δ KPQ but not WT mice hearts. Top: β adp1 had no effect on APD30 and APD90 in WT hearts (n=8). **Bottom:** β adp1 decreased APD30 and increased APD90 significantly in Δ KPQ hearts (n=8). *p<0.05 (paired Student's *t* test). ns, not significant.

WT



ΔKRPQ

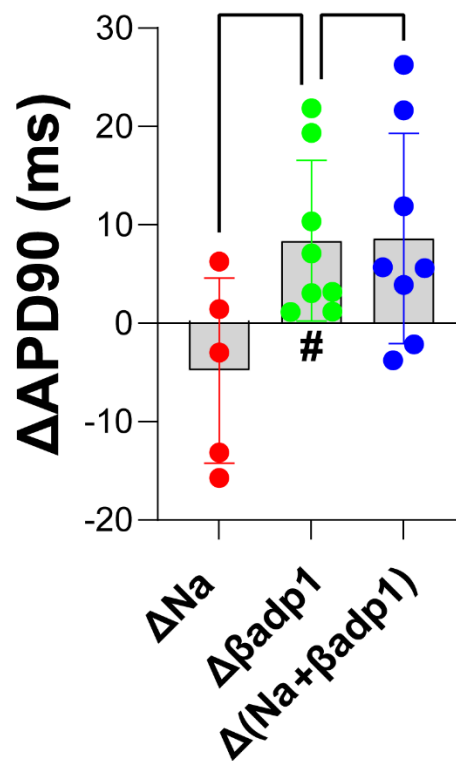
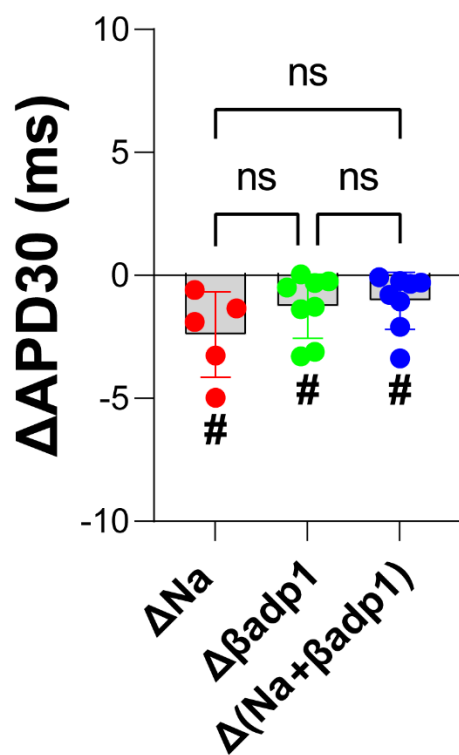


Figure 4. Combination of high sodium and β adp1 has no synergistic effect on APD in Δ KPQ mice hearts. **Top:** combination of high sodium and β adp1 (n=7) decreased APD30, not APD90 in WT hearts. **Bottom:** combination of high sodium and β adp1 (n=8) decreased APD30 and marginally increased APD90 in Δ KPQ mice hearts. *p<0.05 (One-way ANOVA corrected with Bonferroni). #p<0.05 (One sample *t* test). Δ Na, ^{160}Na relative to ^{145}Na ; $\Delta\beta$ adp1, $^{145}\text{Na}+\beta$ adp1 relative to ^{145}Na ; $\Delta(\text{Na}+\beta$ adp1), $^{160}\text{Na}+\beta$ adp1 relative to ^{145}Na .

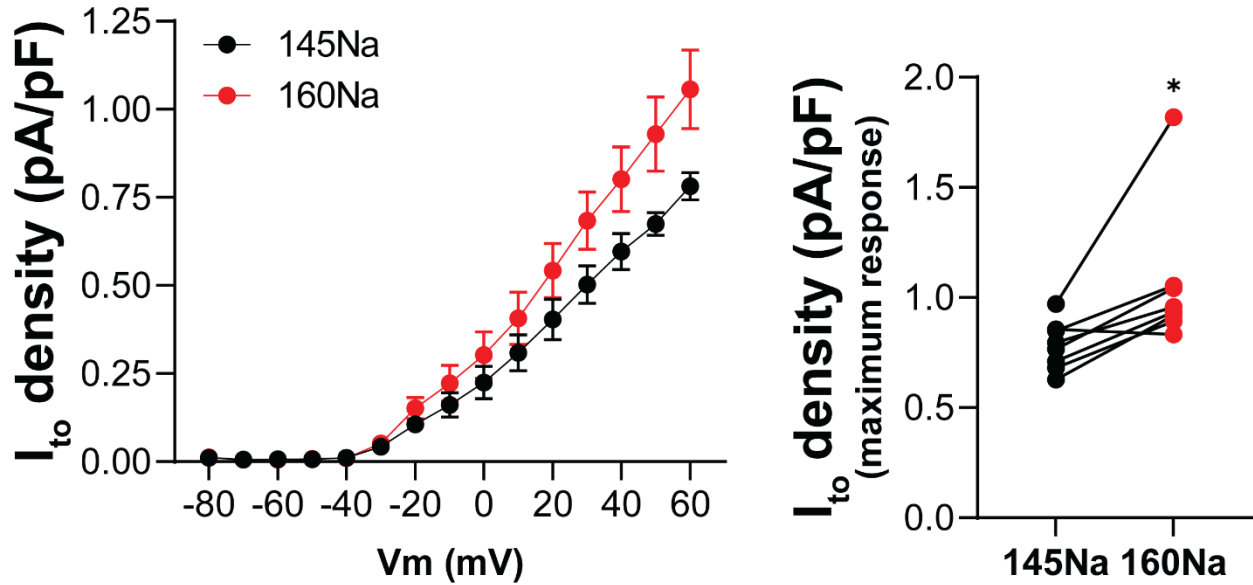


Figure 5. High sodium increases the transient outward potassium current (I_{to}). **Left:** the I-V curves of I_{to} in the conditions of different sodium concentrations from isolated ventricular cardiomyocytes ($n=8$) of a ΔKPQ heart. **Right:** I_{to} at 60mV was significantly increased by high sodium. * $p<0.05$ (paired Student's t test).

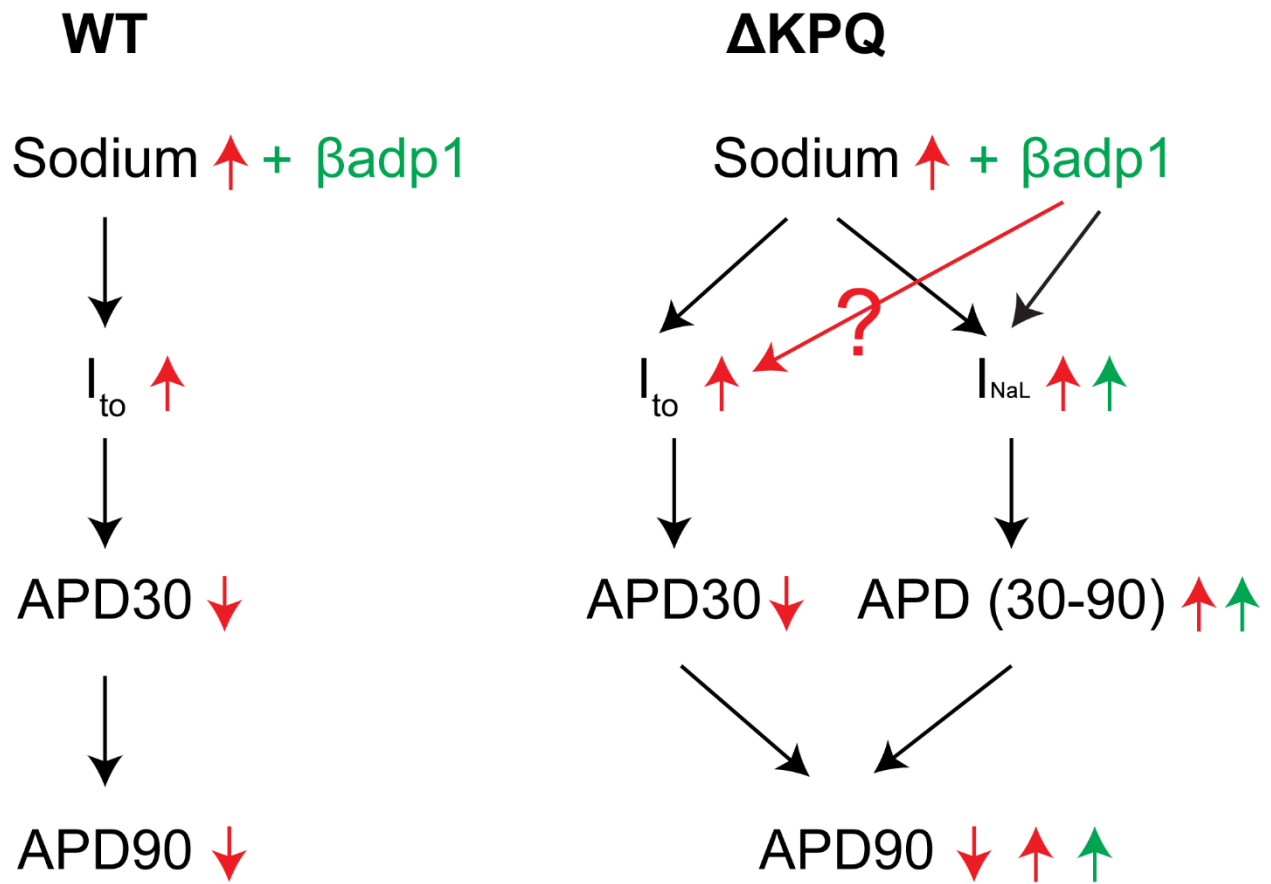


Figure 6. The proposed mechanism of the individual and combined effects of high sodium and β adp1 on APD in WT and Δ KPQ hearts.

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Chapter 4: Age-associated perinexal narrowing masks Long QT Syndrome Type 3 phenotype

Running title: Perinexus masks Nav1.5 gain of function during aging

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Abstract

Background: The Long-QT Syndrome Type 3 (LQT3) is characterized by action potential duration (APD) prolongation due to cardiac voltage-gated sodium channel ($\text{Na}_v1.5$) gain of function. Arrhythmia risk increases with age in LQT3 patients, but it is unknown whether this is due to progressive APD prolongation. Recent studies have shown that the LQT3 phenotype can be exacerbated by widening the perinexus, an intercalated disc nanodomain adjacent to gap junctions. However, it remains unclear if the perinexus mediates the LQT3 phenotype during aging.

Objective: To investigate the role of the perinexus during aging in LQT3.

Methods: Ventricular epicardial APD was obtained by optically mapping Langendorff-perfused adult (3-month) and aged (15-month) guinea pig hearts. LQT3 phenotype was induced with sea anemone venom (ATXII), and the perinexus was widened with an intercalated disc adhesion inhibitor (βadp1). Perinexal width (W_p) was quantified by transmission electron microscopy. Susceptibility to arrhythmia was assessed with burst pacing in the absence and presence of βadp1 and evaluated from electrocardiograms (ECG). A computational model simulating LQT3-associated mutation was conducted to investigate the effects of aging and perinexal separation on LQT3 phenotype.

Results: APD was not different between adult and aged hearts at baseline, with ATXII, or ATXII+ βadp1 . W_p was significantly wider in adult LQT3 hearts either with or without βadp1 relative to aged hearts. These data suggest the perinexus narrows with aging and attenuates previously reported cellular APD prolongation such that there is no difference in whole-heart, epicardial APD between adult and aged LQT3 hearts. However, ECG

$T_{\text{peak}}-T_{\text{end}}$ interval was not prolonged in adult, but significantly prolonged in aged hearts after treatment with ATXII+ βadp1 , suggesting that βadp1 increases transmural dispersion of repolarization during aging in LQT3. Furthermore, ATXII+ βadp1 did not induce arrhythmias in adult but was arrhythmogenic in aged hearts. Simulations were consistent with experiments and illustrated changes underlying APD prolongation.

Conclusions: Perinexal narrowing associated with aging attenuates APD prolongation and reduces arrhythmic risk in aged LQT3 hearts. Loss of perinexal adhesion in aged LQT3 hearts results in significant APD prolongation and increased risk of arrhythmia.

Introduction

The cardiac voltage-gated sodium channel subunit, Nav1.5, supports electrical propagation of action potentials in the heart.⁶⁸ Gain-of-function (GOF) mutations in SCN5A encoding Nav1.5 disrupt sodium channel inactivation, which results in a small persistent sodium influx known as late sodium current (I_{NaL}) and thereby prolongs action potential duration (APD) and QT interval in electrocardiograph manifested in Long QT syndrome Type 3 (LQT3).^{68, 85} However, LQT3 patients could have normal corrected QT interval (QTc) while the related mechanism is unclear^{4, 5}. Recently, our group demonstrated that LQT3 phenotype could be mediated by the ventricular perinexus located in the intercalated disc.^{7, 8, 10, 72} The perinexus is an intercellular nanodomain adjacent to the gap junctions with high density of Nav1.5 expressed in it.¹³ In spite of nanometer scale of the perinexal width, widening the perinexus can significantly exacerbate APD prolongation in LQT3 due to increased sodium ion availability in this nanodomain.^{7, 72}

It has been well known that APD prolongation is an arrhythmic substrate. Prolonged APD is associated with increased early afterdepolarization in Nav1.5 GOF.^{2, 3} We have reported in a drug-induced aged LQT3 guinea pig model, that perinexal expansion induced by mannitol prolonged APD and induced intrinsic arrhythmia.⁷ Therefore, these findings suggest that exacerbation in APD prolongation is an arrhythmic risk in LQT3. However, widening the perinexus with mannitol did not induce arrhythmia in a drug-induced adult LQT3 guinea pig model.⁷² We also found in this adult LQT3 model that widening the perinexus and increasing sodium concentration, also do not induce arrhythmia even if they synergistically exacerbated APD prolongation.⁷² Therefore, we reasonably assume

that the effect of perinexal expansion on arrhythmogenesis may be associated with aging. With the clinical findings that aged LQT3 patients is associated with increased cardiac death,⁸⁶ we hypothesize that the increased age-associated cardiac death in LQT3 is associated with the perinexal expansion.

In the present study, we investigated the effect of perinexal expansion on APD and arrhythmogenesis in adult and aged drug-induced LQT3 isolated guinea pig hearts. We found that the perinexus narrows with aging and APD does not change between adult and aged LQT3 hearts in any condition, which suggest that narrow perinexi observed in aged hearts may attenuate the reportedly increased I_{NaL} in isolated aged cardiomyocytes and thereby restrict APD prolongation in intact hearts. We also found that perinexal widening significantly induced cardiac arrhythmia in aged LQT3 hearts but did not induce arrhythmia in adult LQT3 hearts. This increased susceptibility to arrhythmia after perinexal widening in aged LQT3 hearts was not associated with the change in conduction velocity. Furthermore, we found that perinexal widening prolonged heart rate corrected $T_{peak}-T_{end}$ interval in ECG in both age groups but exacerbated in aged LQT3 hearts, which suggests that perinexal widening may increase transmural dispersion of repolarization to induce arrhythmias. Therefore, we concluded that narrow perinexi during aging may play a cardioprotective role in aged LQT3 hearts and perinexal expansion in aged LQT3 hearts is an arrhythmic risk.

Methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised

1996). All animal study protocols were approved by the Institutional Animal Care and Use Committee at the Virginia Polytechnic Institute and State University.

Establishment of Langendorff-perfused heart model

Adult (3 months, n=15) and aged (15 months, n=16) male albino Hartley guinea pigs (Hilltop Lab Animals, Scottsdale, PA) were involved. All animals were anesthetized with isoflurane inhalation. Hearts were rapidly excised, cannulated (<5 minutes) and retrogradely perfused with a crystalloid perfusion solution, consisting of (in mM) 1.25 CaCl₂, 139.5 NaCl, 4.56 KCl, 5.5 dextrose, 0.7 MgCl₂, 10 HEPES; and 5.5 NaOH (pH=7.4 measured at 36°C), and heart temperature was maintained at 36°C in a three dimensional (3D) printed polylactic acid bath.²² Atria were removed in order to induce ventricular bradycardia. The perfusion pressure was maintained at 50 mmHg by adjusting the flow rate of perfusion solution. Each heart was stabilized for 15 mins before the experiment started.

Optical mapping

Optical mapping of Langendorff-perfused guinea pig hearts has been extensively described by our group.^{22, 23, 72} Briefly, the voltage-sensitive fluorophore, di-4-ANEPPS (15 µM, Biotium, CA, USA), was perfused into the heart, and the heart position was visually adjusted to move the left anterior descending artery (LAD) in the center of the field of view so that the anterior epicardial surfaces of both ventricles were mapped during external pacing to record optical action potentials with a MiCAM HS02-CMOS camera (SciMedia: 92 x 80 pixels, field of view = 14.02 x 12.19 mm). The electromechanical uncoupler, blebbistatin (10 µM, ApexBio Tech LLC, TX, USA), was added to the perfusate

to reduce cardiac motion. An I_{NaL} agonist, sea anemone venom ATXII (7 nM, alomone labs, Jerusalem, Israel), was perfused to induce the phenotype of Long QT Syndrome Type 3 (LQT3). A sodium channel β 1-subunit de-adhesion peptide^{16, 72}, β adp1 (1 μ M, LifeTein LLC, NJ, USA), was perfused to induce perinexal expansion.

In order to investigate the age-associated difference in epicardial action potential duration (APD), the heart was paced with a bipolar plunge electrode that was placed in the septum near the atrioventricular node to stimulate the natural electrical propagation. The final stimulation current was set at 1.5X the minimum stimulation threshold which was tested with a cycle length (CL) of 300 ms (pulse width = 5ms). Each heart was perfused with baseline (BL), ATXII and ATXII+ β adp1 solutions in order. The perfusion duration of each solution was 20 mins. Optical signals were recorded during steady-state pacing at progressively increasing CLs of 200, 300, 400, 500, 600, 700 and 800 ms until intrinsic activity prevented 1:1 capture. For analysis, APD of one optical action potential in each of 1840 pixels was calculated after 2x2 spatial binning by using a custom MatLab program. The average APD across the array was considered a single data point for a heart at one pacing CL. APD was measured as the difference between the time of the maximum upstroke velocity and 90% repolarization (APD90) of the optical action potential. APD90 was only reported when both adult and aged hearts were successfully paced at the same CLs.

In order to investigate if epicardial conduction velocity (CV) changes with perinexal expansion, a different set of adult and aged hearts were paced with a single pacing electrode that was placed on the left ventricular wall close to LAD on the anterior epicardium. The reference electrode was placed in the bath. Each heart was perfused

with ATXII and ATXII+ β adp1 (same doses as above) in order and the perfusion duration of each solution was also 20 mins. After each solution, optical signals were recorded during steady-state pacing with a CL of 300ms (pulse width =5ms). For analysis, optical action potential signals were spatially binned 2 X 2 and subsequently analyzed by using a custom MatLab program to determine action potential activation time and then calculate CV at transverse (CVT) and longitudinal (CVL) directions and anisotropy ratio (CVL/CVT).

Susceptibility to arrhythmia

To investigate if the susceptibility to arrhythmia is associated with perinexal expansion, both adult and aged hearts that involved CV measurements above were paced with the same electrode using two modified stimulation protocols²⁵ of S1S2 and burst pacing after optical mapping. S1S2 is made of 10 stimulation pulses (S1) and 1 extra stimulation pulse (S2) with the pulse width of 2ms. All hearts were stimulated with S1 with time interval of 600ms, and the S2 was progressively reduced by as low as 1 ms until loss of 1:1 capture or arrhythmia induction. Burst pacing is made of 50 consecutive stimulation pulses with a 2-ms pulse width. The time interval of burst pulses was initialized with 300ms and then was progressively reduced by as low as 1 ms until loss of 1:1 capture or arrhythmia induction. Each heart was stimulated with S1S2 followed by burst pacing after each solution. Percentage of hearts with induced arrhythmias was quantified.

Transmission Electron Microscopy

In order to measure the perinexal width (W_p), left ventricular tissues were collected and fixed with glutaraldehyde as previously described for transmission electron microscopy (TEM).²⁷ A minimum of 20 images of gap junctions and the associated perinexus were

obtained at 150,000X magnification for each heart. For each image, i.e. each perinexus, the mean W_p was calculated over the distance of 30-105 nm from the edge of the gap junction plaque by a Matlab algorithm developed in our lab²⁸. The average W_p for each heart was obtained by averaging the measured W_p from each image for a given heart.

Tissue staining

Sections (10- μ m thickness) of left ventricular tissues were fixed with 2% paraformaldehyde for 60 minutes and then washed with Phosphate Buffered Saline (PBS) three times (5 minutes for each time). After that, sections were stained with primary antibody N-cadherin (1:500) and then placed in 4 °C room for overnight. After washout with PBS three times (5 minutes for each time), sections were then stained with secondary antibody Alexa fluor 488 goat anti-mouse (1:500, A11029, ThermoFisher Scientific) and wheat germ agglutinin conjugated with Alex fluor 488 (1:100, W11261, ThermoFisher Scientific) and DAPI (1:500, D1306, ThermoFisher Scientific) for 60 minutes at the room temperature in PBS. After washout with PBS three times (5 minutes for each time), section slides were mounted with ProLong Diamond mounting media and then stored in 4 °C room before imaging.

Simulations

We simulated a 50-cell cable (one-dimensional tissue) comprised of individual myocytes coupled via gap junctions and ephaptic coupling, as in prior work by us and others.^{7, 9, 29-31, 72} We accounted for non-uniform sodium channel subcellular localization by spatially discretizing each cell into multiple¹⁰ axial membrane patches along the length of the cell and two intercalated disc (ID) membrane patches at the ends of the cell. It was assumed

that 90% of sodium channels were localized in the ID patches. A T-shaped network of cleft axial resistances and a radial resistance, proportional and inversely proportional, respectively, to the intercellular cleft width (W_p), governed extracellular potentials at the ID and cleft. ID sodium currents and diffusion with the bulk extracellular space governed the extracellular cleft sodium ion concentration ($[Na^+]_e^{cleft}$) dynamics, which also depended on the cleft volume.

We utilized an established ventricular guinea pig myocyte ionic model ³²representing individual ion channel dynamics, and incorporated a Markov model of a LQT3-associated $Na_v1.5$ GOF mutant ³³which reproduces a pronounced I_{NaL} . Specifically, the mutant channel model exhibited two modes of gating - a “background mode” and a “burst mode,” the latter of which represented a population of channels that transiently fail to inactivate. Adult and aged conditions were represented by modifying myocyte size parameters. Specifically, the length and radius of adult myocytes were defined as 110 μm and 7 μm , respectively, and of aged myocytes as 130 μm and 11 μm , respectively. Full model equations, parameters, and simulation codes can be found in our prior work.^{7, 72}

The cable was paced at one end with a CL of 900ms. For each simulation, APD and CV were calculated as a function of varying values for W_p under adult and aged WT and mutant conditions. APD was calculated by taking the difference between the action potential repolarization time (defined as the time V_m crossed below -60 mV) and activation time (above -60 mV) for each cell and averaging the values for cells 15 to 35. CV was computed by linear regression of the activation times (defined as the time when V_m crosses above -60mV) of cells 15 to 35.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). The results are presented as mean $\pm$ standard deviation. Comparisons of QT interval and APD90 as a function of CLs in both age groups were analyzed with two-way ANOVA followed by Bonferroni correction for multiple comparisons (Figure 1A, 1B and S2). Comparisons of W_p , QT interval, APD90 and cell size in both age groups were analyzed with one-way ANOVA followed by Bonferroni correction for multiple comparisons (Figure 1C, S1 and 4). Fisher's exact test was used to analyze the susceptibility to arrhythmia after β adp1 (Figure 2). The unpaired Student's t-test was performed for statistical analysis of CVT, CVL, CVL/CVT and heart rate-corrected $T_{peak}-T_{end}$ interval (T_{pTe-c}) before and after β adp1 in each age group or same conditions (Figure 3, S3 and 5). A $p < 0.05$ was considered statistically significant.

Results

We have demonstrated that widening the perinexi with the sodium channel β 1 subunit de-adhesion peptide (β adp1) prolongs action potential duration (APD) in adult drug-induced LQT3 guinea pig hearts.⁷² Consistent with the previous study, β adp1 prolonged APD in both age groups in the presence of ATXII (Figure S1). For comparison of the difference in LQT3 phenotype between these two age groups, APD and QT interval during pacing with a series of cycle lengths (CLs) were reported (Figure 1A and 1B). We found that APD and QT interval as a function of CLs have no difference between these two age groups under same conditions at baseline (BL), with ATXII and ATXII+ β adp1, respectively. Interestingly, perinexal width (W_p) in aged hearts is significantly smaller than adult ones in either condition of ATXII or ATXII+ β adp1 (Figure 1C) suggesting that the perinexus

narrows with aging. Since the narrow perinexus facilitates depletion of local sodium ions during $\text{Na}_v1.5$ activation to enhance self-attenuation and thereby decreases I_{NaL} to shorten APD,^{7, 72} the findings suggest that narrow perinexi in aged hearts may conceal increased I_{NaL} in aged cardiomyocytes and thereby to cause no difference in APD and QT interval between these two age groups (3 vs 15 months). Therefore, APD in aged LQT3 hearts should be greater than adult ones if W_p is similar in both age groups. We then compared APD in adult hearts after ATXII and aged hearts after ATXII+ βadp1 because of comparable W_p in these two groups (Adult hearts after ATXII vs aged hearts after ATXII+ βadp1 : 29.6 ± 2.5 vs 29.9 ± 1.2 nm, Figure 1C). The comparison showed that APD is significantly larger in aged hearts than adult ones with matched W_p (Figure S2). Very importantly, a previous study demonstrated that I_{NaL} increases with aging and is primarily responsible for APD prolongation in isolated aged guinea pig cardiomyocytes.⁸⁷ Notably, the guinea pigs involved in this study have very similar ages (1 vs 12 months) compared with the current study (3 vs 15 months). Therefore, the results suggest that the perinexus narrows with aging and may conceal age-dependent cellular APD prolongation in LQT3.

Widening the perinexus with mannitol induced intrinsic arrhythmia in aged LQT3 guinea pig hearts.⁷ However, the current study did not observe intrinsic arrhythmia in adult and aged LQT3 hearts in the presence of ATXII and βadp1 . That is probably due to βadp1 and mannitol widening the perinexus with distinct mechanisms.^{16, 72} In order to investigate if perinexal widening contributes to cardiac arrhythmia in aged hearts, two stimulation protocols of S1S2 and burst pacing were applied to adult and aged LQT3 hearts with ATXII to test susceptibility to arrhythmia in the absence and presence of βadp1 (Figure 2). S1S2 pacing did not significantly induce arrhythmias in both adult and aged LQT3

hearts even with β adp1 (Figure 2A). In contrast, burst pacing significantly induced cardiac arrhythmias in 3 of 4 aged hearts after β adp1 relative to one aged heart (1/5) before β adp1 (Figure 2B). Still, no arrhythmias were induced in adult hearts (0/6) before and after β adp1 with burst pacing. Therefore, widening the perinexus in aged LQT3 hearts is susceptible to arrhythmias.

Both animal experiments and computational model have demonstrated that perinexal expansion could lead to conduction velocity (CV) slowing due to reduced ephaptic coupling.^{7, 16, 29} Importantly, wider perinexus is associated with slower CV and increased cardiac arrhythmias without modification in sodium channels.¹⁶ Therefore, in order to investigate if the increased arrhythmias after β adp1 in aged LQT3 hearts are associated with CV slowing, all hearts prior to S1S2 and burst pacing were optically mapped with epicardial pacing at CL=300ms to analyze CV. The transverse CV (CVT) and longitudinal CV (CVL) before and after β adp1 in both age groups were quantified (Figure 3A). Surprisingly, β adp1 significantly increased CVT in both age groups (Figure 3B). β adp1 also increased CVL in adult hearts while did not significantly increase it in aged hearts. The effect of β adp1 on CVL in aged hearts showed an increasing trend with a marginally significant difference ($p=0.0617$) probably due to under-powered size of hearts. We also found that β adp1 did not alter CVL/CVT in either age group. Overall, widening the perinexus in the presence of ATXII didn't decrease CV, which may not be responsible for the increased arrhythmias after β adp1 in aged hearts.

We also observed that CVL was significantly higher in aged hearts relative to adult ones with either ATXII or ATXII+ β adp1 (Figure 3B). Since there is no significant difference in CVT, CVL/CVT showed significant increase with aging. In order to demonstrate if the

increase in CVL is related to ATXII, we also analyzed CVT and CVL in both age groups in the absence of ATXII in a different set of experiments. The results showed that there is no significant difference in CVT between these two age groups while CVL increases with aging (Figure S3) suggesting that CV increases with aging regardless of ATXII or ATXII+ β adp1.

The increase in CV with aging may be associated with cellular hypertrophy.⁸⁸ In order to investigate if cell size increases with aging, left ventricular tissues collected from both age groups after ATXII and ATXII+ β adp1 were stained to measure cell area, length and width (Figure 4A). We found that cell area is significantly larger in aged hearts and β adp1 does not affect cell area (Figure 4B). Interestingly, cell length does not change with aging although there was an increasing trend (Figure 4C). Therefore, the significantly greater cell width may primarily lead to larger cell area in aged hearts relative to adult hearts (Figure 4D). Overall, these data suggest that the increased CV in aged hearts may be associated with increased cardiac hypertrophy.

However, it remains unclear why perinexal expansion in aged LQT3 hearts is associated with increased cardiac arrhythmias. The reports of Nav1.5 heterogeneity across the ventricular wall⁸⁹ and larger I_{NaL} in M cells⁹⁰ suggest electrical heterogeneity across the ventricles. Importantly, preferential prolongation of M-cell APD in LQT3 produced an increase in transmural dispersion of repolarization (TDR)^{91, 92}, which is associated with increased arrhythmia. Therefore, we wonder if the increased arrhythmias after β adp1 in aged LQT3 hearts is related to increased TDR. Due to un-feasibility of TDR measurement in the current experimental setting, heart rate corrected $T_{peak}-T_{end}$ interval (TpTe-c) using Bazett formula on ECG was evaluated as TpTe is a reliable indicator of TDR⁹³ and TpTe-

c is more precise than TpTe.⁹⁴ TpTe-c was measured right before arrhythmia was induced or 1:1 capture was lost with burst pacing. We found that there is no significant difference in TpTe-c between these two age groups in the absence of β adp1, while β adp1 increased TpTe-c in both age groups and exacerbated TpTe-c prolongation in aged hearts relative to adult hearts (Figure 5). Therefore, widening the perinexus in aged LQT3 hearts may increase TDR and thereby lead to increased arrhythmias.

Finally, we performed simulations in a strand of myocytes with a LQT3-associated sodium channel mutant (Y1795C). In these simulations, we generated adult and aged conditions based on changes in myocyte size and investigate if intercellular cleft (equivalent to the perinexus) expansion leads to early afterdepolarization (EAD) incidence in aged, not adult mutant myocyte strands. Simulations results show that all types of myocyte strands with narrow cleft ($W_p=20\text{nm}$) did not produce EADs, although both presence of the mutation and aged conditions prolonged APD (Figure 6A, left). In contrast, only aged mutant myocyte strands produced EADs for a wide cleft ($W_p=40\text{nm}$, Figure 6A, right). Importantly, these results are consistent with our prior work simulating neonatal and adult myocytes that found that a wide cleft is associated with EAD formation in larger cells.⁹ Although no EADs occurred in the mutant adult myocyte strands due to different simulation conditions compared with our prior work (slightly narrower cleft and faster pacing rate), these results suggest that perinexal expansion in aging LQT3 hearts increases arrhythmic risk.

In order to explore the mechanism of EAD formation due to wide cleft in aged mutant myocytes strand, sodium channel open probability, sodium current and sodium reversal potential were tracked during the action potential. In the presence of narrow cleft ($W_p=20\text{nm}$), sodium channel open probability during repolarization in aged mutant is

slightly lower than adult mutant (Figure 6B, left) and there is a clear lower disc I_{NaL} in aged mutant (Figure 6C, left), which can be explained by reduced sodium reversal potential (Figure 6D, left). By putative mechanism, Nav1.5 activation rapidly depletes the local sodium in the narrow cleft while slow pacing allows sodium to continuously refill the cleft and maintains at a high and stable level. Therefore, the inherently larger I_{NaL} current (due to the age-dependent increase in cell size) decreases sodium reversal potential, which in turn attenuates sodium driving force and thereby reduces I_{NaL} with a negative feedback. Furthermore, the prolonged sodium channel opening duration (Figure 6B, left) in aged mutant allows sodium ions continuously flowing into the myocytes, which leads to APD prolongation (Figure 6A, left). By contrast, wide cleft increases sodium reversal potential (Figure 6D, right), due to a mitigation of the local sodium depletion, and thereby increases I_{NaL} in both adult and aged mutants to prolong APD (Figure 7A).

The experimental results showed that CV increases with both aging and perinexal expansion in drug-induced LQT3. However, the association between CV and genotype is unclear. Therefore, the simulations in adult and aged WT and mutant (Y1795C) myocyte strands were performed to explore the impact of cleft width, genotype, and aging on CV (Figure 7B). The simulation showed that CV increases when the cleft widens in all strands, suggesting that wide cleft enhances ephaptic coupling regardless of genotype and age. We also found that mutants are associated with increased CV, which also has been found in our prior study on neonatal and adult myocytes,⁹ and may be caused by increased peak sodium current (15-20% increase in the current study). Furthermore, the increase in CV with aging regardless of genotypes may be associated with increased cell size.^{9, 95} Therefore, CV can be impacted by cleft width, genotype and aging.

Discussion

Perinexal expansion exacerbates Nav1.5 gain of function through reduced self-attenuation. In LQT3, widening the perinexus allows increased sodium ion availability in it to enhance I_{NaL} during Nav1.5 inactivation and thereby to prolong APD.^{7, 8, 72} Therefore, perinexus narrowing during aging observed in the current aging LQT3 heart models should decrease cellular I_{NaL} to shorten APD. Although the direct measurement of effect of perinexal widening on cellular I_{NaL} in the tissue level is unavailable, similar APD between adult and aged hearts, reportedly prolonged APD due to increased I_{NaL} in isolated aged cardiomyocytes⁸⁷ and together with results from computational LQT3 models^{7, 8, 72} suggest that perinexi narrowing with aging conceals prolonged cellular APD and thereby plays a cardio-protective role in aging LQT3 hearts. Furthermore, frequent induction of cardiac arrhythmias after perinexal widening in aged LQT3 hearts is not associated with CV changes, but may be associated with increased transmural dispersion of repolarization (TDR) indicated by prolonged $T_{peak}-T_{end}$ interval. However, the relationship between perinexal widening and TDR needs to be further investigated. Overall, we concluded that the perinexus narrows with aging, and perinexal expansion is an arrhythmic risk in aged LQT3.

The ventricular perinexus narrowing with aging with the same experimental procedures was reported in the current study. Ventricular perinexal width has been separately reported in both adult and aged hearts with the similar ages by different experimenters.^{7, 8, 16, 72} Although these studies have been done independently, the reported perinexal width shows smaller in aged hearts than adult ones. Therefore, these studies consistently suggest that the perinexus narrows during aging. However, the perinexal separation may

vary with locations. Our previous study reported that the atrial perinexus widens with aging in patients.⁴⁷ Although it is unclear why atrial and ventricular perinexi change in opposite directions during aging, the investigation of ventricular perinexal separation is the key to study the effect of the perinexus on LQT3 phenotype.

It has been demonstrated that incidence of cardiac death increases with aging in LQT3 patients,⁸⁶ but the associated mechanism is still unknown. Prolonged QT interval/APD has been commonly treated as an arrhythmic substrate,^{2, 3} and QT interval serves as a good indicator of risk of cardiac death in uncontrolled LQT3 population.^{86, 96, 97} Interestingly, the risk of cardiac death is not significantly associated with QT interval in aged LQT3 patients ($QTc \geq 530$ vs $QTc < 530$).⁸⁶ Furthermore, QT interval prolongs during normal aging in patients;⁹⁸ however, it is challenging to determine if QT interval also prolongs with aging in LQT3 as QT interval varies with different LQT3 mutants.⁹⁹ Notably, full spectrum of QT interval in lifespan in LQT3 patients has not been completely reported except specific age ranges.^{100, 101} In the current controlled study, QT interval and APD have no difference between 3 and 15-month hearts with and without ATXII or ATXII+ β adp1 respectively, suggesting that the increased incidence of cardiac arrhythmias in aged LQT3 hearts after β adp1 may not be associated with QT interval or APD prolongation.

It remains unknown why the perinexus narrows with aging. It has been demonstrated that application of ATXII in isolated hearts has no effect on the perinexal separation.⁸ Therefore, the perinexus narrowing should be a consequence of natural aging. Aging has been associated with multiple structural remodeling including gap junction reduction,¹⁰² fibrosis¹⁰³ and hypertrophy.¹⁰⁴ However, although gap junctions are adjacent to the

perinexus, gap junction reduction does not change the perinexal width.¹⁴ The fibrosis is associated with intercellular widening,¹⁰⁵ but obviously narrow perinexi in aged hearts are not associated with increased fibrosis. Furthermore, the induced cardiac hypertrophy is associated with intercalated disc widening. However, chronic cardiac hypertrophy with aging reflects the molecular adaptation over time and the molecular changes leading to induced and natural cardiac hypertrophy may generate different effects on intercalated discs.^{104, 106} Therefore, it remains unknown if cardiac hypertrophy with aging contributes to perinexal narrowing.

Increased transmural dispersion of repolarization (TDR) is associated with the development of cardiac arrhythmias. Previous studies have shown that epicardial pacing prolongs TDR relative to endocardial pacing by altering the transmural sequence of activation of intrinsically heterogeneous ventricular myocardium. Direct measurement of TDR during epicardial pacing in intact hearts is difficult due to unavailable action potential measurement across ventricular wall in relatively small hearts like guinea pigs, but TpTe-c, which stably indicates TDR, measured right before arrhythmia inductions and 1:1 capture loss before and after β adp1 in both age groups suggests that prolonged TDR after β adp1 is an arrhythmic substrate that predisposes to dangerous ventricular fibrillation. Furthermore, a TDR threshold was suggested to be associated with inducing arrhythmias, which may interpret the increased arrhythmias after β adp1 in aged LQT3 hearts which presents exacerbated TpTe-c interval relative to adult LQT3 hearts after β adp1.

Widening the perinexus decreases conduction velocity due to reduced ephaptic coupling in hearts without ATXII. However, with the same experimental setting, the current study

demonstrated that widening the perinexus increases CV in the presence of ATXII suggesting that ATXII may interfere with the effect of the perinexal widening on CV slowing. The main effect of ATXII on cardiomyocytes is to increase intracellular sodium and thereby calcium ions by decreasing forward mode of sodium-calcium exchanger without altering sodium channel activation. Addition of non-conductive β adp1 in the presence of ATXII should greatly increase intracellular sodium and calcium ions by enhancing I_{NaL} . It is well known that the sodium and calcium ions are charge carriers so that increasing both ions in the cytoplasm should decrease membrane resistance¹⁰⁷⁻¹⁰⁹ and thereby increase CV. Although measurement of tissue bioimpedance was not included in the current study, at least increased induction of arrhythmias after perinexal widening in aged LQT3 hearts is not associated with CV changes.

Limitations

There are several limitations in the current study. First, only 3 and 15-month animals were included, so the data may only represent cardiac electrical and structural changes in this age range. Aging-associated changes such as connexin 43 (Cx43) expression and fibrosis which impacts CV are not measured. Although previous studies have demonstrated that decreases in Cx43 and increase in fibrosis leads to conduction slowing or delay,¹¹⁰⁻¹¹⁴ the current study did not observe conduction slowing suggesting that both changes in the current age groups may not produce significant impact on CV. Secondly, only male animals were included. Further work is needed to investigate if sex matters in the effect of the perinexus on LQT3 phenotype. Third, it is not confirmed yet if I_{NaL} increases with aging in isolated cardiomyocytes between these two age groups with and without ATXII. Since increased I_{NaL} has been observed in isolated cardiomyocytes from

different aging animals,⁸⁷ no difference in APD in intact hearts must indicate that perinexal narrowing conceals the increased I_{NaL} in aged cardiomyocytes.

Conclusions

In conclusion, the perinexus narrows with aging in LQT3 hearts to conceal cellular $Na_v1.5$ GOF which enhances with aging. Perinexal expansion in aged LQT3 hearts is associated with increased cardiac arrhythmias, which suggests prevention of extracellular edema should benefit LQT3 patients.

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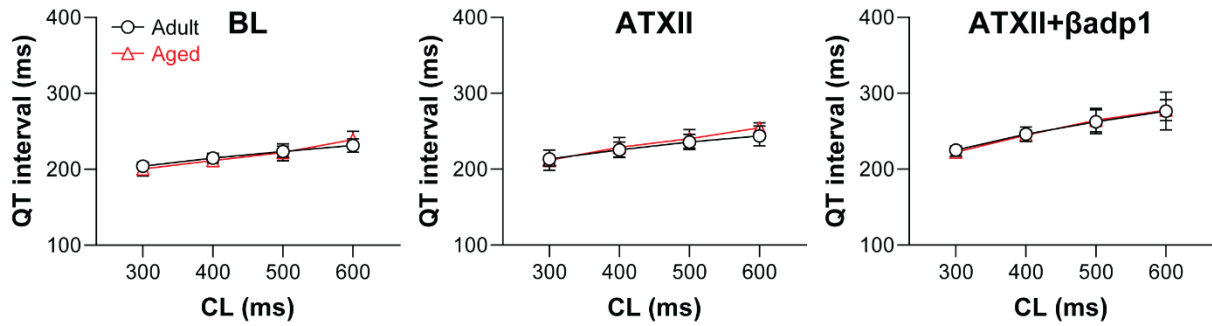
This work was supported by the National Institutes of Health Grants: R01HL138003 (SP and SHW), R01HL141855 (SP), R01HL102298 (RGG and SP), and R01HL056728 (RGG).

Disclosures

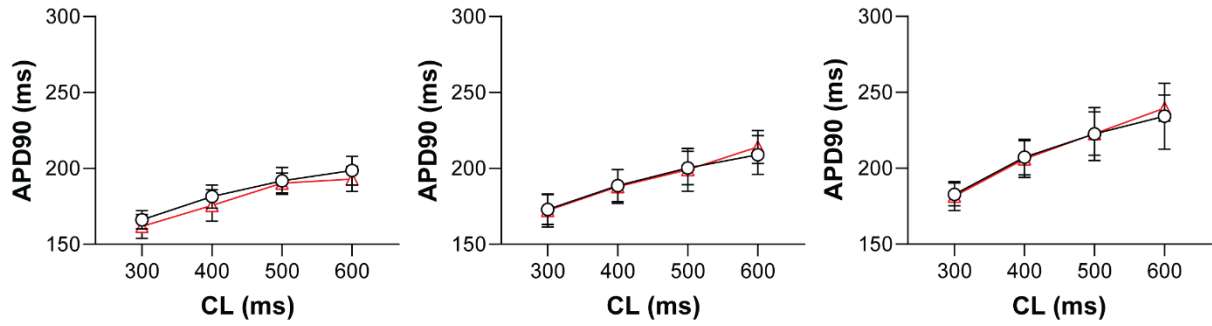
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Figures

A



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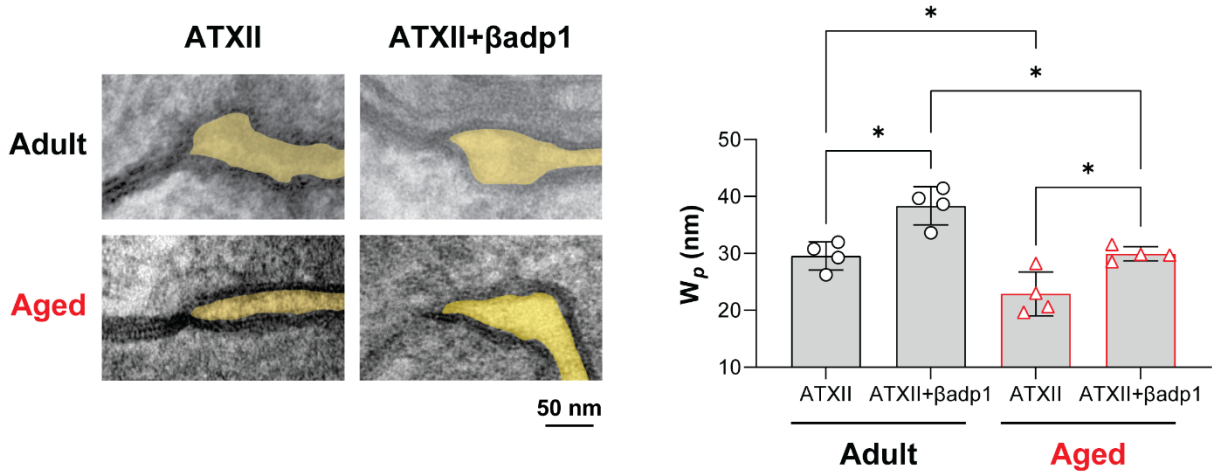


Figure 1: Narrow perinexi observed in aged hearts conceal APD prolongation. A.

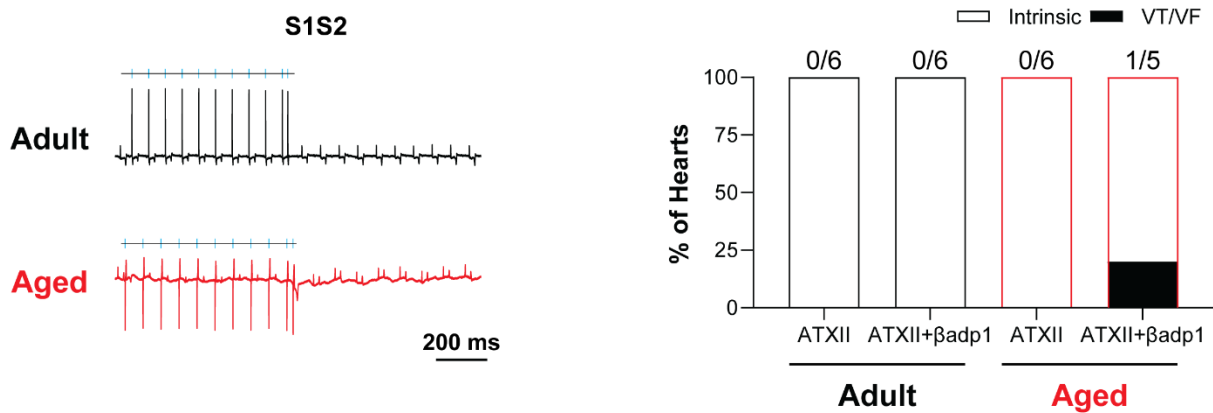
QT interval as a function of cycle lengths (CLs) from both adult (n=5) and aged (n=6) hearts at baseline (BL), with ATXII and ATXII+βadp1, respectively. **B.** APD at 90%

repolarization (APD90) as a function of CLs from both adult (n=5) and aged (n=6) hearts.

C. Left - The representative perinexi (highlighted with yellow) from adult and aged hearts after ATXII and ATXII+ β adp1, respectively. Right - Quantified perinexal width (Wp) of both adult and aged hearts after ATXII and ATXII+ β adp1 (n=4 per group), respectively.

*p<0.05.

A



B

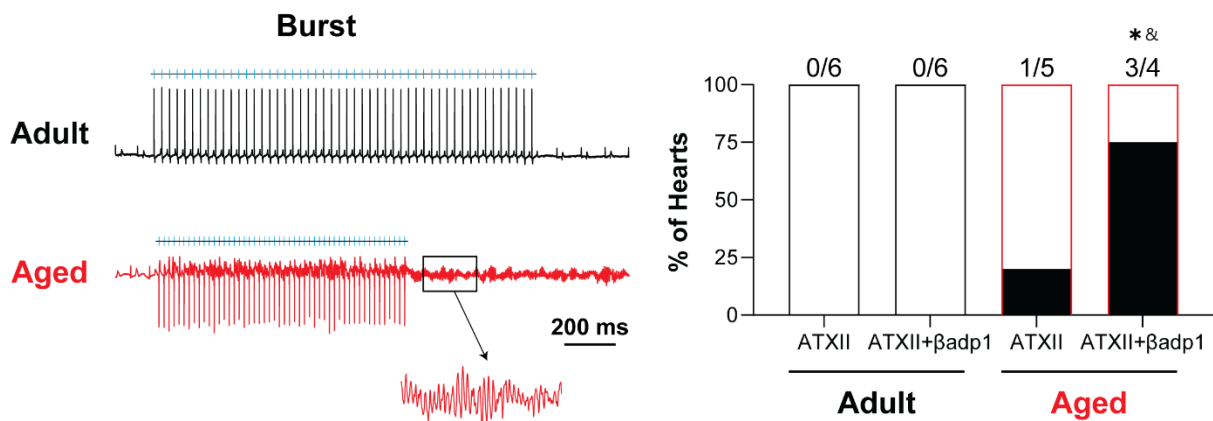
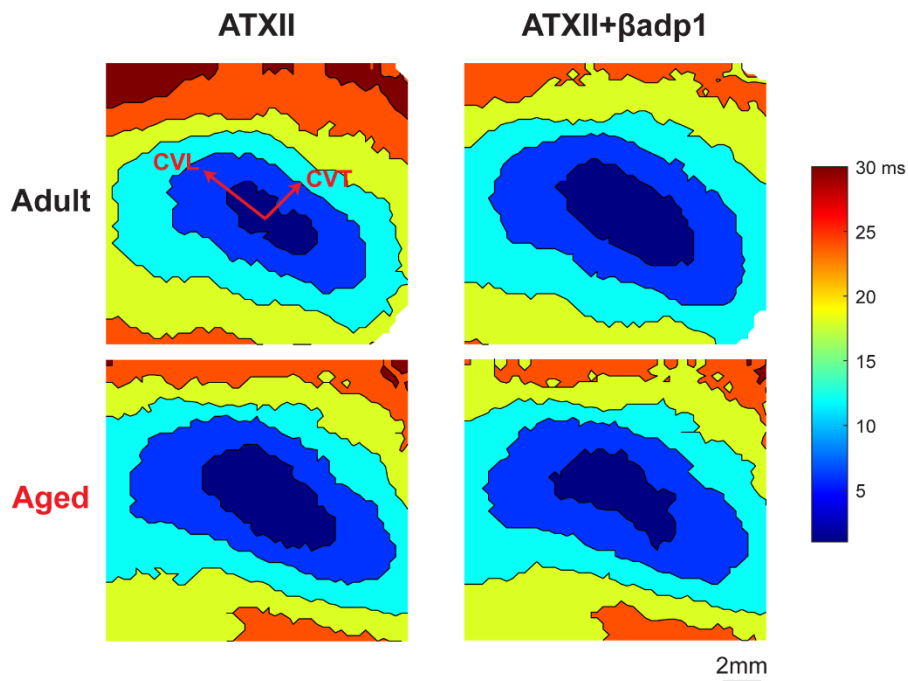


Figure 2: Perinexal widening increases arrhythmias in aged LQT3. **A.** Left – representative ECG waveforms with S1S2 pacing in both age groups under ATXII+βadp1; Right - Percentage of hearts (n=6 per age group) with arrhythmias induced by S1S2. **B.** Left – representative ECG waveforms with burst pacing in both age groups under ATXII+βadp1; Right - Percentage of hearts (n=6 per age group) with arrhythmias induced by burst pacing. *p<0.05 relative to adult hearts with ATXII+βadp1; &p<0.05 relative to aged hearts with ATXII.

A



B

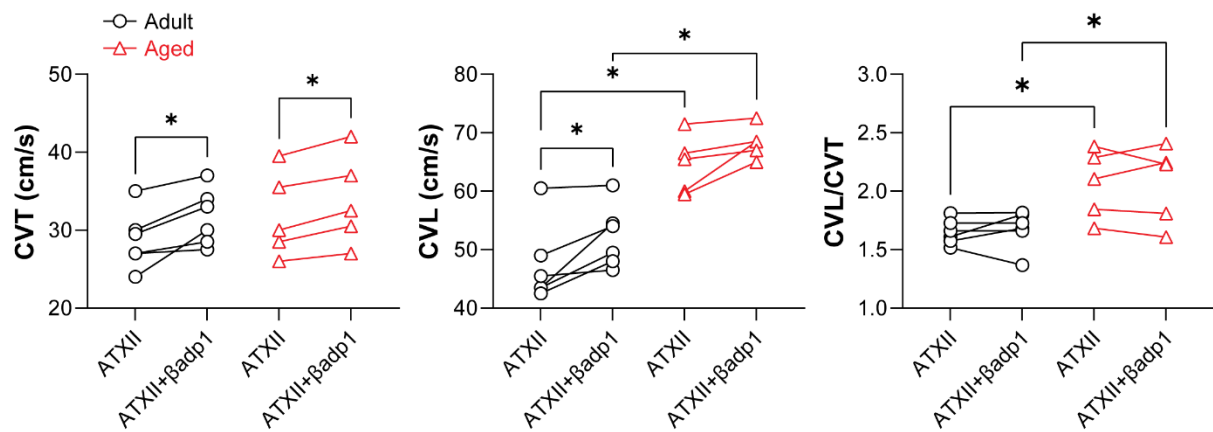


Figure 3: Perinexal widening increases CV in both age groups. A. The representative activation time maps from two age groups with ATXII and ATXII+ β adp1, respectively. **B.** Transverse CV (CVT), longitudinal CV (CVL) and CVL/CVT before and after β adp1 in both age groups (adult hearts: n=6; aged hearts: n=5). * $p < 0.05$.

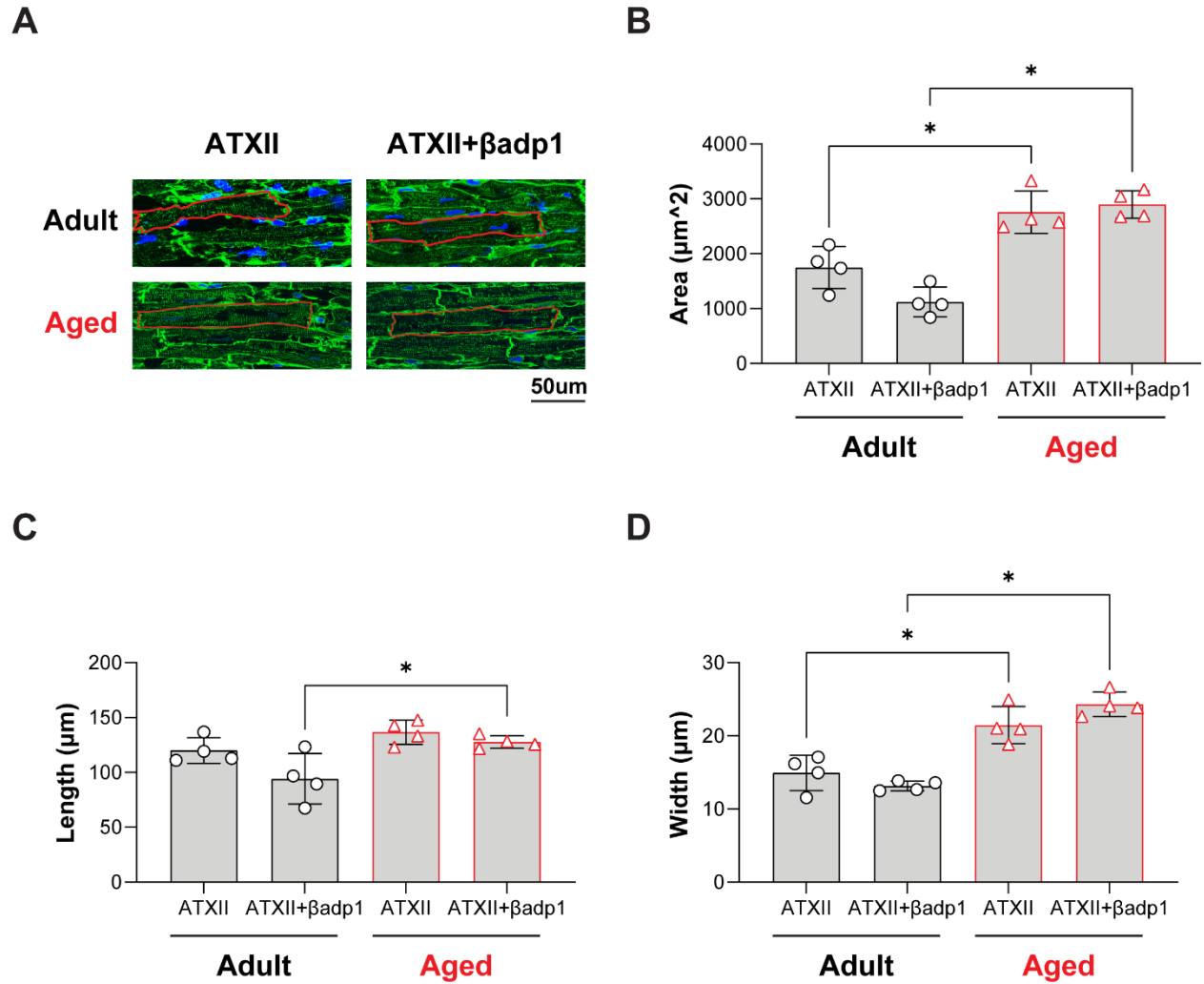


Figure 4: Cell size increases with aging. **A.** The representative left ventricular tissue images from two age groups. **B, C and D:** Cell area, length and width in two age groups (n=4 per age group) before and after βadp1, respectively. *p<0.05.

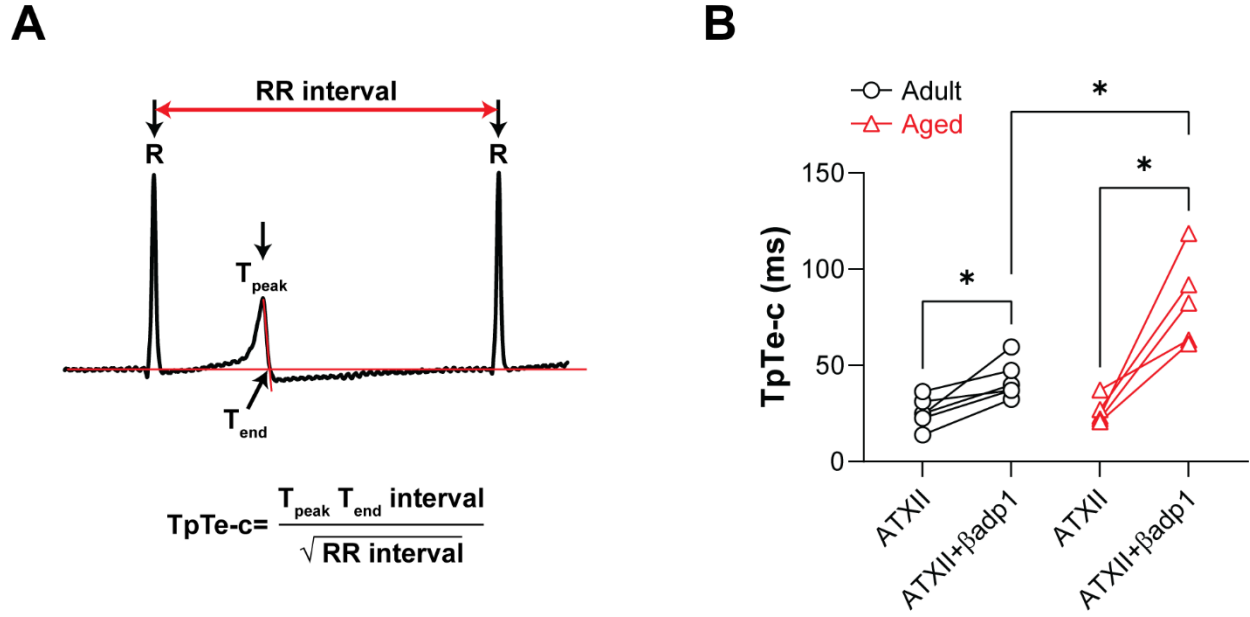


Figure 5: Perinexal widening exacerbates Tpeak-Tend interval in aged LQT3. A. A representative guinea pig ECG tracing showing Tpeak-Tend interval corrected by heart rate (TpTe-c). **B.** TpTe-c was measured before induction of arrhythmia or 1:1 capture loss in both age groups (adult hearts: n=6; aged hearts: n=5) in the absence and presence of βadp1. *p<0.05.

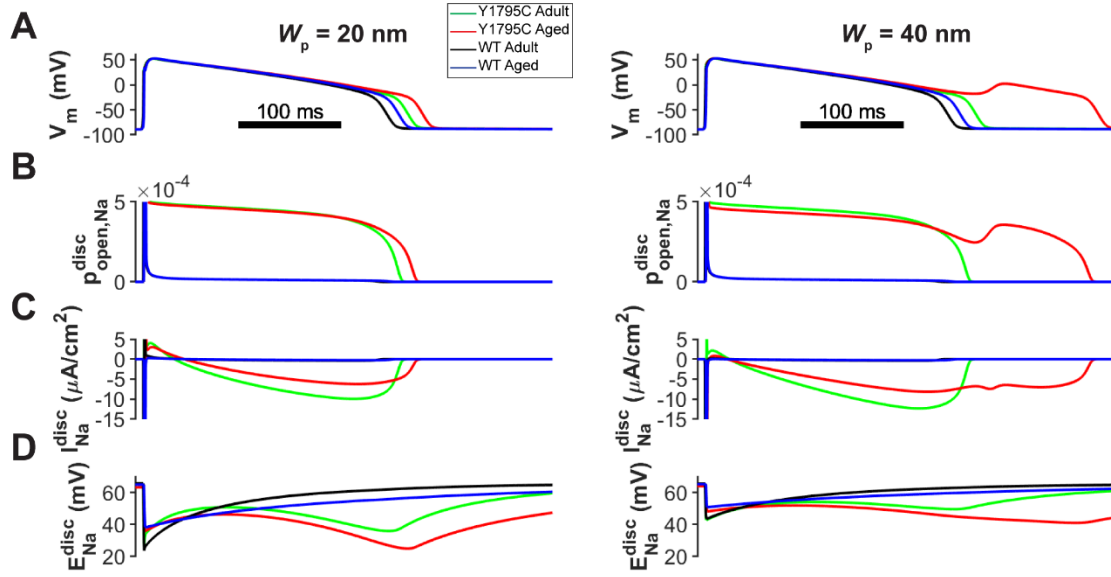


Figure 6: Early afterdepolarizations are masked by narrow cleft in mutant aged myocyte strands. **A.** Transmembrane potential (V_m). **B.** Sodium channel open probability ($p_{open,Na}^{disc}$). **C.** Sodium current (I_{Na}^{disc}). **D.** Sodium reversal potential (E_{Na}^{disc}). Traces are shown for wild-type (WT) and mutant (Y1795C) adult and aged myocyte strands with narrow ($W_p=20\text{nm}$) and wide ($W_p=40\text{nm}$) cleft width, respectively.

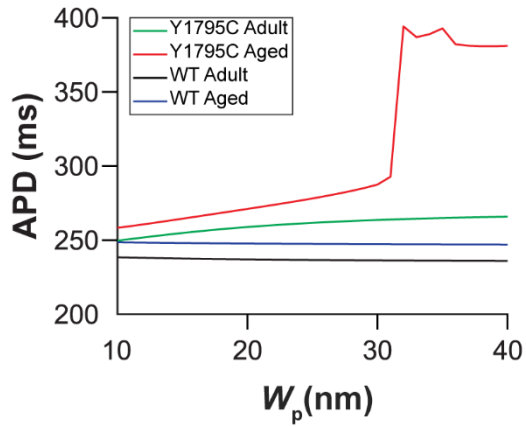
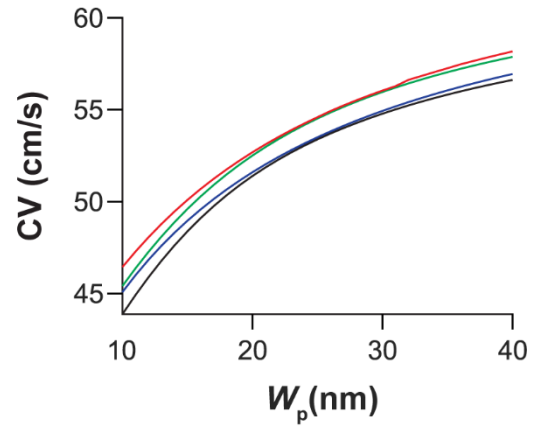
A**B**

Figure 7: Action potential duration (APD) and conduction velocity (CV) are greater in mutant aged myocyte strands with matched cleft widths. A&B. APD and CV as a function of cleft widths for WT and mutant (Y1795C) adult and aged myocyte strands, respectively.

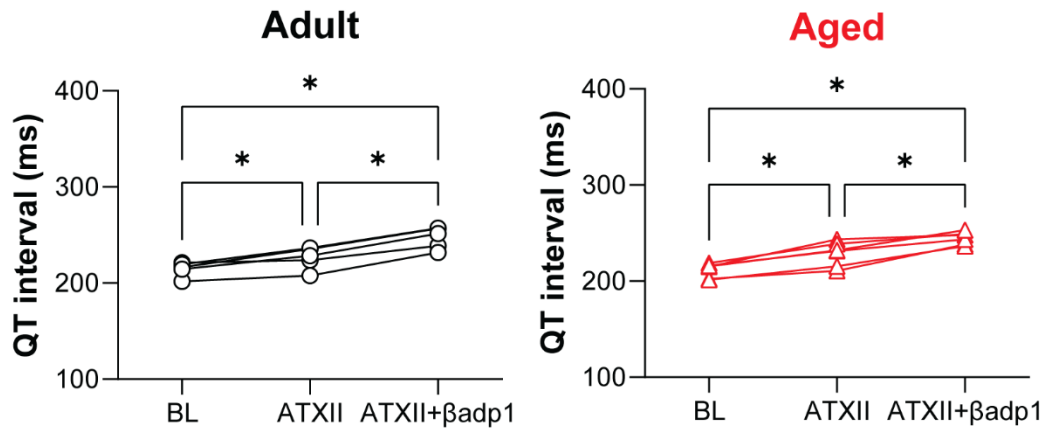
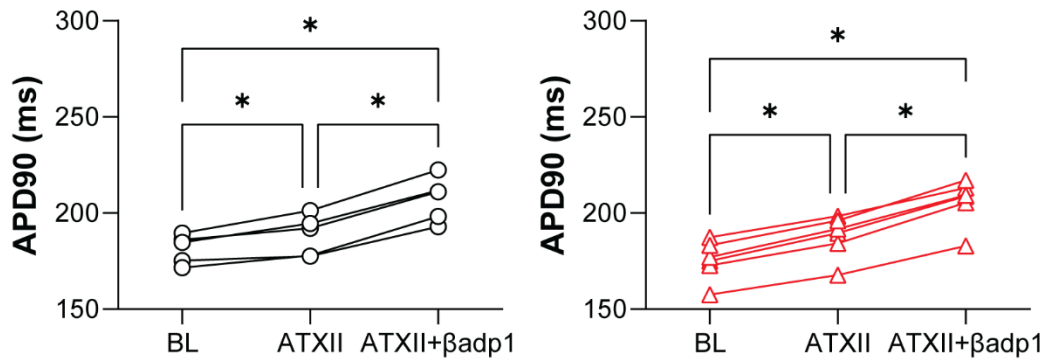
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Figure S1. Perinexal widening with β adp1 exacerbates LQT3 phenotype in both adult and aged LQT3 hearts. A. QT interval during pacing with cycle length (CL) at 500ms at BL, with ATXII and ATXII+ β adp1 in both age groups. **B.** Epicardial APD90 at CL=500ms at BL, with ATXII and ATXII+ β adp1 in both age groups. * $p < 0.05$ (One-way ANOVA).

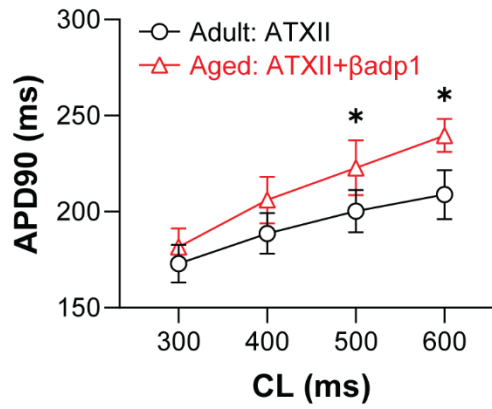


Figure S2. APD in aged LQT3 hearts is greater than adult ones with matched W_p .

APD90 as a function of CLs in adult hearts after ATXII and aged hearts after ATXII+βadp1.

* $p < 0.05$ relative to adult hearts after ATXII (two-way ANOVA). W_p , perinexal width

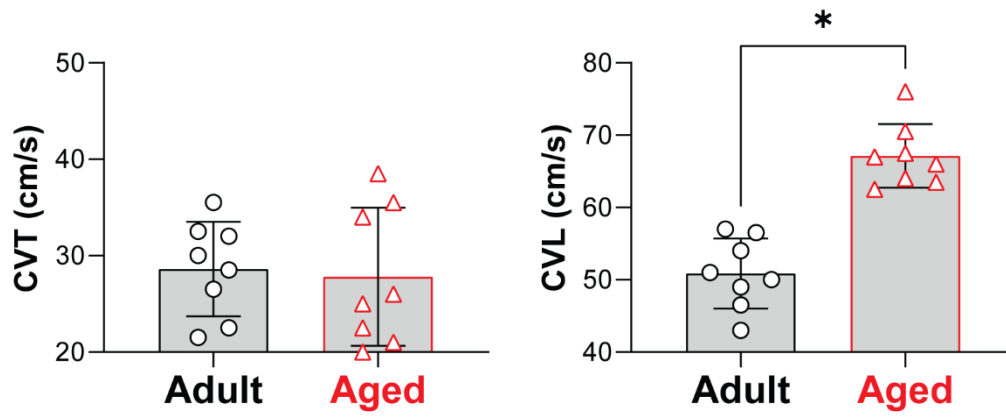


Figure S3. CVL increases in aged hearts. CVT and CVL with epicardial pacing with CL of 300ms in both age groups without ATXII. * $p < 0.05$ (unpaired T-test).

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Chapter 5: Conclusions

The whole research project investigates the impact of a nano-scale intercellular space, termed as the perinexus, on the phenotype of Long QT Syndrome Type 3 (LQT3) under different conditions including sodium stress, aging and species. All findings in this project support the ‘butterfly effect’ theory proposed by Dr. Lorenz ²that *The Flap of a Butterfly’s Wings in Brazil Set off a Tornado in Texas*, which means that a small change (perinexal widening) can result in large differences (LQT3 phenotype).

Synergistic effect of perinexal expansion and high extracellular sodium

Perinexal widening reduces ‘self-attenuation’ due to increased amount of local sodium ions, which increases the sodium driving force during repolarization to increase late sodium current (I_{NaL}) and then prolong action potential duration (APD) in drug-induced LQT3 guinea pig hearts.³ The most important prerequisite for this effect is highly expressed Nav1.5 in the perinexus. Following this finding, we designed the experiments to prove that APD prolongation is caused by increased sodium ions in the perinexus.¹ Specifically, we found that elevating sodium concentration alone or widening the perinexus alone prolongs APD and the combination of both synergistically exacerbates APD prolongation. These findings support the idea that elevating sodium concentration in the presence of perinexal widening greatly increase the amount of local sodium ions and thereby robustly enhance I_{NaL} . Translationally, LQT3 patients should benefit from managing sodium intake and preventing cardiac edema.

Involvement of transient outward potassium current in the regulation of high sodium

Following the previous findings above, we tested the effect of high sodium and perinexal widening on APD in a genetically-modified LQT3 (Δ KPQ) mouse model. We found that high sodium produced the shortening effect on APD in both WT and Δ KPQ mouse hearts. Although perinexal widening still prolongs APD, consistent with the previous finding,^{1, 3} the effect of perinexal widening and high sodium fails to produce synergistic effect on APD. These data suggest that the effect of high sodium on APD in mouse hearts should involve a different mechanism. Since it is well known that the guinea pig heart doesn't functionally express transient outward potassium (I_{to}) channels but the mouse heart does,⁴ the effect of different sodium concentrations on I_{to} was evaluated in isolated mouse cardiomyocytes. We found that high sodium significantly increases I_{to} , which may be responsible for APD shortening with high sodium and disappearance of synergistic effect of high sodium and perinexal widening as well. The electrophysiological properties of mouse hearts are very different to human when compared with other animals.⁴ Therefore, these findings suggest that it is very critical to use a proper animal model for translational purpose.

Impact of perinexal expansion in aging LQT3 hearts

We have demonstrated that perinexal expansion exacerbates LQT3 phenotype, but it remains unclear the role of the perinexus during aging in LQT3. Clinical studies have shown that aged LQT3 patients are associated with increased cardiac death.⁵ We wonder if it is associated with perinexal change. Interestingly, we found that the perinexus

significantly narrows with aging and APD doesn't change with aging from 3 to 15 months. Since I_{NaL} increases with aging, ⁶our data suggest that narrow perinexus in aged hearts may play a cardio-protective role in concealing LQT3 phenotype. Importantly, we discovered that widening the perinexus in aged LQT3 hearts increases the susceptibility to cardiac arrest, which suggests that perinexal expansion is very arrhythmogenic in aged LQT3 hearts. However, the increases arrhythmogenesis due to perinexal widening cannot be interpreted by the change in conduction velocity (CV) as the perinexal widening in both adult and aged LQT3 hearts increases CV. Furthermore, we found that perinexal widening in aged LQT3 hearts exacerbates corrected $T_{peak}-T_{end}$ interval ($TpTe-c$), which reflects transmural dispersion of repolarization (TDR). Studies have demonstrated that increased $TpTe-c$ or TDR is associated with the increase in cardiac arrhythmias.^{7, 8} Overall, this project discovered that the perinexal narrows with aging and widening the perinexus in aged LQT3 hearts is very arrhythmogenic, which suggests that increased incidence of cardiac death in LQT3 patients may be associated with perinexal expansion.

Limitations

Specific limitations of each study have been described in each of Chapter 2, 3 and 4. However, the general limitations in the whole project may have two. The first one is the failure of manipulating the perinexal width. Narrowing the perinexus is very appealing as widening the perinexus in a nano-scale could significantly impact LQT3 phenotype. Several agents ⁹have been tested to narrow the perinexus, unfortunately none of them have shown APD shortening. Despite of the challenge in narrowing the perinexus, the

findings in LQT3 guinea pig hearts suggest an alternative way that managing the sodium intake should benefit LQT3 patients.

The second limitation is discovered in the study involving mouse hearts. The inconsistency in findings between guinea pig and mouse hearts leads to the discovery of involvement of I_{to} in the regulation of high sodium on LQT3 phenotype. The difference in cardiac electrophysiology between these species and also relative to human suggests that selection of an animal model which functionally expresses I_{to} channels and have similar action potential shape as human is very important for the translational purpose.

Overall conclusion

We have demonstrated and confirmed, together with the previous finding, that widening the perinexus leads to APD prolongation in both drug-induced and genetically-modified LQT3 hearts regardless of age and species. The effect of high sodium alone on APD depends on if the heart functionally expresses I_{to} . The synergistic effect of widening the perinexus and elevating sodium on APD can be regulated by I_{to} due to elevating sodium. We also demonstrated that widening the perinexus in aged LQT3 hearts is associated with increased cardiac death, which cannot be interpreted by change in CV.

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