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# The KCNMA1 *Drosophila* Ortholog *Slowpoke* in *Drosophila melanogaster* Cardiac Function and Human Disease

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Biomedical Sciences

By

Santiago Pineda

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Professor Karen Ocorr

2016

The Dissertation of Santiago Pineda is approved, and it is acceptable in quality and form for publication on microfilm and electronically

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University of California San Diego

2016

# DEDICATION

To my incredibly supportive parents and siblings: I love you and thank you for everything you have done for me.

To my friends who kept me sane during this time and helped me grow in so many different ways.

I would like to thank Drs. Rolf Bodmer and Karen Ocorr for their support, guidance and mentorship. I learned an amazing amount from our weekly meetings. Additionally their patience while I explored different avenues of research was a crucial part of my development through graduate school.

I would also like to acknowledge all of my Bodmer lab mates past and present that made this an incredibly experience. Dr. Georg Vogler deserves particular credit for his patience while teaching me microscopy and while I fumbled with molecular biology. Dr. Ryan Birse was an unbelievable font of guidance and always provided a unique and helpful perspective.. Finally again, everyone else and the immeasurable help they have provided.

I would also like to acknowledge my thesis committee for their patience and help in making my work and thesis better.

I am a better person for having known you all.

# EPIGRAPH

That was a memorable day to me, for it made great changes in me. But it is the same with any life. Imagine one selected day struck out of it, and think how different its course would have been. Pause you who read this, and think for a moment of the long chain of iron or gold, of thorns or flowers, that would never have bound you, but for the formation of the first link on one memorable day."

-Charles Dickens, Great Expectations

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LIST OF ABBREVIATIONS

AF= Atrial Fibrillation

BK- Big K Channel

EAD= Early After Depolarization

$K^+$= Potassium

$Ca^{2+}$= Calcium

Iks= Delayed Rectifier Current

Ikr= Rapid Rectifier Current

DD= Diastolic Depolarization

Cx43= Connexin-43

RyR2= Ryanodine Receptor

SA= Sinoatrial

UAS=Upstream Activation Sequence

HP= Heart Period

DI=Diastolic Interval

SI= Systolic Interval

AI= Arrhythmia Index

FS= Fractional Shortening

GS= GeneSwitch

AP= Action Potential

IC50= Half-maximal Inhibitory Concentration

EC50= Half-maximal Effective Concentration

MMode= Motion Mode

LQT= Long QT Syndrome

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Dr. Vesna Nikolova-Krstevski, did immunofluorescence and electron microscopy in Chapter 3. Various members from Dr. Diane Fatkin's lab helped in developing the mutation used in Chapter 4. The dissertation author performed all other experiments.

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The large conductance calcium-activated potassium channel, $K_{Ca}1.1$, is a determinant of heart function and arrhythmia risk

ABSTRACT OF THE DISSERTATION

The KCNMA1 *Drosophila* Ortholog *Slowpoke* in *Drosophila melanogaster*

Cardiac Function and Human Disease

by

Santiago Pineda

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2016

Professor Rolf Bodmer, Chair

Professor Sylvia Evans Co-Chair

Aging leads to increases in the incidence and severity of cardiac arrhythmias. The most common of these is atrial fibrillation (AF), which is a major health issue contributing to mortality and affecting some 33.5 million people worldwide (Mozaffarian et al., 2015). The changes with age that cause

increases in AF and how they contribute to the pathophysiology of the disease are questions that are still unanswered. However, researchers long identified a strong heritability component to the disease. A host of different monogenetic candidate gene, linkage analysis and Genome Wide Association Studies (GWAS) followed. These studies led to the discovery of many important cardiac function and disease genes. Yet owing to the inherent complexity of cardiac disease with a wide range of heterogeneic environmental factors, a large portion of the heritable component of disease was still unexplained (Sinner, Ellinor, Meitinger, Benjamin, & Kaab, 2011). One of the first genetic studies looking at AF was linkage analysis study mapping a familial atrial fibrillation locus to chr10q22-q24. However, the study was not able to discern any AF causative genes (Brugada et al., 1997). Recently, our collaborator, Dr. Diane Fatkin, carried out a screen of affected AF patients and found an association between a case of familial AF and the *KCNMA1* gene, which actually spanned the 10q22-q24 locus originally found in the Brugada study. The gene encodes the Big K (BK) channel which was not previously known to have a function in the cardiac action potential. However, examination of this channel in mice has been hampered due to the very rapid kinetics of cardiac repolarization in the mouse versus human heart. Many of the $K^+$ channels underlying the major repolarizing currents in the human do not make key contributions to adult mouse cardiac physiology (Nerbonne, 2004). In contrast,

these channels contribute significantly to *Drosophila melanogaster* cardiac function (Ocorr et al., 2007).

This increased similarity in the channels underlying repolarization between the *Drosophila* and human cardiac action potential made it an ideal model to study the role of the KCNMA1 homolog *slowpoke* in the cardiac action potential. In Chapter 3 of this thesis I used *Drosophila* genetics to temporally and spatially control expression of *slowpoke* in conjunction with in-house assays to measure cardiac function. The two main assays were video recording and intracellular current clamping of *ex-vivo* hearts. Both of these showed that *slowpoke* is important for cardiac function and its absence causes bradyarrhythmias in a cell autonomous fashion. Additionally, I saw that knockdown of *slowpoke* seemed to downregulate many of the major depolarizing and repolarizing channels in the heart. In Chapter 4 we looked at how the aforementioned human mutation would affect the integrated cardiac system of the fly heart. Finally in Appendix 1 we look at the synergistic phenotypes when different voltage-activated $K^+$ channels, including *slowpoke,* were knocked down in conjunction. This research points us in the direction to take further steps in dissecting out the repolarizing phase of the Drosophila cardiac action potential and how these mechanisms could translate to human cardiac function and disease.

# CHAPTER 1 INTRODUCTION

## 1.1 Historical Perspective of Arrhythmias and Atrial Fibrillation

The ancient world from China in 500 B.C. and Egypt in 3000 B.C. recognized the importance of the heart and peripheral pulse as indicators of disease (Janse & Rosen, 2006). The measurement of abnormal strength or rhythm in the peripheral pulse led to identification of arrhythmias as a concomitant disease. Yet, it was not until the 19th century that a series of rapid scientific advancements helped propel and define the electrical study of the heart. It was, for example, in the 19th century that disorders of the heartbeat were termed arrhythmias, that scientists published a cardiac myogenic cause for the peripheral pulse and that it was the electrical activity of the heart that engendered this myogenic action. At the tail end of this movement the technological advances of Einthoven's string galvanometer propelled the electrocardiogram as the method of measuring the electrical activity of the heart. With the measure of the electrical activity of the heart, they were able to better connect electrical dysfunction with irregular heartbeat as the newly termed arrhythmias (Janse & Rosen, 2006; Surawicz, 2003).

## 1.2 *Drosophila* as a Model for Studying Voltage-Activated $K^+$ Channels

Discerning that dysfunction of the electrical activity of the heart is the major cause of arrhythmias was significant but the mechanism of function for the electrical activity was not known. It was associated work in the nervous

system by Hodgkin and Huxley on the squid giant axon that highlighted ion channels as the units of function for electrical impulses in the nervous system (Hodgkin & Huxley, 1952). Ion channels are pore proteins that make up a core component of cell signaling and are classified according their method of opening/closing and the specific species of ions that flow through them. The flow of ion species across them creates currents and subsequent electrical impulses across not just the nervous but also musculoskeletal and cardiac systems. Their discovery as the basic unit of function for electrical activity in the heart opened up the avenue to identify specific types of channels acting in the changes of electrical potential during contraction and relaxation. In the heart, the typical ventricular cardiac action potential consists of 5 phases: a resting membrane potential, depolarization, rapid but brief repolarization, a plateau phase, and another rapid repolarization towards the resting membrane potential. Excitatory $Na^+$ and $Ca^{2+}$ channels work to depolarize the membrane while repolarizing $K^+$ channels bring the membrane back to resting potential for another depolarization.

Being able to associate the ion channels according to their specific species with different cardiac phases was another huge step, but the identification of the genes encoding the specific channels had to wait until the modern era of molecular genetics. The identification of the specific genes allowed the study of cardiac abnormalities related to ion channels called “channelopathies”, which underlay many of the previously identified cardiac

arrhythmias. As a group cardiac arrhythmias are a heterogenous set of ailments with a varied set of molecular causes. Yet, the molecular basis of many of these arrhythmias was loss of function mutations in the $K^+$ channels which could lead to a prolonged action potential duration in which an early after depolarization (EAD) can lead to arrhythmias (Shimizu & Horie, 2011). These Potassium ($K^+$) channels are the most diverse and complex category of ion channels. Yet, their study in the heart has been confounded due to a lack of appropriate genetic models. The mouse is one of the main biomedical genetic models but it has a very rapid heartbeat with different kinetics of cardiac repolarization as compared to humans. Specifically, the KCNQ and hERG channels which underlie the major repolarizing currents (delayed (Iks) and rapid (Ikr) rectifier currents respectively) in humans do not make major contributions to adult mouse cardiac physiology (Nerbonne, 2004).

The need for other genetic models to study the function cardiac $K^+$ channels in normal and disease states drove the return to *Drosophila melanogaster.* The fly heart is a mono layer of 52 pairs of cardiomyocytes pumping in an open circulatory system and has long been a crucial model organism for understanding cardiac development. One of the first cardiogenic transcription factors NKX 2.5 as well as the first cloned $K^+$ channel were originally discovered in *Drosophila* (Bodmer, 1993) (Papazian, Schwarz, Tempel, Jan, & Jan, 1987). In the heart there exists amazing conservation of basic characteristics from fly to human hearts in its role as a structural motor,

ion channel conduction, developmental pathways and the whole of the cardiac proteome (Cammarato et al., 2011) (Ocorr, Vogler, & Bodmer, 2014).However, the advantages of this model extend past its robust genetic tools and role as a developmental model; it can also tell us something about cardiac physiology. In regards to cardiac electrical function and physiology, $K^+$ channel studies in our lab show that the same channels that belie the important repolarizing currents in humans but not in the mouse (KCNQ and hERG) are important for *Drosophila* cardiac physiology. In fact, mutations in the KCNQ *Drosophila* homolog gene, which in humans are responsible for Long QT Syndrome, cause an age dependent cardiac arrhythmia in flies (Ocorr et al., 2007) (Unpublished work).

### 1.3 BK Channel History and Novel Function in the Heart

The similarity in the repolarizing mechanisms between humans and *Drosophila* make the model perfect for probing the function of novel cardiac $K^+$ channels. Collaborators in Dr. Diane Fatkin's lab at the Victor Chung Cardiac Research Institute found a connection between one such channel called KCNMA1 or BK channel and familial atrial fibrillation (AF). The BK channel was first discovered through its *Drosophila m.* ortholog *slowpoke* in the dorsal longitudinal flight muscles. There its contribution to the action potential was a fast transient voltage and $Ca^{2+}$ dependent $K^+$ outward current (Atkinson, Robertson, & Ganetzky, 1991; Elkins, Ganetzky, & Wu, 1986). The channel has the typical voltage-activated $K^+$ channel features such as the 6

transmembrane domains that come together in a tetrametric structure to make up the $K^+$ pore and an adjacent transmembrane voltage sensitive domain. It has several additional domains such as the intracellular $Ca^{2+}$ bowl and an extra 7$^{th}$ transmembrane domain, all of which separate the channel from other voltage-activated $K^+$ channels. Both the human and *Drosophila* channels have the largest single-channel conductance of any $K^+$ channel (Salkoff, Butler, Ferreira, Santi, & Wei, 2006). Phenotypically, mutant *slowpoke* flies were temperature sensitive paralytics unable to sustain flight and sensitized to sedative compounds. Although the human ortholog shares only a 52.5% protein sequence identity to the fly ortholog (UniProtKB.com) both have remarkably similar protein domains in the important ion sensing regions and the $Ca^{2+}$ sensitive bowl. The similar human and *Drosophila m.* molecular determinants of repolarization along with human BK and *Drosophila m. slowpoke* channel similarities made the *Drosophila m.* heart tube a perfect model to explore the role of this channel in cardiac physiology and possible connection to disease.

This channel has not previously been thought to contribute to cardiac arrhythmias. However, its $Ca^{2+}$ and voltage activation along with its position spanning a familial AF locus (Brugada et al., 1997) make it an attractive candidate as an emerging $K^+$ channel important in cardiac physiology. Previous studies identified channel isoforms in the mitochondria of mammalian cardiomyocytes where it seems to have a cardioprotective effect during

ischemia reperfusion. The exact mechanism of action for this function is unknown, however, and is still undergoing study. Yet, there was no focus on cardiomyocyte plasma membrane as earlier studies had failed to find the channel there (Balderas, Zhang, Stefani, & Toro, 2015). Work from our collaborators in Chapter 3 and 4 seem to refute this and suggest that this may be an overlooked aspect of channel function.

The initial genetic studies knocking out the BK channel ortholog in the mouse showed moderate ataxia, incontinence and hypertension but no overt cardiac phenotype (Meredith, Thorneloe, Werner, Nelson, & Aldrich, 2004; Sausbier et al., 2005). However, pharmacological inhibition of the channel decreased heart rate in wild type but not null mice. Additionally, they were able to find the channel in the sinoatrial node and saw an increase in the diastolic depolarization (DD) of genetic null mutants (Imlach, Finch, Miller, Meredith, & Dalziel, 2010). These results suggest that the BK channel in the mouse heart has a redundant role or that sympathetic compensatory mechanisms are abolishing cardiac phenotypes in the knockout mice. Mitochondrial BK channels open during hypoxia and confer a cytoprotective effect during ischemia-reperfusion by counteracting excessive and damaging $Ca^{2+}$ motive force into the mitochondria via $K^+$ efflux (Balderas et al., 2015). This BK channel role and the crucial role of mitochondrial ATP production for cardiac contraction and relaxation could underlie some of these cardiac phenotypes. Yet, knockout of the major $Ca^{2+}$entry mitochondrial uniporter did not change

the maximum DD in pacemaker cells (Wu et al., 2015). If the mitochondrial BK isoforms were wholly responsible for cardiac phenotypes and one of their main roles in the mitochondria were to regulate $Ca^{2+}$ homeostasis within the mitochondria, then it would be expected that knockout of the major entry $Ca^{2+}$ uniporter would also similarly change the action potential shape. The unique effect on action potential shape due to BK channel knockout in mouse pacemaker cells and *Drosophila* whole hearts suggests that there is a separate cardiac specific function for the BK channel.

One area that the channel's role as attenuator of electrical excitation is more firmly established is in the neural tissues. Here, it has a variety of roles relating to timing bursts of $Ca^{2+}$ action potentials, regulating synaptic transmission, and compensatory reflexes to hypoxia (Brenner et al., 2005; Du et al., 2005; Gu, Vervaeke, & Storm, 2007). The new research localizing the channel to the sinoatrial node and our collaborators own work finding subcellular localization to the sarcolemma points to this channel perhaps fulfilling a similar role in cardiac tissues. Ubiquitous channel expression in the whole body and importance in a variety of physiological processes suggest the channel could play an important $Ca^{2+}$ feedback regulation role in almost every tissue of the body.

## **1.4 Rationale and Aims**

A genetic mutation screen of affected familial AF cohorts looking at genes spanning a familial AF locus identified possible disease causing mutations in

KCNMA1. This gene encodes a voltage and $Ca^{2+}$ activated $K^+$ channel that did not have a known role in the cardiac action potential and warranted further study. Yet, the distinct electrical repolarizing mechanisms between the mouse and human limit the use of this genetic model to study this novel connection. *Drosophila m.* shares many of the same human molecular determinants of the repolarizing currents and as such is a great model to study a possible role for this channel in the heart. I hypothesize that this channel functions to modulate the repolarizing phase of *Drosophila m.* cardiac action potentials. Additionally, this system can act as an exogenous genetic model to observe the effect of genetic mutations with possible applications to human cardiac function. My thesis work addresses the following specific aims:

**Aim 1: Determine the functional effect of BK channel knockdown and overexpression in the Drosophila heart**. If the Bk channel homolog *slowpoke* is important for *Drosophila m.* cardiac function, then constitutive and cardiac specific knockdown should have a negative effect.

**Aim 2: Determine the effect of the human KCNMA1 mutation in the fly heart.** Overexpress either the human mutant or wild-type KCNMA1 gene in order to test whether a human wild-type KCNAM1 gene is able to rescue *Drosophila* null mutant. Test whether a 'mutant' human variant form of KCNMA1 derived from an AF patient perturbs cardiac function and is unable to rescue the *Drosophila* null mutant.

## Aim 3: Determine *slowpoke* channel interactions with other $K^+$ channels and its role in the cardiac action potential

# CHAPTER 2 EXPERIMENTAL METHODS AND MATERIALS

## 2.1 *Drosophila* Stocks

The fly lines *slo*$^4$ (gift from Barry Ganetzky), *slo*$^1$ (stock number 4587), a *Df (3R) BSC 397* (stock number 24421), *w*$^{1118}$, *pan-neural driver ElAV-Gal4* (stock number 458) and *w; Cyo/Sco; TM3/TM6B* (cross between chromosome 2 balancer stock 2555 and chromosome 3 balancer 2537) were obtained from the *Drosophila* Stock Center (Bloomington, IN;http://flystocks.bio.indiana.edu/). *Slowpoke* UAS-RNAi line (construct ID: 108671) and control RNAi insertion *y,w[1118];P{attP,y[+],w[3`]}* (Stock Number and Transformant ID: 60100) were from the Viennna *Drosophila* RNAi Center (VDRC) (Dietzl et al., 2007). The cardiac tissue-specific *Hand*-Gal4 driver was a gift from Zhe Han and Eric Olson (Han, Yi, Li, & Olson, 2006). UAS-KCNMA1 flies were generated from cloned human cDNA that was cloned into pcDNA3.1, then the pUAST vector, and finally PhiC31 mediated transformation via injection in *w*$^{1118}$ strain. PBac slo genomic rescue flies were generated using a *slowpoke* genomic BAC library (gift of Hugo Bellen, library clone ID CH321-61O01) (Venken et al., 2009) which was injected in a *w*$^{1118}$ strain and transformed via PhiC31. Flies were reared on a standard yeast/molasses/cornmeal diet and maintained at 25°C. Flies were collected within 24 h of eclosion and placed into vials with 20-25 same sex cohorts. Vials were changed every 2-3 days. The cardiac-specific inducible HandGS-Gal4 driver(Osterwalder, Yoon, White, & Keshishian, 2001) (Monnier et al., 2012) was a gift from Laurent Perrin. Flies

were collected two days from eclossion and kept on transgene inducing RU486 (100ug/mL  working concentration) food for 21 days to a 3 week age time point.

## 2.2 Optical Heartbeat Analysis of Adult *Drosophila* Hearts

Adult heart parameters were prepared and analyzed and as described previously (Fink et al., 2009; Ocorr et al., 2007).  Briefly, flies were dissected in an artificial hemolymph containing 108 mM $Na^+$, 5 mM $K^+$, 2 mM $Ca^{2+}$, 8 mM $MgCl_2$, 1 mM $NaH_2PO_4$, 4 mM $NaHCO_3$, 10 mM sucrose, 5 mM trehalose, and 5 mM Hepes (pH 7.1). All reagents were obtained from Sigma Chemical (St. Louis, MO). Movies of beating hearts were recorded using a Leica (model) with a water immersion lens, high-speed digital camera (Hamamatsu EM-CCD) using HC Image image capture software (Hamamatsu Corp.). Heart function parameters including heart period, heart diameters, systolic and diastolic intervals, fractional shortening and arrhythmia index were quantified using our semi-automated optical heartbeat analysis software (Fink et al., 2009). All genotypes used in our experiments had N=15 to 30

## 2.3 Electrophysiology of Adult Hearts

Semi-intact heart preparations were incubated in artificial hemolymph containing 10 µM blebbistatin (Sigma Aldrich) and equilibrated with oxygenation in the dark for 45-60 minutes until the hearts stopped beating.

The preparation was then supplied with fresh saline without blebbistatin and electrical potentials were recorded from the conical chamber using sharp glass electrodes (20-50 MΩ) filled with 3M KCl and standard electrophysiological techniques. Data were acquired using an Axon-700B multiclamp amplifier, signals were digitized using the DIGIDATA 1322A and data were captured and analyzed using PClamp 9.0 and Clampfit 10.0 software respectively (all from Molecular Devices). Data was quantified from representative 30s recordings where the resting membrane potential had remained stable for at least 30s.

### 2,4 Immunofluorescent Imaging

Adult hearts were labeled with phalloidin and visualized using an Apotome Microsocope Z1 (Zeiss) and an AxioCam MRm (Zeiss) microscope as previously described (Alayari et al., 2009). Fluorescent probes Alexa488-phalloidin (Invitrogen, Carlsbad, CA) and goat-anti-rabbit-Cy3 (Chemicon, Temecula, CA) were used.

### 2.5 Statistical Analysis

Differences between groups were assessed using Student's t test or ANOVA followed by Tukey's or Newman-Keul's Multiple Comparison test. Data are expressed as mean ± SEM. A p value <0.05 was considered statistically significant. All values are expressed as mean ± standard error of mean (SEM).

Survival was assessed using the Kaplan-Meier method and logrank test. $p<0.05$ was considered significant.

**2.6 Lifespan Assay**

Female and male progeny were collected for 3 days. Then, they were briefly anaesthetized and separated in groups of 25 flies in each vial. The flies were kept at 25 °C, and the dead flies were counted every 2 days after transfer. The experiment was performed on 200 flies. Data were analyzed using Prism 5.0 (GraphPad Software, La Jolla, CA, USA).

**2.7 Nanofluidic qPCR of *Drosophila* hearts**

5 fly hearts were isolated and snap frozen in 10µl of water. Each sample was lyophilized and brought up to a final volume of 40µl of lysis buffer (LB) (0.25% NP40 in water). Lysis was carried out by heating individual hearts in LB for 2 minutes at 98$^{o}$C. The lysed hearts were then briefly centrifuged, and 30µl transferred to a fresh tube. Reverse transcription was carried out by using 3.3µl of lysed heart (~8% of a single heart) in a 5µl final reaction volume using the VILO Reaction mix as per the manufacturer's instructions (Life Technologies), resulting in a final volume of 6µl of RT cDNA per heart. For use with the BioMark platform, we then carried out pre-a amplification for 10 genes using a Fluidigm Master Mix (Fluidigm corp) (3µl), 10x single target amplification (STA) (1.5µl of 10X STA - 100µM of the 48 primers listed in Supp. Table X), 0.5 M EDTA(pH 8) (0.075µl), and water, to a final volume of

9µl. Pre amplification was then carried out in a volume of 15 µl (pre-amp cycling - 95$^{o}$C for 2 minutes, and 20 cycles of 96$^{o}$C 5 seconds, 60$^{o}$C for 4 minutes). For removal of single stranded DNA prior to nanofluidic cycling, 6 µl of Exosap solution (4.2µl of water, 0.6µl of Exonuclease 1 Rn Buffer, Exonuclease 1 (20 units/µl, New England Biolabs) was added to the 15µl final reaction volume of the RT step. The resulting 21 µl final volume was then incubated at 37$^{o}$C for 30 minutes, and then heat inactivated at 80$^{o}$C for 15 minutes. The pre-amplified volume (21µl) was then diluted 10 fold in DNA suspension buffer (Teknova), and stored at -20$^{o}$C prior to running on chip. Pre-amplified products for each individual fly heart was then assayed using Fluidigm's 48.48 nanofluidic qPCR arrays on a Biomark system (Fluidigm), according to their protocols. Biotium's EvaGreen DNA binding dye was used to detect amplified product according to Fluidigm's protocols. The primers used for amplification were the following:

Ca-alpha1D- Forward: CTACGTCCACTGCGACTTGTA Reverse: AGTGGCACCATGGCCTTTAA

Elk- Forward: CTGCCCTTTGATCACCTGTAC Reverse: CAGGAGACGCGTCAATTTCA

Ih- Forward: ACAACCGACTGGCCATGAA Reverse: GTGCCCGGAAGTTTTCTGAC

Irk1- Forward: GCAACGTTGTGCAGGGAAA Reverse: CGTCAACCAGGGTGGTGAA

KCNQ- Forward: TGAAGCCCTACGACGTCAA Reverse:

GCATTTTAACGCGACCCAAC

Seizure- Forward: AATCCAGAGAGCCGGCAATA Reverse:

CCGACCGTTGGGTAAATACAC

Shaker- Forward: CCGAGCTTCGATGCGATTTTA Reverse:

GGGACATTGACCGGTCTCC

Slowpoke- Forward: TCATCCAGCTGATGCAGTACC Reverse:

ATCGTCGCCCTGTTTCCAA

SUR- Forward: GCAGCTGAAGGAGTTTGTCA Reverse:

AGGTTTAGCCCTCCATCACA

## 2.8 Pharmacology Administration

Flies were dissected into a semi-intact prep and filmed as previously described. We diluted drug and vehicle control into artificial hemolymph at the indicated concentrations and incubated the dissected flies for 10 minutes under drug or vehicle before filming once more. We then incubated the flies in drug free hemolymph for 10 minutes. We repeated this process twice more and filmed a final post treatment time point.

# CHAPTER 3 *SLOWPOKE DROSOPHILA* CARDIAC FUNCTION

## 3.1 ABSTRACT

The KCNMA1 *Dm* homolog *slowpoke* has important functions in neural and muscle tissues in the fly. However, its role in adult cardiac function has not been explored. Here I show that it is expressed in the heart and that its function there is important for control over cardiac chronicity. We then show that cardiac specific and constitutive knockdown both slow down the heart rate and increase the incidence or irregular beats as measured visually and electrically.

## 3.2 INTRODUCTION

The study of ion channels in the fly has a storied past from the discovery of temperature sensitive mutants in the neuromuscular region (Papazian et al., 1987) (Atkinson et al., 1991)to the modern era of molecular genetics and identification of specific genes which led to the discovery of mechanism of action for such diseases as LQTS (Spears & Gollob, 2015) (Ocorr et al., 2007). Into this backdrop, the KCNMA1 BK channel emerged as a unique yet confusing channel. It was first discovered and cloned in the fly yet researchers found that in both fly and human the channel had the largest single-channel conductance of any $K^+$ channel and could be activated by voltage changes and increases in intracellular $Ca^{2+}$. These two properties highlight the channel's physiological role in a wide variety of cells as an important negative-feedback regulator of $Ca^{2+}$ signals. In addition to playing a

part in this important regulatory mechanism, and perhaps in part because of it, the channel is ubiquitously expressed throughout the body. However, a main focus of research has been on its roles in hearing, neuronal function, and smooth muscle tone. The loss of BK channels caused an age-related decrease in hearing that may be due to the channel's role in signal transduction of the cochlear inner hair cells and partly due to the maturation these cells during development. In neural tissues it has a variety of roles ranging from timing bursts of $Ca^{2+}$ flow in cells during  the action potential, regulating synaptic transmission, and compensatory reflexes to hypoxia(Salkoff et al., 2006). Interestingly, there is also a disease association where a gain of function mutation in humans increased channel activity and caused a non-specific epileptic disorder (Du et al., 2005).  This channel also plays a prominent role in the the smooth muscle. Mice without the channel exhibited hypertension, incontinence, frequent urination and erectile dysfunction (Salkoff et al., 2006). Though this has been an immense amount of work, the research is only beginning to elucidate more roles for the channel, indeed it has emerged as a potential therapeutic target for everything from asthma to cancer. Additionally, the work here and from our collaborators underscores its possible importance in regulating cardiac rhythm.

## 3.3 RESULTS

### *3.3.1 Cardiac Specific Expression of the BK Channel in Human and Drosophila Hearts*

In regards to cardiac research, the BK channel had heretofore not been identified in the plasma membrane of cardiomyocytes. Instead, the focus has been on the peripheral vascular tissues where channel activity reduced vascular tone and decreased blood pressure. However, a cardioprotective role for the channel emerged when it was discovered in cardiac mitochondria. Here it seems that it contributed hypoxic preconditioning of the heart; particularly in ischemia-reperfusion injury models (Xu et al., 2002) (Balderas et al., 2015). However, our collaborator Dr. Diane Fatkin, using standard immunostaining and electron microscopy techniques, found cardiac expression of the channel in human atrial and ventral cardiomyocytes (Figure 3.1, 3.2). Specifically they were found in the sarcolemma (3.2 B) intercalated discs (3.2 D) and co-localized with the gap-junction protein connexin 43 (CX43) and ryanodine receptor (RyR2) in human atrial tissues (Figure 3.1 A). Out of all the structures in the heart that they looked at, they found the highest level of expression at the sinoatrial node (SA) (Figure 3.1). When they looked at subcellular expression within cardiac cells with immuno-gold labeling and transmission electron microscopy, they found the channel localized at the sarcoplasmic reticulum and T-tubules (Figure 3.2). The BK channel cardiomyocyte localization at the sarcolemma and localization with $Ca^{2+}$ homeostasis structures had never been observed before. It suggests that this channel could have a role in the action potential in $Ca^{2+}$ homeostasis past the previously reported mitochondria bioenergetics. The strong expression in the SA node

also suggests that the BK channel functions in the pacemaker region of the heart, possibly to set the heart rate.

The functional consequences of the channel localization raised some of the questions I looked at in the physiological studies. Due to the aforementioned rapid heartbeat of the mouse, we used the fly as genetic model to elucidate a cardiac function for this channel. First, we looked at expression in the *Drosophila* heart. We were able to first show cardiac specific expression through RT-PCR (Figure 3.3).We also saw a stronger qualitative expression in the head of the fly versus the heart, but we still saw expression of not only *slowpoke* but the other major voltage-activated $K^+$ channels. We then examined expression using Fluidigm micro-fluidic technology. We looked at cardiac specific expression of different ion channels in a wild-type, transheterozygote *slowpoke* mutant (*slo$^4$* and *Df (3R) BSC 397*). We saw a steady expression of the *slowpoke* channel in the wild type control and strong downregulation in the transheterozygote mutant (Figure 3.4).

### *3.3.2 Cardiac Specific slowpoke Knockdown Causes Abnormal Cardiac Function*

After finding cardiac expression of *slowpoke* in the *Drosophila m.* cardiac tube, we tried to identify a cardiac role for the channel by genetically ablating it. We used the bipartite Gal4-UAS system (Duffy, 2002) to reduced expression of *slowpoke* specifically in the cardiac tube. I used a published Gal 4 cardiomyocyte specific driver, Hand 4.2, crossed with UAS *slowpoke*

channel RNAi from the Vienna RNAi Consortium (Han et al., 2006). Through our SOHA analysis we looked at young (1 week), middle aged (3 weeks) and old (5 weeks) knockdown flies (Figure 3.6). For our control crosses we used the RNAi line outcrossed to a wild-type strain and the Gal4 line crossed to Vienna RNAi consortium control; an RNAi control line with an attp insertion at the same landing site as the UAS-RNAi. Interestingly, cardiac specific RNAi knockdown of *slowpoke* resulted in a significantly increased heart period (HP) at 3 and 5 weeks of age that was due to significant increases in both diastolic (DI) and systolic intervals (SI). The increase in SI is interesting as this is rarely changed and indicative of long fibrillatory events. This was accompanied by a significantly increased arrhythmia index (AI) at 3 weeks of age in the knockdown versus controls. When we looked at fractional shortening (FS) as a measure of cardiac contractility, we did not see a significant difference between the knockdown and both controls. Although, the actual diameters of the knockdown fly were significantly smaller at 1 and 5 weeks of age as compared to controls (Figure 3.6). Hence, although the heart overall was smaller at these ages, the relative of both the diastolic and systolic diameters maintained a normal value for FS.

I used a different cardiac specific driver (Hand2-Gal, made in-house from Hand 4.2 in order to limit expression only in cardiomyocytes and not in pericardial cells) crossed to the RNAi lines described above to confirm my initial results. I found that Hand2 driven knock down had a significantly

increased HP at 5 weeks of age due to significantly increased DI and SI (Figure 3.8). We also combined the stronger Hand 4.2 cardiac Gal4 with another RNAi line (GD slo). The effect of the new RNAi line mirrored the previous ones (Hand 4.2> KK slo), cardiac knockdown exhibited significantly increased HP,and AI at 3 weeks of age. One difference was a significantly increased HP at five weeks of age although the increase in the AI was not significantly different from controls at that age (Figure 3.8).

To distinguish if this was a cell autonomous effect we used a panneural Gal4 driver (ELAV-Gal4) in combination with our previously used KK RNAi line. When we examined 3 week old flies we found that neural knockdown of the channel only had a minor effect if at all on cardiac parameters (Figure 3.9). Since our Hand 4.2 Gal4 driver is strongly expressed in the *Drosphila* pericardial cells, we wanted to differentiate any pericardial knockdown phenotype. We drove *slowpoke* knockdown specifically in the pericardial cells using pericardial specific Dorothy Gal4 and measured cardiac function. We found no significant effect on heart period or arrhythmia index at 3 weeks of age (Figure 3.9). These results suggest that our phenotype was a cardiac cell autonomous effect.

***3.3.3 Cardiac Specific Knockdown Causes Cardiac Electrical Dysfunction***

Having quantified the effects of knockdown on mechanical contraction, we then used intracellular current clamping to record the electrical activity of *slowpoke* knockout hearts versus control.  The representative 10 second

electrical trace of a control fly shows a typical *Drosophila* cardiac action potential. The *slo* knockout action potentials (APs) exhibit a bursting phenotype with more peaks per event and also significantly longer individual event duration shown in Figure 3.10, quantified in 3.11 and Table 3.1. The increased number of peaks is reminiscent of early after depolarization (EAD) seen in human arrhythmias and likely associated with the observed longer systolic interval (Fig 3.7 C).

The relaxation period between events quantified as the inter-event interval was also significantly longer in the knockdown genotypes as compared to controls. A longer inter-event interval was previously seen in mouse pacemaker cells when KCNMA1 was knocked down (Lai et al., 2014) However, this same effect was also observed in human induced pluripotent stem cells (iPS) when the SK (small $K^+$) $Ca^{2+}$ sensitive was knocked down (Weisbrod, Khun, Bueno, Peretz, & Attali, 2016). Although, neither study was conclusive, it suggests that one or both channels could be acting in concert to regulate the pacemaker firing rate and diastolic depolarization. The resting membrane potential (RMP) of a cell denotes a period when no dynamic voltage change occurs and is crucial for cellular excitability. The knockdown had a lower RMP but both knockdown and control where within normal values seen in other control genotypes (-40mV-60mV). Additionally, although the control had higher maximum AP amplitude, both control and knockdown

values were still within normal functional ranges. Overall, the observed extra peaks and event length is likely due to the absence of *slowpoke*.

While we sometimes saw extra peaks in the electrical activity of the control genotypes, we always saw a greater number of peaks per burst for the *slo* knockdown (Figure 3.9). However, we wanted to compare not just the length and number of electrical events, but also how the overall depolarization and repolarization curves were affected. In order to try to determine this, we average all traces for each of the 30 second record per genotype, and are able to see the strongest signal within some arrhythmic traces (Figure 3.10, C). When we match the average traces the depolarization curve was not affected but the knockdown genotypes have a repolarization curve that takes a longer period of time to reach resting RMP.

### *3.3.4 Slowpoke Knockdown Causes Mild Morphological Remodeling*

We quantified the diameters during relaxation and contraction and while found some significant differences compared to control, overall cardiac contractility (FS) was not affected (Figure 3.6). However, since we did have an effect on cardiac function, we wanted to determine if this effect carried over to a strong morphological phenotype. While from our functional measurement the diameters were significantly smaller than controls (Figure 3.6), Immunofluorescent staining of myofibril revealed only moderate myocardial disorganization was observed (Figure 3.13). This minor disorganization was characterized by somewhat loose arrangement with greater gaps between

myofibrils compared to controls and does not relate to diameters or FS. The slower heart rate, arrhythmia and longer individual electrical events suggest that this channel is important for regulating the heart rate but does not cause a strong structural abnormality.

### *3.3.5 Constitutive Loss-of-Function of Slowpoke Recapitulates Cardiac Knockdown Effects*

To further verify the age related bradyarrhythmic effects of the cardiac specific *slo* knockdown were not due to artificial transgenic interactions, we tested the cardiac function of *slowpoke* genomic mutants $slo^4$, $slo^1$, and a deficiency line- *Df (3R) BSC 397.* We chose the $slo^4$ EMS inversion mutant due to its viability and since it was one of the classical genomic mutants. The *Df (3R) BSC 397* line has a complete deletion of the *slowpoke* locus and is homozygous lethal, so in combination with a more classical genomic mutant, allowed us to completely abolish any *slowpoke* expression or compensatory mutations. We used this exact strategy to combine *Df (3R) BSC 397* with $slo^4$ ($slo^4$ and *Df (3R) BSC 397*) as transheterozygotes to help eliminate genetic background or compensatory mutation effects,

The genomic mutants $slo^1/slo^1$ and $slo^4/$ *Df (3R) BSC* at 3 weeks of age all had a significantly higher HP, DI, and SI and AI compared to wild-type and heterozygote controls (Figure 3.14 A, B, C). By five weeks of age, however, this effect was diminished and these mutants only had significantly larger values than control $W^{1118}$ but not in comparison with the transheterozygote

controls. The *slo⁴/+* heterozygote control in particular had a longer SI, DI and HP than the other controls although not significant, and by five weeks it had a huge increase of the DI, SI, and HP (Figure 3.14 A, B, C). Without this control the aforementioned genomic mutants *$slo^1/slo^1$* and *$slo^4$/ Df (3R) BSC* have significantly larger AI,DI, and SI. The reason for the huge increase in cardiac parameters in the *$slo^4$/+* heterozygote is unclear. The *$slo^4/slo^4$* homozygous genomic mutant in fact is not heavily affected and has the lowest AI, HP, DI and SI of all the other genomic mutants (Figure 3.14 A, B, C). I was not able to find an exact reason for this discrepancy but as will be discussed in 3.3.7 associated lethality with *slo* knockdown could be hiding a worse cardiac phenotype of homozygous genomic mutants. Similar to the cardiac specific knockdown (Figure 3.6, 3.7, 3.8) we did not see a significant effect on cardiac function at 1 week of age or an effect on FS at any age. The bradyarrhythmic effect of the genomic mutants was similar but generally stronger than that of the cardiac specific knockdown.

In the same methodology as the cardiac specific knockdown, we measured the voltage across cardiomyocyte membrane of genomic knockdown and controls. Electrically, the event duration and peaks per burst were all significantly longer in the mutants measured as compared to controls and again reminiscent of EADs (Figure 3.10, 3.11 Table 3.1). The event duration was significantly greater than the cardiac specific knockdown and although it was not significant, there was a trend towards greater peaks per

burst in the genomic mutants. When we normalize the repolarization curve of the transheterozygote, *slo*$^{4}$ homozygous, and *slo*$^{4}$ heterozygote we do see increase in the period of time to RMP. However, the lengthening of the repolarization curve is not longer at every single period on the curve. The *slo*$^{4}$/*slo*$^{4}$ mutants do take longer to reach RMP but are closely matched for most of the curve with the control lines. Meanwhile the other transheterozygote has curves that are wholly separate and take longer to repolarize at every point in the curve (Figure 3.12 A, B). The RMP of the mutant and control were similar but we did see somewhat abnormally high peak amplitude for the transheterozygote compared to controls. Overall, the genomic mutants have a more extreme but similar phenotype as the cardiac specific knockdown. The replication of the bradyarrhythmic phenotype over different UAS-RNAi lines, Gal4 drivers, and genomic mutants in both suggest that the *slowpoke* channel functions to regulate heart rate.

#### ***3.3.6 An Extra Genomic Copy of Slowpoke Rescues Mutant Cardiac Phenotype***

We wanted to test the effect of overexpressing *slowpoke* and whether we could rescue the cardiac phenotype, so we developed a transgenic fly line that adds extra copies of the endogenous *slo* locus. We used a bacterial artificial chromosome construct containing the *slo* locus developed by the Hugo Bellen lab and injected this into wild type flies to get the extra copies (Venken et al., 2009). When used in concert with genomic mutants, this line

(called PBac Slo) was able to rescue all of the genomic mutant cardiac phenotypes (3.15). It is worth noting however, that Individually, the PBac slo line had a negative effect on cardiac function as either one or two extra genomic copies in a wild-type background elicited a huge increase in heart period due to both systolic and diastolic intervals. This effect could underscore the importance of genetic dosing as too little or too much of the channel was detrimental to cardiac function.

#### *3.3.7 Slowpoke Knockdown Affects Lifespan*

While carrying out the cardiac specific knockdown, we noticed that a higher proportion of the knockdown flies died as compared to the control. Additionally, we noticed that it was more difficult to age the genomic mutants to the 5 week time point. In order to try to quantify this, we carried out a survival assay with 100 flies each of cardiac specific knockdown and control. We saw that more than %50 of the knockdown flies died within the first week (Figure 3.16). We kept flies in vials on their side to avoid possible drowning in food, but still had this die off. In order to examine these early deaths, we looked at 2, 3 and 4 days post-eclosure knockdown flies in or to determine if there was an early, lethal cardiac phenotype that we were missing. However, none of the parameter s we looked at were significantly different than the 1 week knockout parameters (Figure 3.17). Additionally, although we saw moderate myofibrillar disorganization, we did not think that it was sufficiently worse than the 3 week knockout time point to explain the early lethality. The

associated early lethality with cardiac specific knockdown of *slo* made us think there could be some developmental or nonspecific genetic effect responsible for lethality.

#### *3.3.8 Adult Specific Knockdown Greatly Increases Arrhythmias*

In order to rule out developmental effects, we knocked *slo* down in adult only flies. This was performed using a conditional Gal4 cardiac specific driver called Hand-GeneSwitch (GS). The GS system functions by administering Mifepristone (RU-486) directly to the food of the flies for transgene induction (Osterwalder et al., 2001). Similar to our initial knockdown, we used UAS-RNAi and control RNAi lines in combination with the Hand-GS inducible driver. We further stratified these groups into induced and noninduced (+ or No RU486). At 1 week of age the induced knockdown had a very strong fibrillatory phenotype and the induced control line was phenotypically normal (Figure 3.18). The induced knockdown had significantly higher HP (A), DI (B), SI (C) than either the non induced lines RU genetic control. At an early age the RU adult knockdown strongly increased the bradyarrhythmic phenotype we saw before. The *slo* adult specific cardiac knockout showed that our bradyarrhythmic phenotype was not due to developmental effects using both genetic and drug controls.

At 3 weeks of age we again saw an increase in HP, DI, and SI in RU fed *slo* knockdown hearts compared to No RU486 flies. However, in RU486 fed control flies (Hand GS> RNAi Control+ RU486) I saw a longer heart

period. The induced adult *slo* knockdown did not have a significantly longer HP (A), DI (B) or SI (C) than this control. Nevertheless, there was a very significant increase in the arrhythmia index of the induced knockdown flies (Figure 3.18). Importantly, we never saw the fibrillatory events that characterized the induced knockdown in the control lines (Figure 3.18).

The arrhythmic phenotype also had a pronounced effect on the electrical function. We saw significant increase in the number of peaks per burst and long event duration reminiscent of the cardiac and genomic *slowpoke* knockdown (Figure 3.19, Figure 3.20,Table 3.2). While the peak amplitude was within normal range, the RMP was significantly lower than any other genotype that we looked at. Additionally, due to the strong arrhythmicity of the induced knockdown, the average repolarization curve in the knockdown was strongly irregular and took longer to reach RMP versus the control (Figure 3.21).

### *3.3.9 Expression Profile of Genomic slo Mutants and Wild-Type Controls*

We next looked at the expression profile of *Drosophila m.* cardiac ion channel homologs in a transheterozygote *slo$^4$/ Df (3R) BSC* and wild-type Canton-S (CS) *Drosophila.* hearts. The differences between wild-type and transheterozygote could highlight compensatory mechanisms. We used nanofluidic qPCR arrays on a Biomark system (Fluidigm), according to their protocols; figure 3.5 and a larger part of the data set from Figure 3.4 reported in 3.3.1. The panel of channel genes that we looked at carry many of the

important cardiac electrical currents. We quantified expression of the *CACNA1S* homolog *Ca-α1D*, the *hERG* (*KCNH2*) channel homolog *seizure*, *KCNQ1* homolog *KCNQ1*, IRK (*KCNJ12*) homolog *IRK*, *HK2* (*KCNA5*) homolog *shaker*, sulfonylurea receptor (*SUR*) homolog *SUR*, the *HCN4* homolog *Ih*, and the *KCNMA1* homolog *slowpoke*. We saw a downregulation of gene expression in all of these channels (Figure 3.5). As expected *slowpoke* had the strongest expression reduction in the transheterozygote compared to the wild type control. The *hERG* and *KCNQ1* genes, which encode the major delayed rectifier currents, appeared to be downregulated in the transheterozygote mutant compared to the CS control. We also quantified expression of the *KCNH1* homolog *elk*, a voltage-activated $K^+$ channel that to date has no cardiac function. We found a slight upregulation of this gene but this change was not significant when calculated from the ΔCt values. Additionally, we looked this panel expression in *seizure* mutant *Seizure*$^{ts1}$. Although not as strong, we a found a similar downregulation of these channels compared to control (CS).

***3.3.10 Pharmacological Manipulation of Slowpoke Channel was inconclusive***

We sought to confirm the results of our genetic manipulations pharmacologically using specific agonists and antagonists. We set up an acute assay that consisted of adding a concentration of either an antagonist or agonist to a dissected *in vivo* prep. We tested 1uM, 5uM, 10uM and 20uM

concentrations of NS11021 BK agonist and Iberiotoxin antagonist. We set up incubation periods ranging from 0, 1min, 5min, and 10min. From these pilot experiments, a 10uM concentration and 10min incubation period for both agonist and antagonist changed HP without inducing ultra slow heartbeat or periods of fibrillatory action in both knockdown and control. While some results were encouraging, I found that the effect on the cardiac parameters we measured was just too varied and we unable to confirm any phenotype (Figure 3.22). Initial studies reported that neither iberiotoxin or another BK channel blocker charybdotoxin were effective in the fly (Toro, Wallner, Meera, & Tanaka, 1998). Yet, these results came from heterologous expression systems and the studies looking at the native channel showed charybdotoxin sensitivity in *Drosophila* flight muscle and to both iberiotoxin and charybdotoxin in the cockroach (Derst et al., 2003; Elkins et al., 1986). It is possible that iberiotoxin is either not effective and that our working concentration is too high and/or unspecific. In aforementioned heterologous expression systems the IC50 was around 450nM, yet at both inhibitors in the fly muscle and cockroache were used around 200nm. For the agonist there was no previous data relating specifically to the fly but in *Xenopus* oocytes the EC50 was 0.4uM and concentrations of up to 10uM did not affect other common $K^+$ channels (Bentzen et al., 2007). However, Langendorff perfused hearts using a working concentration of 500nM reported off-target effects (Wojtovich et al., 2013). An alternative approach would have been to try

fungus based BK antagonist such as Paxilline. Additionally, a better approach would be to test both new inhibitors and agonist in a heterologous expression system based off *Drosophila* channels before moving to functional assays.

**3.4 DISCUSSION**

The molecular identity of ion channels driving the cardiac action potential has been a constant process of discovery. The BK channel was originally not thought to contribute to the cardiac action potential due to the lack of any overt cardiac phenotype in genetic mutants. Yet, the genomic location of the gene in a possible AF locus and its $Ca^{2+}$ activation made it an attractive candidate as contributor to cardiac function. Indeed our own collaborator was able to find it localized to human cardiac tissue, with the highest level of expression found in the SA node. They also found an association between the channel and a familial AF. All of which pointed to the importance of the channel in regulating heartbeat.  The discrepancy between mouse and human function could be explained by the different channels in each ornganism that underlie cardiac repolarization. Due their rapid heartbeat, the important repolarizing currents carried through *hERG* and *KCNQ* channels in the human are almost completely absent in the adult mouse heart. Work in our lab has explored the *hERG* and *KCNQ* channels important for human cardiac function and found them to also be important for *Drosophila* cardiac function. In this same fashion we could use *Drosophila m.* as a model to study cardiac function for the BK channel homolog *slowpoke.*

Using this model system we were able to show *slowpoke* gene expression in the *Drosophila* heart. Reverse Transcriptase-PCR, and qPCR indicated cardiac specific expression of this and other voltage activated channels in the *Drosophila m.* heart. This method, however, did not address regional specificity for channel localization. My data indicates that *slo* is important in regulating *Drosophila m.* cardiac contractions and thus could be important in the pacemaker regions. This idea is supported by the high level of expression found in the human SA node but genetic tagging or better antibodies are needed to verify the localization. Functionally, both systemic mutants and cardiac specific knockdown resulted in a significantly longer heart period and arrhythmia index at 3 weeks of age compared to controls. The increase in the heart period was due to increases in both the diastolic and systolic intervals. Thus, these hearts spend a longer period of time during the relaxation period and also have double beats and trains of abortive contractions that lengthen the systolic period and make up some of the variability of the overall heart period and subsequent arrhythmia index. Because, controls also exhibited the normal age related increase in these parameters (Ocorr et al., 2007), which diminished the effect at the 5 week time point.

We saw similar effects play out in the electrical function of the heart as the same knockdown genotypes exhibited significantly longer event durations and more peaks per burst than controls. However, due to the process of

impalement, drug used to stop cardiac contractions, and greater sensitivity relative to the optical assay, we can not exactly match each recording to a Mmode (Motion Mode). When we carry out dual optical and electrical recording that we do match up for small period of time (Figure 3.23). Regardless of experimental effect, in the control recordings we never see the strong fibrillatory events with huge numbers of peaks per burst that characterize the mutants. In the *Drosophila m.* larvae genetic and pharmacological inactivation of *slowpoke* channels had a dramatic effect on heart rate (Johnson, Ringo, Bray, & Dowse, 1998). However, this larval did not address adult cardiac function or probed electrical function whereas our study looked at the adult cardiac autonomous function of *slowpoke.* The slower, arrhythmic heart rate and wider repolarization curve, suggest this channels functions in setting the repolarizing potential of the heart. The changes in cardiac function that we saw could be due to compensatory changes in the expression of other ion channels that we saw and we need more research into the effects of $K^+$ channel knock down in order to determine if this is a global effect of $K^+$ knock down or specific to the *slowpoke* phenotype. Additionally, although we have several lines of evidence that point to the importance of *slo* in the heart, we cannot differentiate between this effect being specific to a mitochondrial bioenergetics or a direct role in the action potential through the cell membrane. One of the ways we could differentiate this and indeed would

be able to further determine the function of *slo* in the heart would be to record currents in the fly heart that may possibly uncover a $Ca^{2+}$ sensitive $K^+$ current.

The early lethality associated with *slowpoke* knockdown suggested a possible development effect. We quantified some 80% die off compared to control in the cardiac specific knockdown and I was able to age very few genomic mutants to 5 weeks of age. I looked at early cardiac phenotypes of function and structure but found that none of these bore out an explanation for the sudden early lethality. We used an adult specific knockdown and saw the same cardiac phenotype of significantly longer HP and AI of the cardiac specific and genomic mutants, but this time it was greatly enhanced and started at the early 1 week time point. Yet, the RU486 drug slowed down the heart by 3 weeks of age so that the prime difference between the genotypes was the strong fibrillatory phenotype in our induced adult knockdown. The fibrillatory, EAD (Early After Depolarization) phenotype is important as it calls to similar phenotypes observed in humans due to dysfunction of important repolarization $K^+$ channels. Additionally, this phenotype was much stronger than that of the early cardiac specific knockdown or genomic mutants. Both of these knockout *slo* during early stages of development and could lead to some compensatory mechanisms to abrogate any *slo* phenotype, which we see emerge in the adult specific knockdown. All of these pieces of evidence point to *slowpoke* being important in control of cardiac rhythm. As to the regional specific importance of the channel for this function, we cannot rule out that this

effect could be due to the channel's role in the mitochondria. However, compounding expression evidence from our collaborators shows strong expression not just in human and mammalian mitochondria but at a host of important $Ca^{2+}$ regulatory structures such as the sarcolemma, sarcoplasmic reticulum, T-tubules, and L-type $Ca^{2+}$ channels. Enrichment in the SA node and our own electrical studies suggest that although it may have electrical properties in the mitochondria, it could have a dual role as a negative feedback regulator of $Ca^{2+}$ homeostasis.

It was not until recently that interest in the human homolog KCNMA1 as a possible determinant of cardiac pacemaker function was rekindled. (Imlach et al., 2010) noted that pharmacological inhibition of *KCNMA1* using paxilline and iberiotoxin reduced heart rates in wild-type mice and in isolated rat hearts but had no effects in *KCNMA1*-/- mice. Moreover, resting heart rates in *KCNMA1*-/- and wild-type mice were similar. Subsequent analysis of isolated sinus node cells from *KCNMA1*-/- mice did show reduced baseline firing rates when compared to wildtype cells and it was proposed that the absence of bradycardia in intact animals was due to compensatory activation of the sympathetic nervous system. The bradyarrhythmic phenotype that we see here is interesting since it stands somewhat in contrast to other voltage-activated $K^{+}$ mutations which primarily associate with bradyarrhythmia, EADs and ventricular tachycardia rather than AF (Wilde & Bezzina, 2005). In our system we show a strong effect on heart rate and rhythm. Additionally, we

were able to knockout *slowpoke* in ancillary tissues of the neural system. This along with the deinnervated methodology of our dissections, points to the bradyarrhythmic phenotype being a cell autonomous phenotype due to a lack of *slowpoke*. Replicating the pharmacologic experiments in (Imlach et al., 2010) would be helpful but our pharmacological manipulations showed too variable of results. However, genetically we were able to show that the cardiac effect we saw was not only specific but could be rescued with an extra endogenous copy of *slowpoke.*

A $Ca^{2+}$ sensitive $K^{+}$ current has recently been looked at as a determinant of firing rate in human cardiac embryonic stem cells. However, the putative channel responsible for this function was the Small K channel (SK). Through both GWAS and candidate gene studies the SK has been identified as a possible cause of AF (Gutierrez & Chung, 2016). The theorized mechanism of function in cultured in the human stem cells was as a fine tuner of diastolic depolarization in concert with $I_f$ current and NCX transporter (Weisbrod et al., 2016). When this channel was blocked it elicited a bradycardia phenotype similar to the knockout phenotype we see in the fly. Perhaps, the BK channel functions in the phase 4 cardiac action potential during a period of higher $Ca^{2+}$ intracellular concentration and more positive membrane voltage. This fine tuning mechanism could also apply to the initial repolarization phase of the pacemaker action potential. This would place this

channel as an important yet finely controlled feedback regulator of $Ca^{2+}$ and membrane voltage.

## 3.5 ACKNOWLEDGEMENTS

Parts of Chapter 3 and Chapter 4 are being preparedfor joint publication with coauthors: Santiago Pineda, BA; Vesna Nikolova-Krstevski, PhD; Christiana Leimena, PhD; Andrew J. Atkinson, MPhil; Arie Jacoby, PhD; Inken G. Huttner, MD; Yue-Kun Ju, MD, PhD; Magdalena Soka, BSc(Hons); Gunjan Trivedi BSc(Hons); Renee Johnson, PhD, MGC; Dennis Kuchar, MD; Jamie I. Vandenberg, MB BS, PhD; David G. Allen, PhD; Halina Dobrzynski, PhD; Karen Ocorr, PhD; Rolf Bodmer, PhD; Diane Fatkin, MD. The working title of the manuscript is: “The Large Conductance Calcium-Activated Potassium Channel, $K_{Ca}1.1$, is a Novel Contributor to Sinus Node Function and Arrhythmia Risk”

Dr. Vesna Nikolova-Krstevski, did immunofluorescence and electron microscopy in Chapter 3. Various members from Dr. Diane Fatkin’s lab helped in developing the mutation used in Chapter 4. The dissertation author performed all other experiments.

## 3.5 Figures

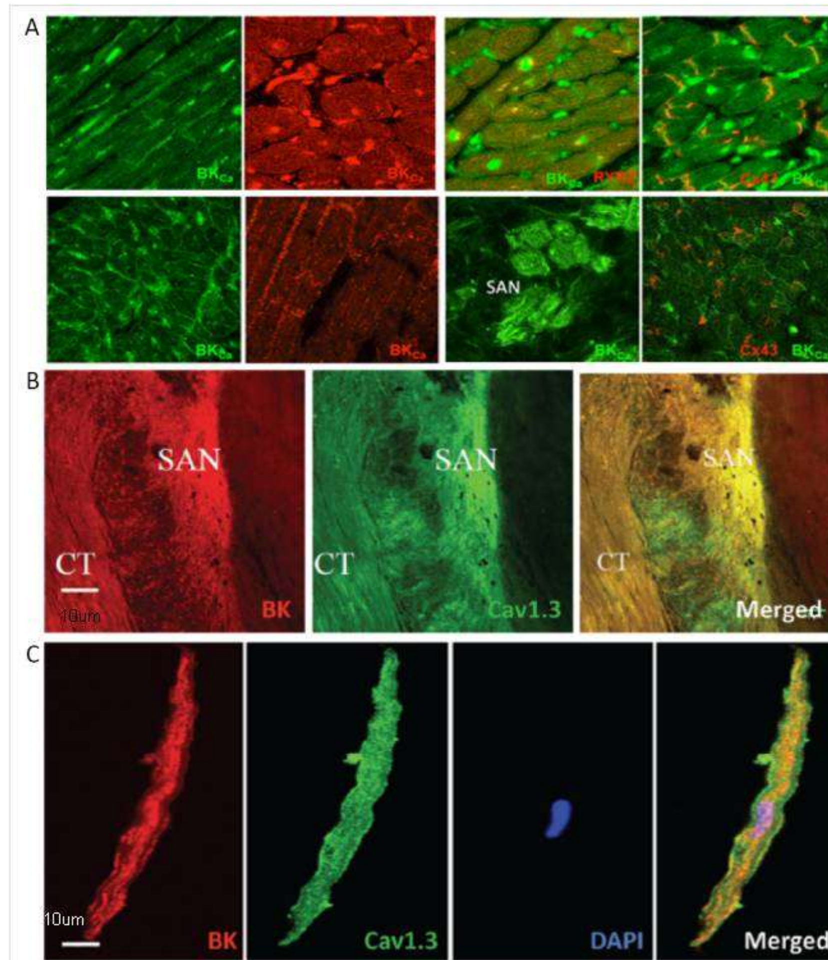



**Figure 3.1. $K_{Ca}$1.1 in the human atrium**. (**A**) Immunostaining of human right atrial tissue sections shows co-localization of $K_{Ca}$1.1 with the ryanodine receptor (RyR2) and connexin-43 (Cx43), with marked $K_{Ca}$1.1 expression in the sinus node (right panel); scale bar = 5 µm. (**B**) Whole mount immunostaining of the murine sinoatrial node (SAN) complex. $K_{Ca}$1.1 is expressed throughout the SAN and paranodal connective tissue (CT) and co-localizes with the L-type calcium channel, $Ca_v$1.3; scale bar = 100 µm. (**C**) Isolated murine atrial cardiomyocytes show $K_{Ca}$1.1 co-localization $Ca_v$1.3; scale bar = 10 µm.

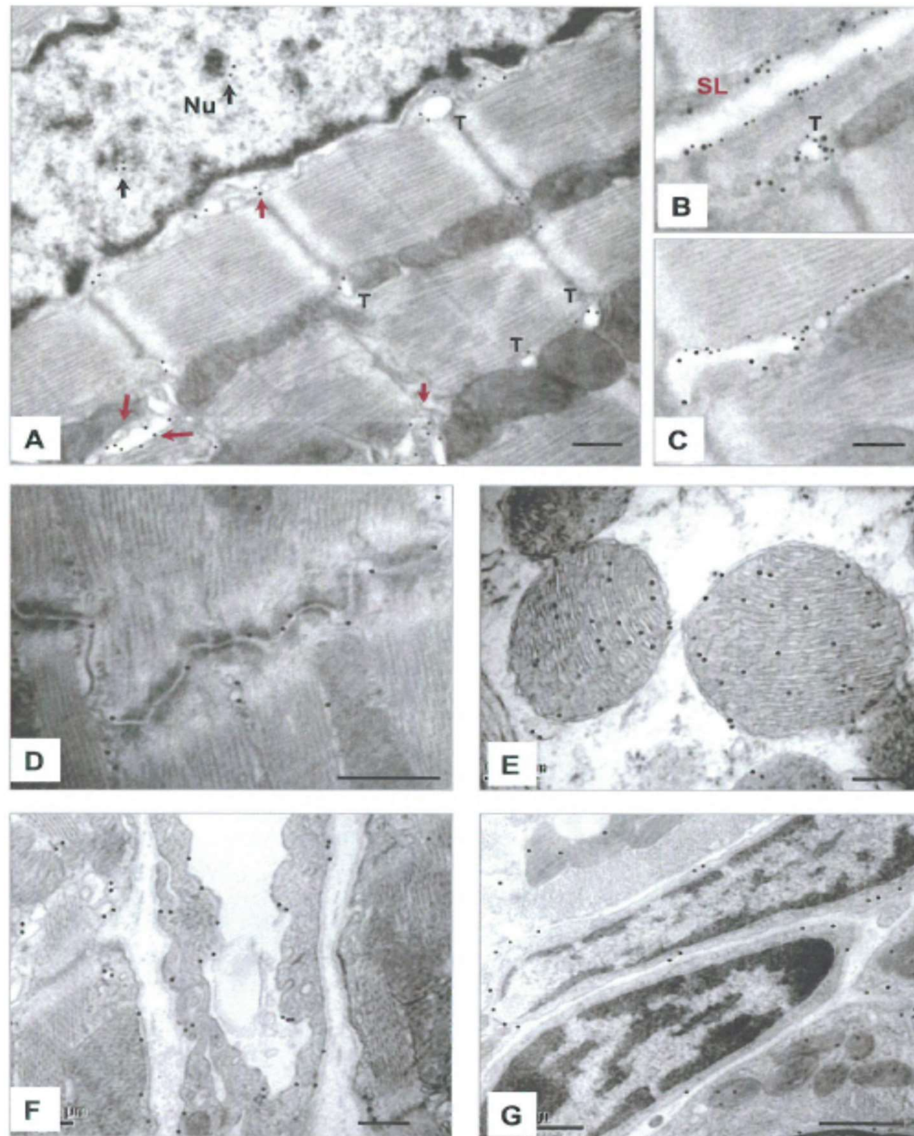
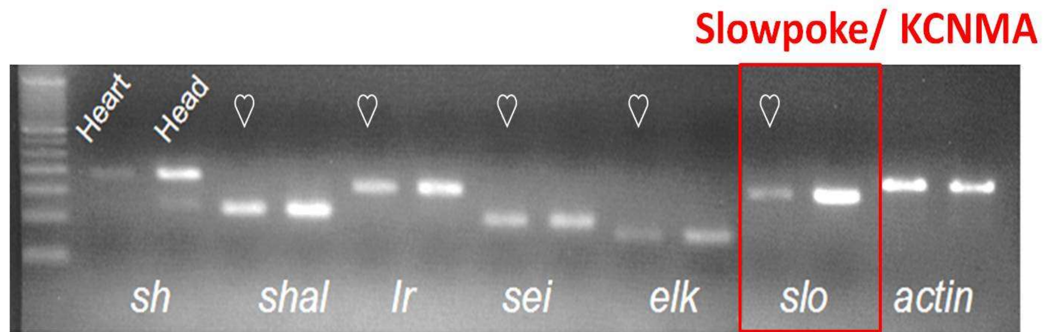



**Figure 3.2. Immunogold electron microscopy of $K_{Ca}$1.1 in human atrial tissue**. (**A**) In atrial cardiomyocytes, colloidal gold labelled $K_{Ca}$1.1 (black dots) was present in the nucleus (nu), T-tubules (T), and sarcoplasmic reticulum (red arrows). Double-labelling of $K_{Ca}$1.1 (15 nm particles, large black dots) and L-type calcium channel, $Ca_v$1.2 or ryanodine receptor-2 (10 nm particles, small block dots), shows $K_{Ca}$1.1 in (**B**) the sarcolemma (SL) and T-tubules (T), and (**C)** sarcoplasmic reticulum. $K_{Ca}$1.1 was also present in (**D**) intercalated discs, (**E**), mitochondria, (**F**) coronary vascular endothelium and (**G**) fibroblasts. Scale bars = (**A,D,E**) 0.2 μm; (**B, C**) 0.1 μm; (**F**) 0.5 μm; (**G**) 1 μm.




*sh* ➔ *shaker*
*shal* ➔ *shaker-like*
*Ir* ➔ *Inward Rectifier*
*sei* ➔ *seizure* (hERG homolog)
elk ➔ ether-a-gogo-like
*Slo* ➔ *slowpoke* ($Ca^{2+}$ - activated $K^{+}$ channel)

**Figure 3.3 RT-PCR of different voltage-activated $K^{+}$ channel homologs**. Expression is shown in both the head and the heart of *Drosophila m.* While these channels are more strongly expressed in the head, they are still present in the heart.

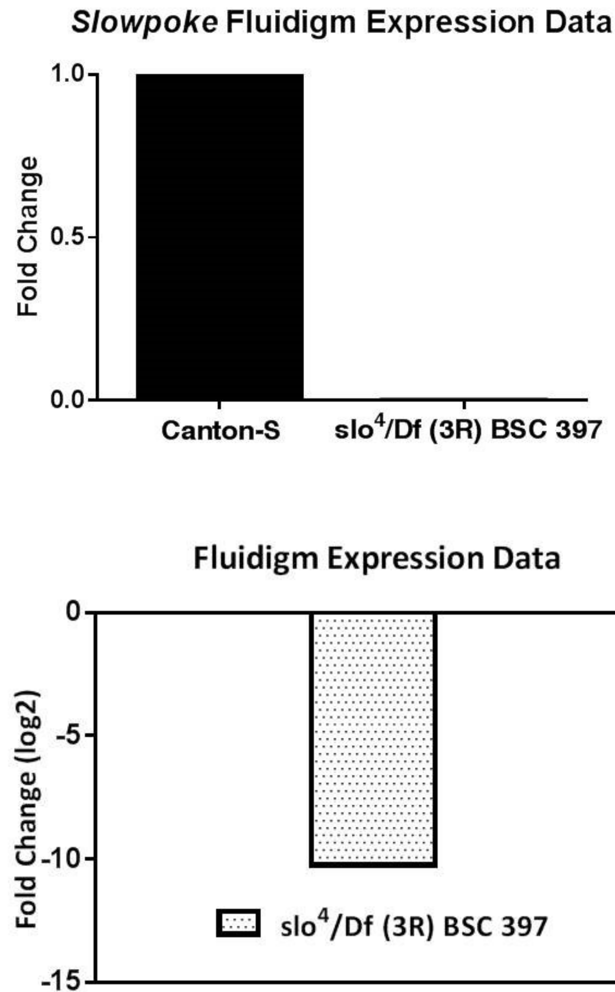



**Figure 3.4 Fluidigm Expression for *slowpoke* in a transheterozygote mutant.** Expression in the heart of *slowpoke* mutant $slo^4$/Df (3R) BSC 397 was downregulated 100 fold in comparison to wild type Canton-S (CS) fly hearts. On the left is the relative expression levelschange with control represented as 1 and on the right a log base2 representation that better shows downregulation of expression with control as 0.

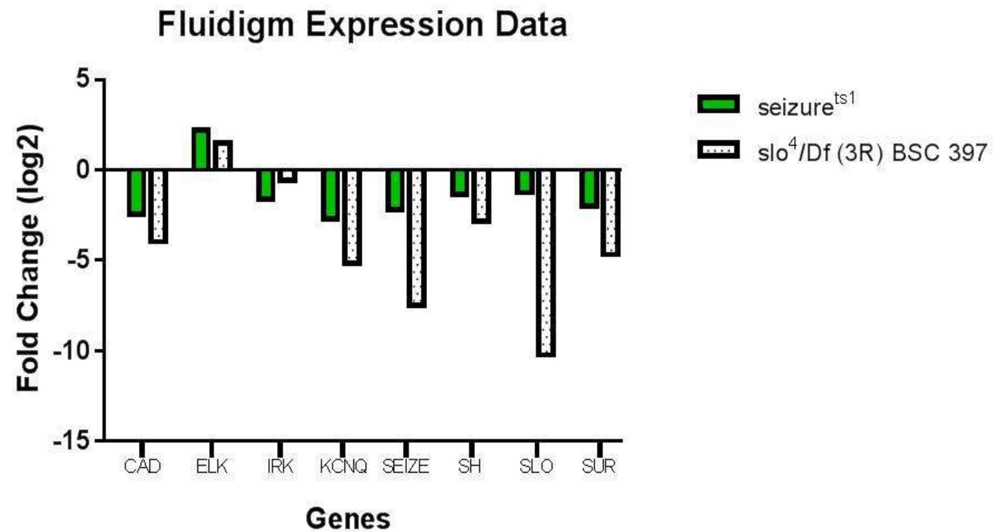



**Figure 3.5 Fluidigm expression data for different ion channels.** An expanded panel from the same experiment in Figure 3.4. We looked at cardiac specific expressionIn two ion channel mutants as compared to a wild type genetic background. The wild-type background was Canton-S. The Seizure ts1 shown in green is a mutant of the hERG Drosophila homolog. The clear dotted bars are expression data for the transheterozygote of the kcnma1 Drosophila homolog slowpoke. We tested for expression of major human channel homologs:
KCNQ- The KvLQT Drosophila homolog
Ca-alpha1D- CAC1AD (L type calcium channel) homolog
Elk- KNH5 homolog
Ir- Inward rectifiying homolog, KCNJ12
Sei- hERG homolog, KCNH2
Sh- KCNA5 homolog, shaker
Slo- slowpoke, KCNMA1 homolog
SUR- sulfonylurea receptor that associates with the ATP sensitive Potassium channel KATP.
The absence of slowpoke caused a general decrease in expression of all the channels except for ELK.

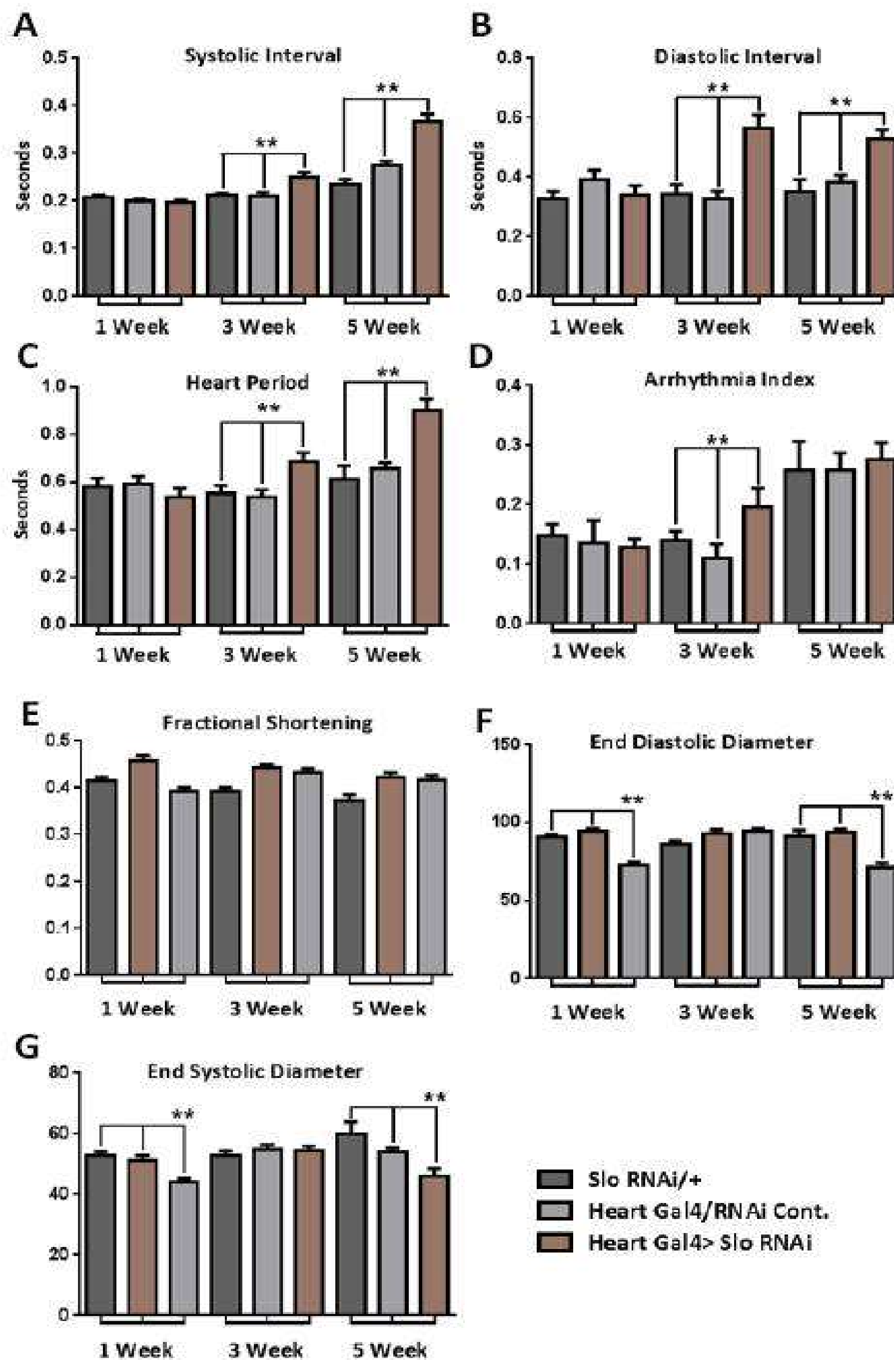



**Figure 3.6 Cardiac specific knockdown of *slowpoke* worsens cardiac function.** Cardiac specific knockdown of *slowpoke* significantly increases SI (A), DI (B), HP (C) at 3 and 5 weeks of age compared to controls. The AI was also increased but only at 3 weeks of age (D). The FS (E) was not affected although there were differences in the DD( F) and SD (G).

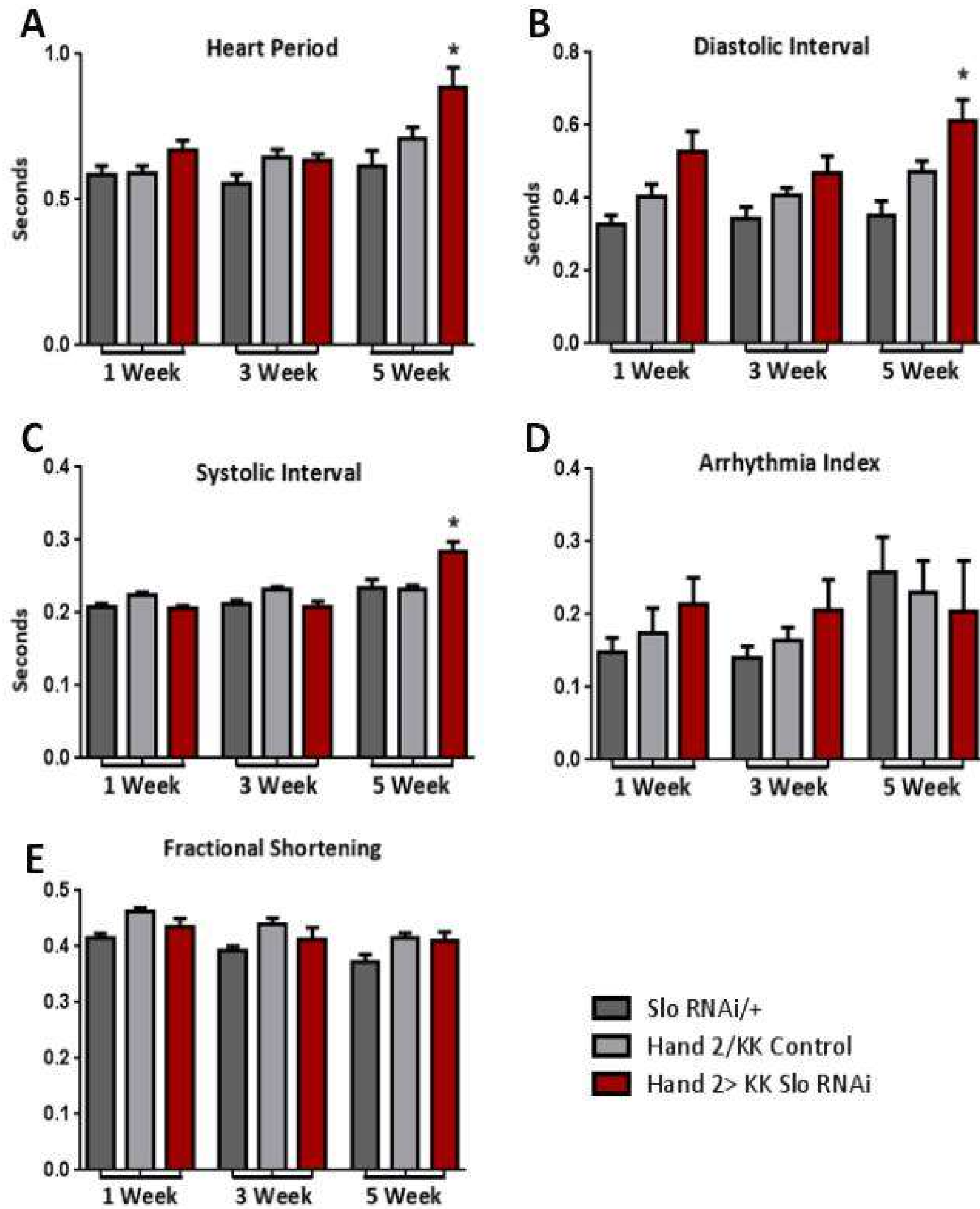



**Figure 3.7 A different cardiac specific Gal4 causes increased heart period.** When we used a weaker cardiac specifc Gal4 (Hand 2) we saw an increased heart period (A), diastolic interval (B), and systolice interval (C) but olnly at 5 weeks of age.

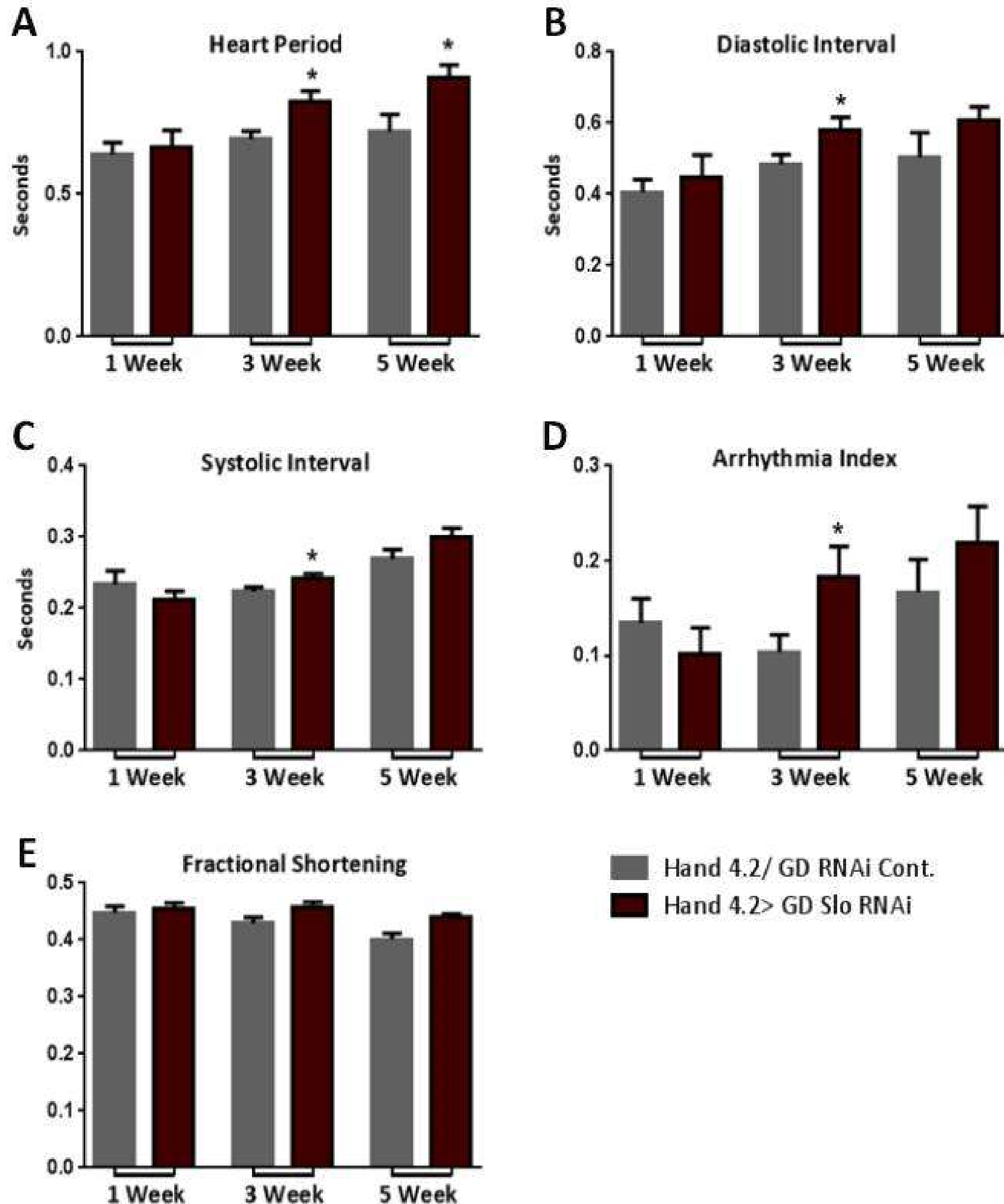



**Figure 3.8 Other RNAi lines replicate cardiac phenotype of *slowpoke* knockdown.** When we use another RNAi line (KK) from the Vienna RNAi consortium we are able to replicate the long heart period (A) and arrhythmia index (D) that we saw with the KK rnai line. Age related increases in the heart period diminished this effect by 5 weeks of age

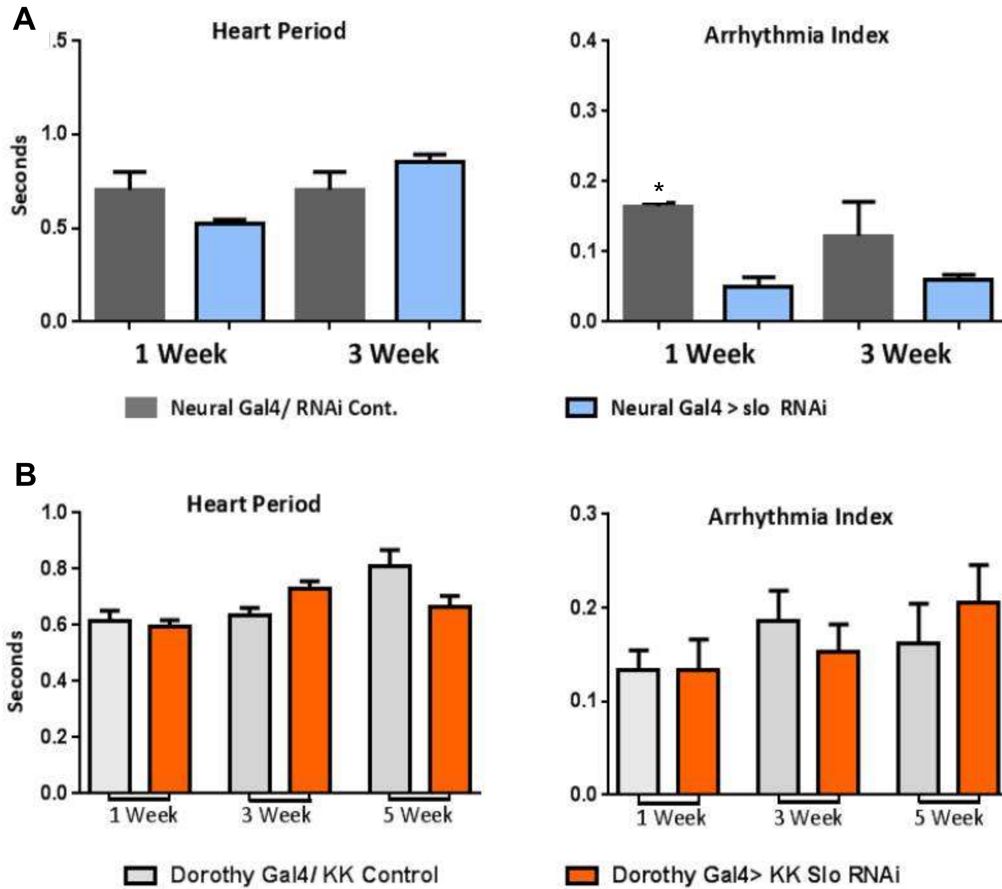



**Figure 3.9 The cardiac effect of *slowpoke* knockdown is cell autonomous.** When we knockout *slowpoke* in either neural tissues or pericardial cells, we do not see a significant effect on arrythmia index. The AI of the pannerual knockdown (ELAV-Gal4) is in fact significantly lower than the neural control.

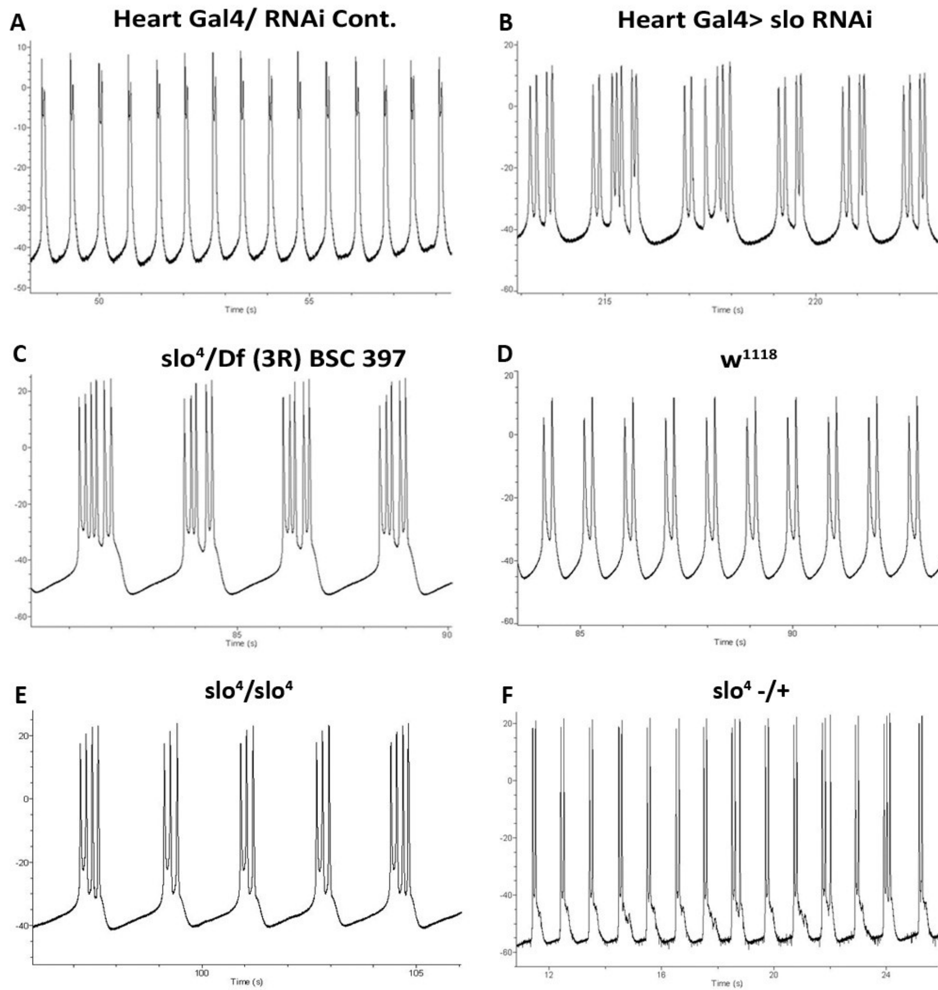



**Figure 3.10 Electrophysiological evaluations of *Drosophila slowpoke* mutants**. Ten second representative traces of the electrical activity within controls (**A**, Heart Gal4/RNAi Cont.; **D**, $w^{1118}$; **F** $slo^4$+/- and , *slowpoke* knockdown (**B**, Heart Gal4> *slo*-RNAi; *(***C**, *$slo^4$/Df(3R)BSC397*), **E**, $slo^4$/ $slo^4$. The Y axis represents voltage in millivolts (mV) and X axis is time in milliseconds (Ms). In all of the *slo* knockdown genotypes we saw increased peaks per event and significantly higher event duration as compared to controls, which is quantified in Table 3.1.

**Table 3.1 Quantitative data from intracellular recording**. The cardiac-specific knockdown, cardiac adult-only knockdown and genomic mutants all had significantly larger peaks/burst and event duration compared to controls. Additionally, the knockdown had longer interevent interval frequency.

| Table 3.1 | N | Resting $V_m$ (mV) | Maximum Amplitude (mV) | Peaks / Burst | Event Duration (ms) | Interevent interval (ms) |
|---|---|---|---|---|---|---|
| Heart Gal4/ RNAi Cont. | 2 | -41.1±.2 | 59.6±.3 | 2±0 | 219.9 ± 6.2 | 583 |
| Heart Gal4> *slo* RNAi | 2 | -48.3±.3 | 47.8±.4 | 3.4±.9 | *601.8± 52.8 | 1198.9 |
| $Slo^4$/ Df (3R) BSC 397 | 3 | -46.8±1.0 | 72.3±.9 | 4.3±.3 | *860.6± 98.2 | 2248 |
| $Slo^4$/ $Slo^4$ | 4 | -44.1 ±.4 | 59.1 ±.2 | 3.4 ±.6 | *862.9 ± 60.2 | 1692.8 |
| $Slo^4$ +/- | 1 | -46.6±0 | 79.0± 0 | 2.3±0 | 317± 17.3 | 1028.7 |
| $W^{1118}$ | 4 | -41.9±.5 | 49.6±.6 | 1.9±.2 | 335± 21.1 | 672.6 |

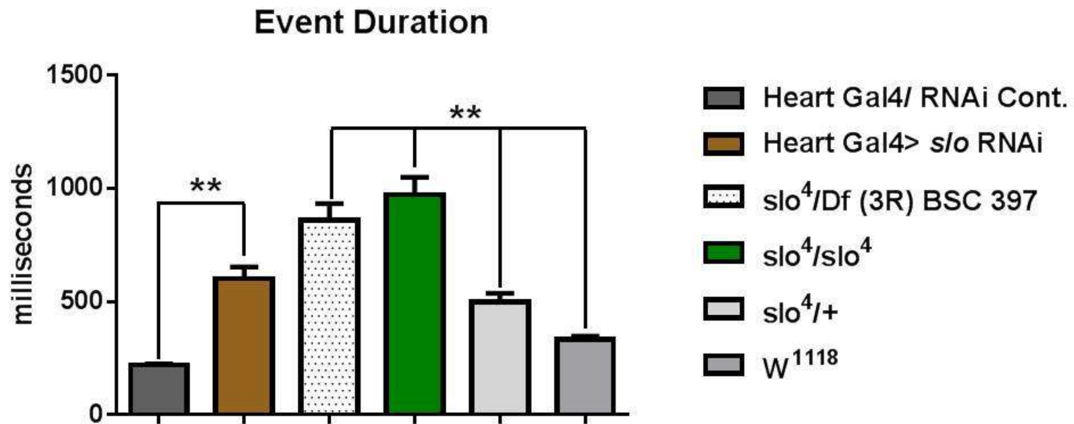



**Figure 3.11 *slo* knockdown has significantly longer event duration compared to controls.** At three weeks of age the *slo* knockdown of Heart Gal4> *slo* RNAi was signifcantly longer than Heart Gal4/RNAi Cont. Additionally the genomic mutants of $slo^4$/DF (3R)BSC 397 and $slo^4/slo^4$ had signifcantly longer event duration than the wild type W1118 and the transheterozygote $slo^4$/+. However, we need higher N for the $slo^4$/+ heterozygote to be sure of this effect.

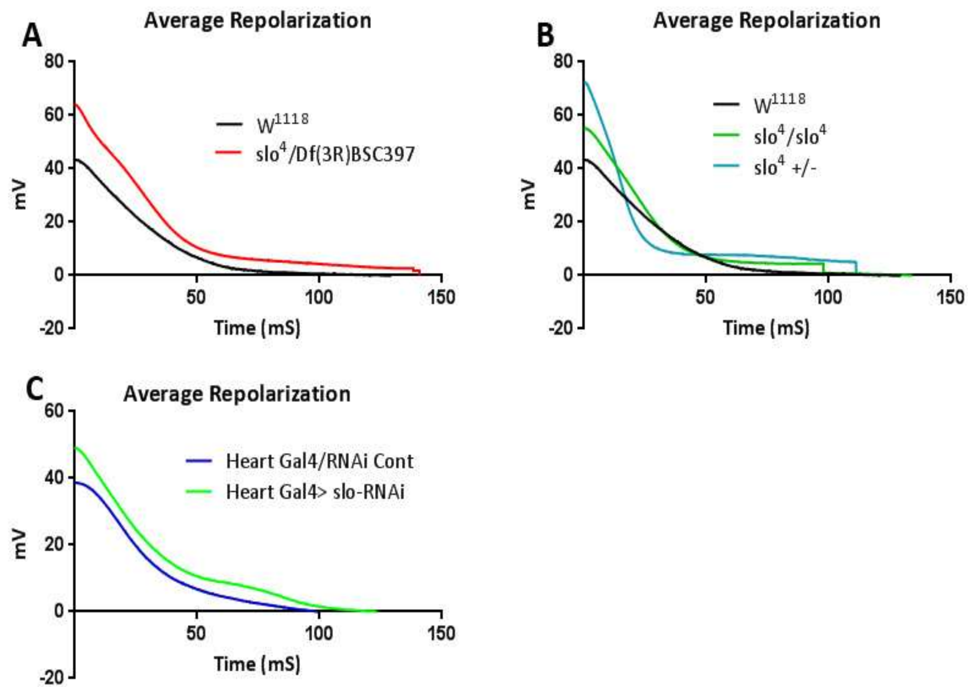



**Figure 3.12 Average trace of repolarization phase.** The *slo* knockdown genotypes of *slo*$^4$*/Df(3R)BSC397, slo*$^4$*/ slo*$^4$ and Heart Gal4> slo-RNAi all had longer repolarization decay of the average trace compared to controls.

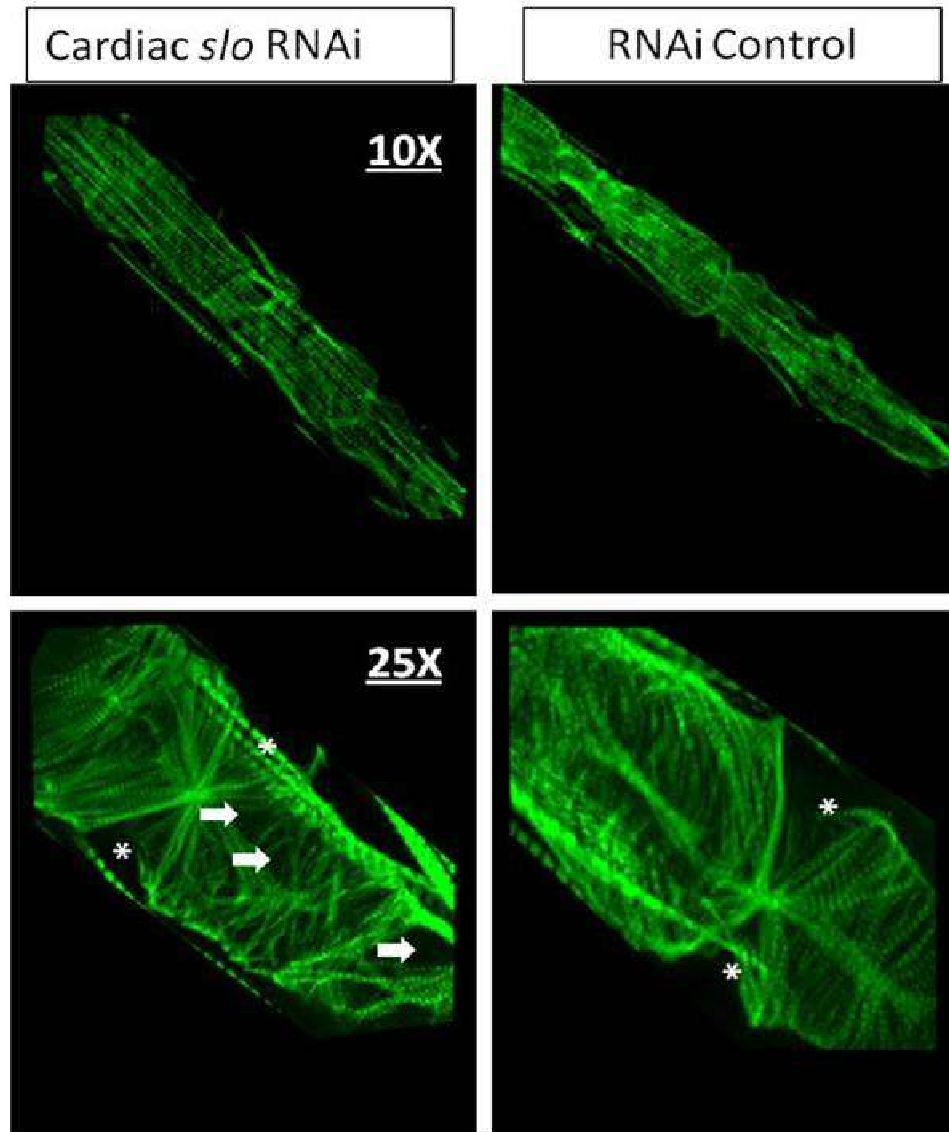



**Figure 3.13 Cardiac staining of *slowpoke* knockdown shows moderate disorganization.** Phalloidin staining of 3 week old cardiac specific knockdown and control reveals moderate myofibrillar disorganization (white arrows). The asterisks denote cardiac ostia.

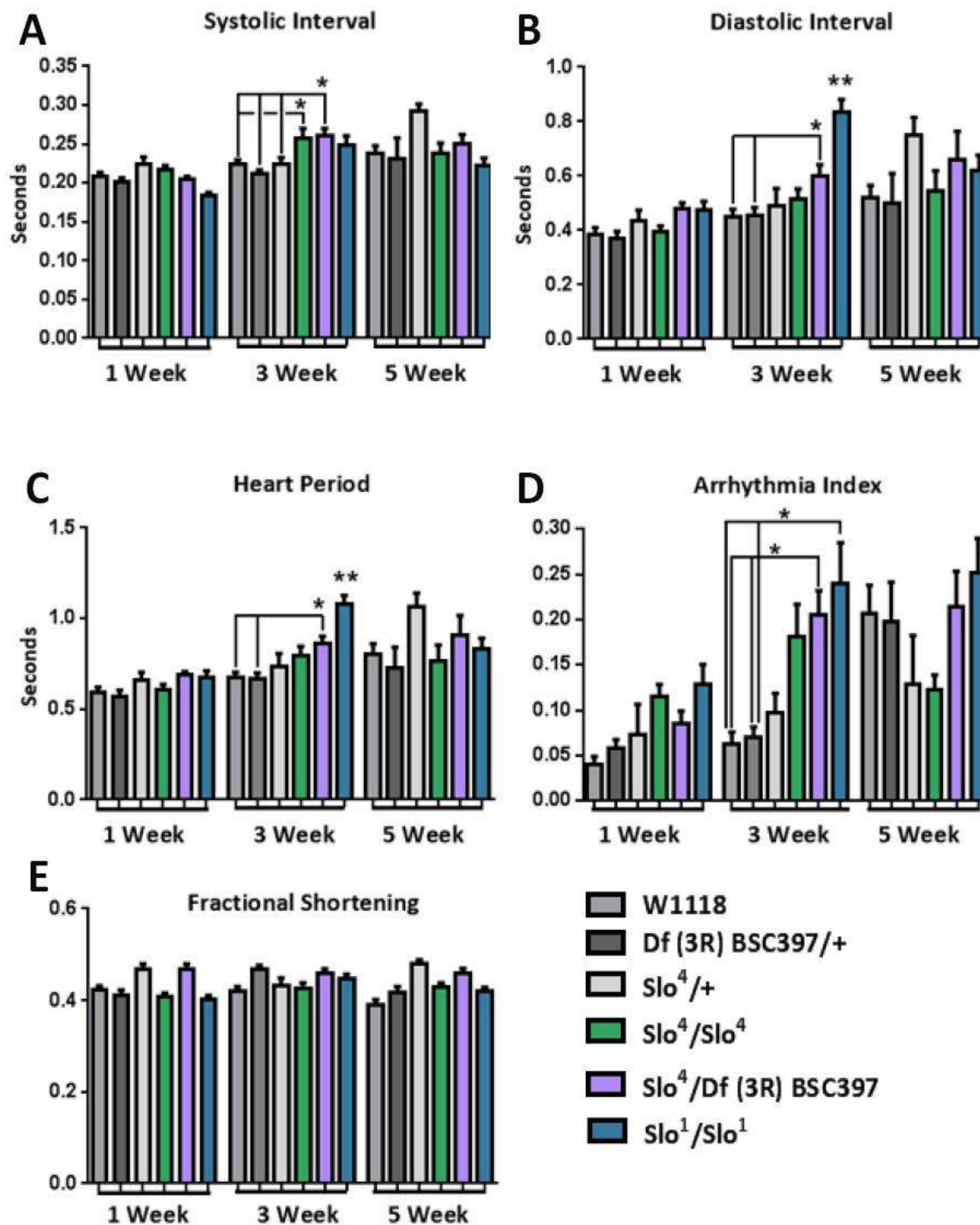



**Figure 3.14 Other genomic mutants of *slowpoke* also have a cardiac phenotype.** Other *slowpoke* genomic mutants induce a longer heart period, systolic interval, diastolic interval, and arrhythmia index (A-D). The differences between the genomic mutant and controls was diminished by 5 weeks of age. In particular, the *slo⁴* heterozygote at 5 weeks of age had a very long heart period, systolic interval, and diastolic interval. On note, the cardiac-specific knockdown flies died off at a higher rate compared to its control

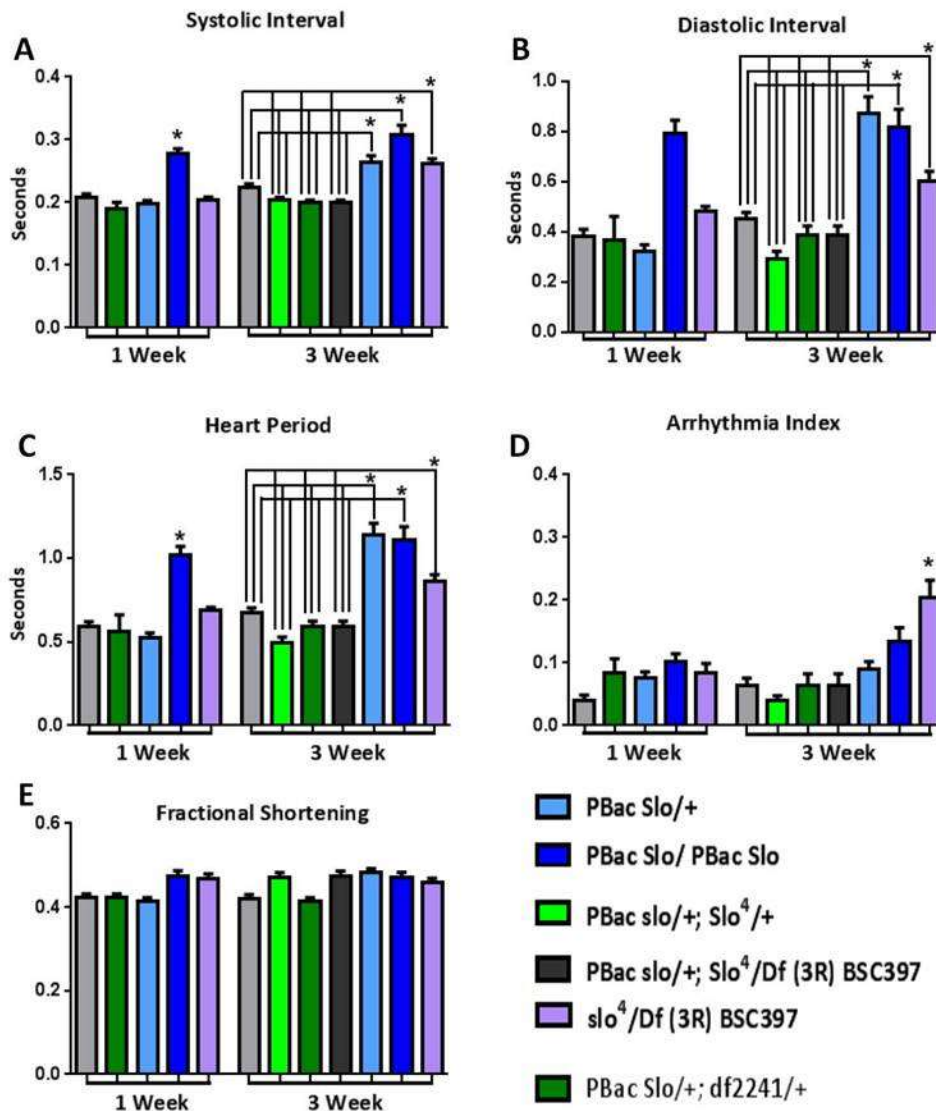



**Figure 3.15 Extra genomic locus of *slowpoke* rescues null cardiac phenotype.** The addition of a single extra genomic *slo* locus (PBac Slo/+) increased the heart period (C), systolic interval (A), diastolic interval (B) compared to a wild type ($W^{1118}$). This was not exacerbated by two extra copies of genomic locus (PBac Slo/PBac Slo). The Extra copies of this locus also did not significantly increase the arrhythmia index. Transgenomic combinations of the genomic mutant (DF(3R)BScS397*)*, $Slo^4$ and PBAC Slo/+ did not have significantly higher arrhythmia index or heart period compared to controls.

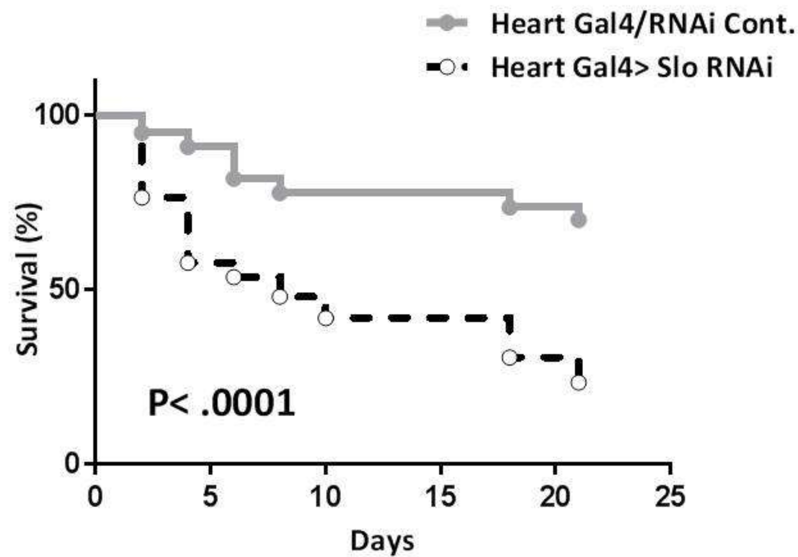



**Figure 3.16 Cardiac specific *slowpoke* knockdown induces early lethality.** Cardiac-specific knockdown flies died off at a higher rate compared to its control. We started with 200 female flies of each genotype spread over 25 flies per vial and counted the number of surviving flies after every two days. Using a log-rank (Mantel-Cox) test, there was a significant difference in the survival curves of control and knockdown.

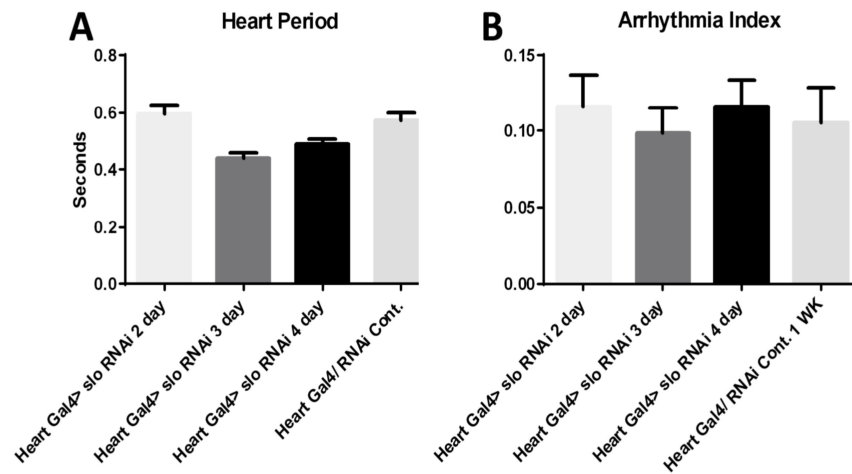



**Figure 3.17 Early *slo* knockdown has no cardiac phenotype.** When we look early aging time period of 2,3, and 4 day knockdown, we see no significant effect on the heart period (A) or arrhythmia index (B), in comparison to the 1 week control.

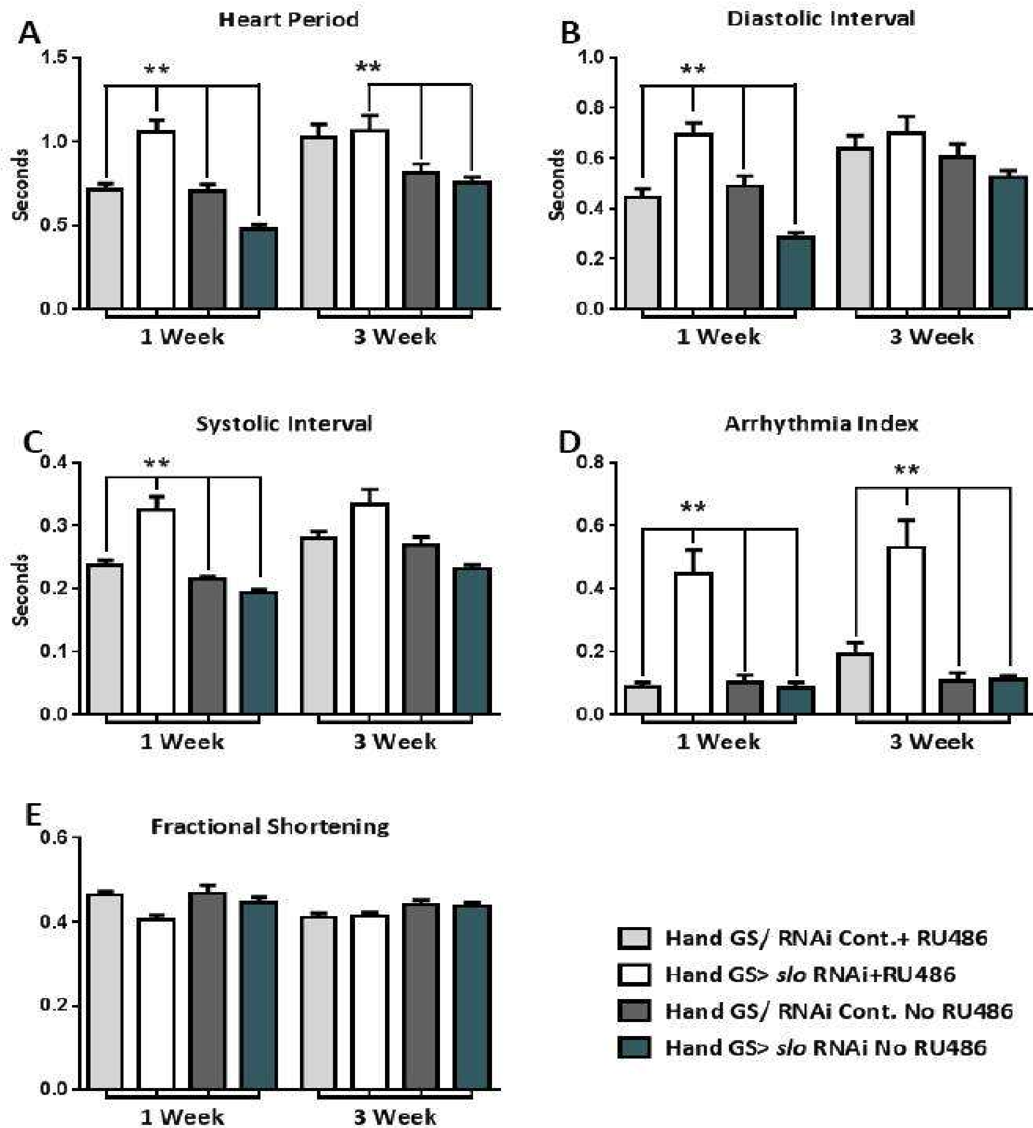



**Figure 3.18. Cardiac specific knockdown in adult flies induces cardiac dysfunction.** Temporal control of cardiac-specific *slowpoke* knockdown with the Hand GeneSwitch system (HandGS-Gal4). RU refers to Mifepristone (RU-486), administered to flies at a 100ug/mL (dissolved in ethanol), thereby inducing the HandGS-Gal4 driver. This in turn induces temporally controlled cardiac-specific expression of *slowpoke* RNAi. Cardiac *slo* knockdown in young adult flies two days after eclosion for 16 days resulted in a dramatic increase in arrhythmia index (D) compared to controls at 1 and 3 weeks of age. The heart period (A) is also increased but only at 1 week of age. At three weeks of age the heart period is only significantly increased compared to controls that did not have drug administered.

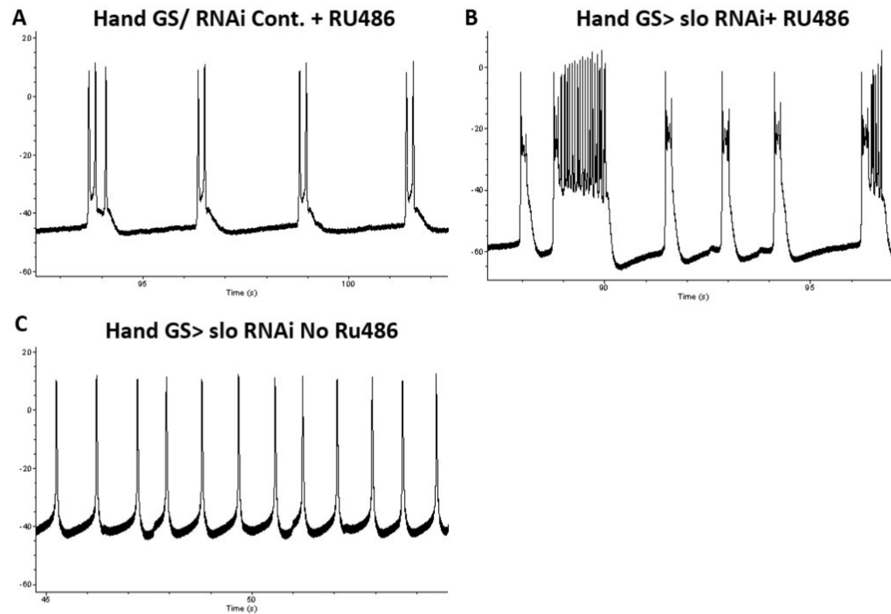



**Figure 3.19 Electrophysiological evaluations of adult *Drosophila slowpoke* mutants**. Ten second representative traces of the electrical activity within controls (**A**, Hand GS/RNAi Cont +RU 486.; **C**, Hand GS> slo RNAi No RU 486; and *slowpoke* knockdown (**B**, Hand GS> *slo*-RNAi +RU486The Y axis represents voltage in millivolts and X axis is time in milliseconds. In all of the *slo* knockdown genotypes we saw increased peaks per event and significantly higher event duration as compared to controls, which is quantified in Table 3.2.

**Table 3.2 Quantitative data from intracellular recording of adult knockdown**. The cardiac-specific knockdown adult knockdown had significantly larger peaks/burst and event duration compared to controls. However, due to drug administration and possible genetic effects, the interevent iinterval was greater in the control with RU (Hand GS> RNAi cont. +RU486), an effect reflected in the SOHA analysis (Figure 3.14)

| Table 3.2 | N | Resting $V_m$ (mV) | Maximum Amplitude (mV) | Peaks / Burst | Event Duration (ms) | Interevent interval (ms) |
|---|---|---|---|---|---|---|
| Hand GS> *slo* RNAi+RU486 | 2 | -74.5±1.4 | 64.4±.8 | 5.1±2.5 | *490.6± 80.9 | 1454.2 |
| Hand GS> *slo* RNAi No RU486 | 3 | -46.5±.5 | 60.3±.8 | 1.9±.5 | 288.6± 15.5 | 1118.7 |
| Hand GS> RNAi Cont. +RU486 | 1 | -46.0±0 | 58.3±.1 | 2±0 | 394.8± 4.6 | 1760 |

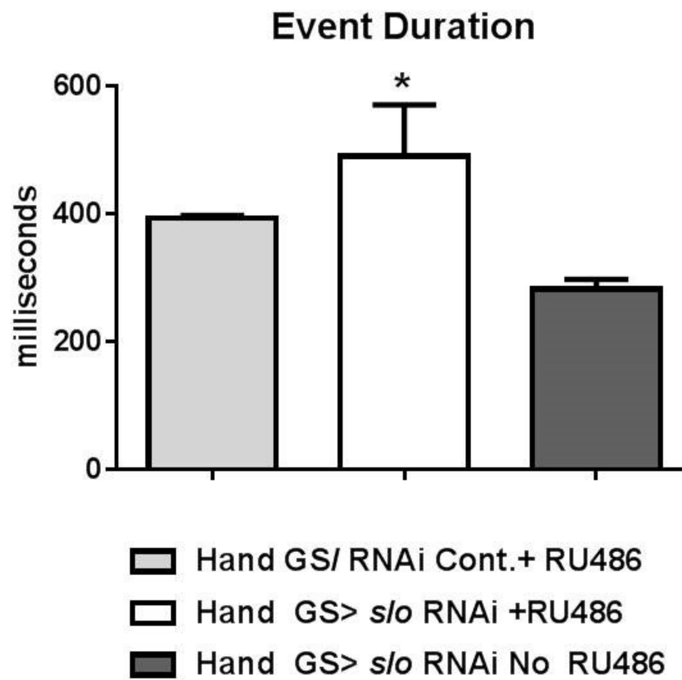



**Figure 3.20 Event duration for cardiac specific adult *slo* knockdown is significantly longer than controls.** The cardiac specific drug induced adult knockdown had significantly longer event duration compared to RNAi control with drug and knockdown without any Gal4 inducing drug.

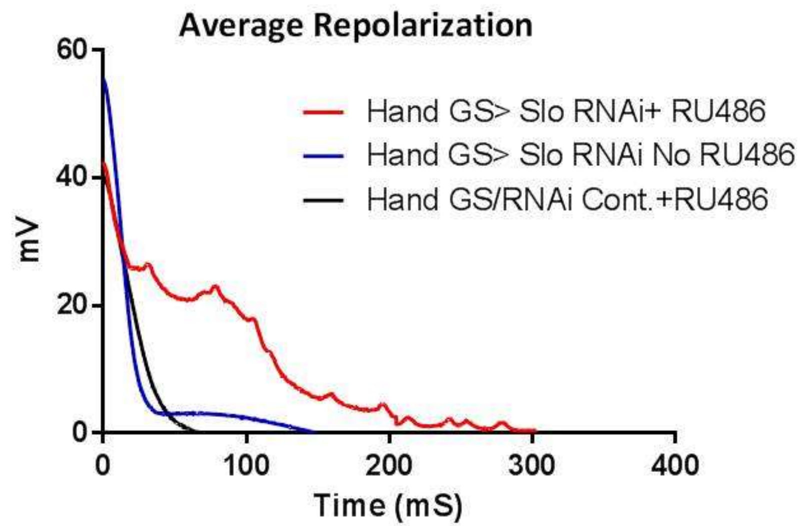



**Figure 3.21 Adult specific average repolarization curve.** Induced adult cardiac specific knockdown has a longer decay curve compared to non induced and genetic controls.

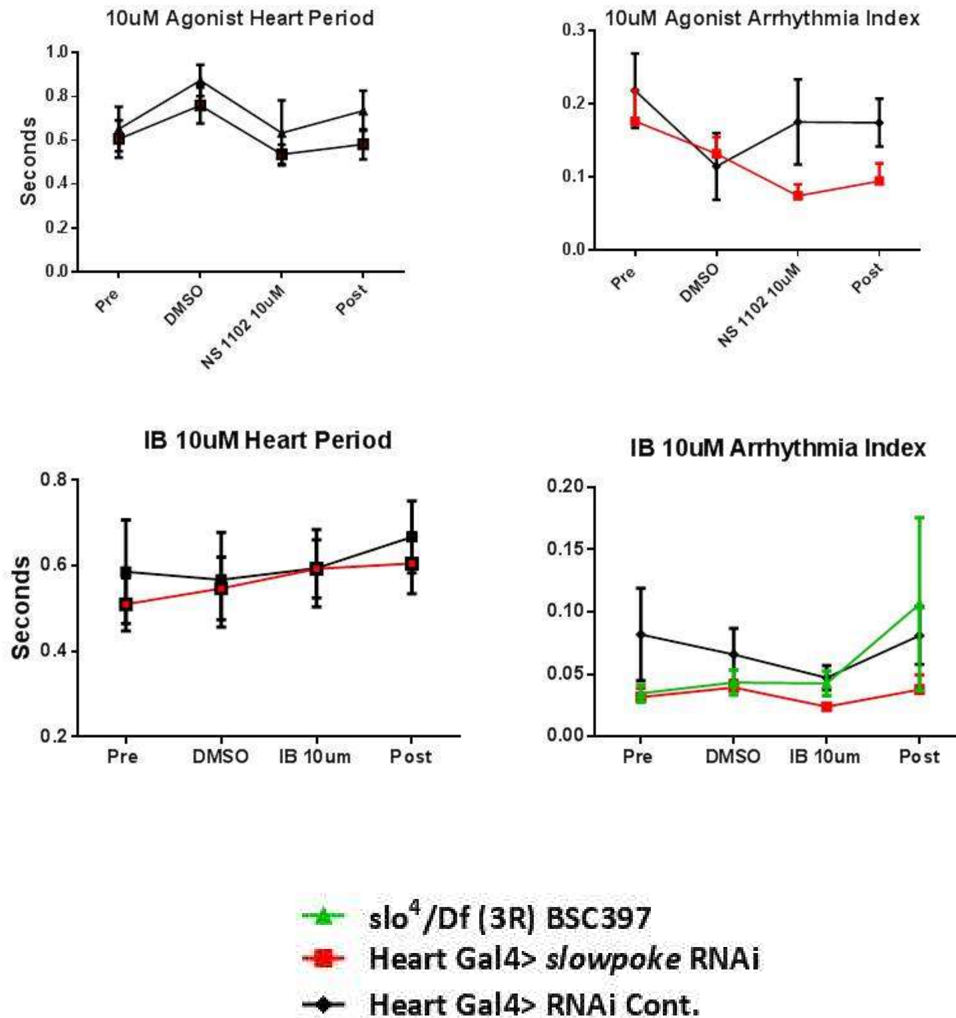



**Figure 3.22 BK channel agonist and antagonist do not significantly affect cardiac function.** We treated *slowpoke* cardiac knockdown and control flies with agonist NS11021 and Iberiotoxin. We used a variety of concentrations and exposure times but did not find any significant differences in cardiac function.

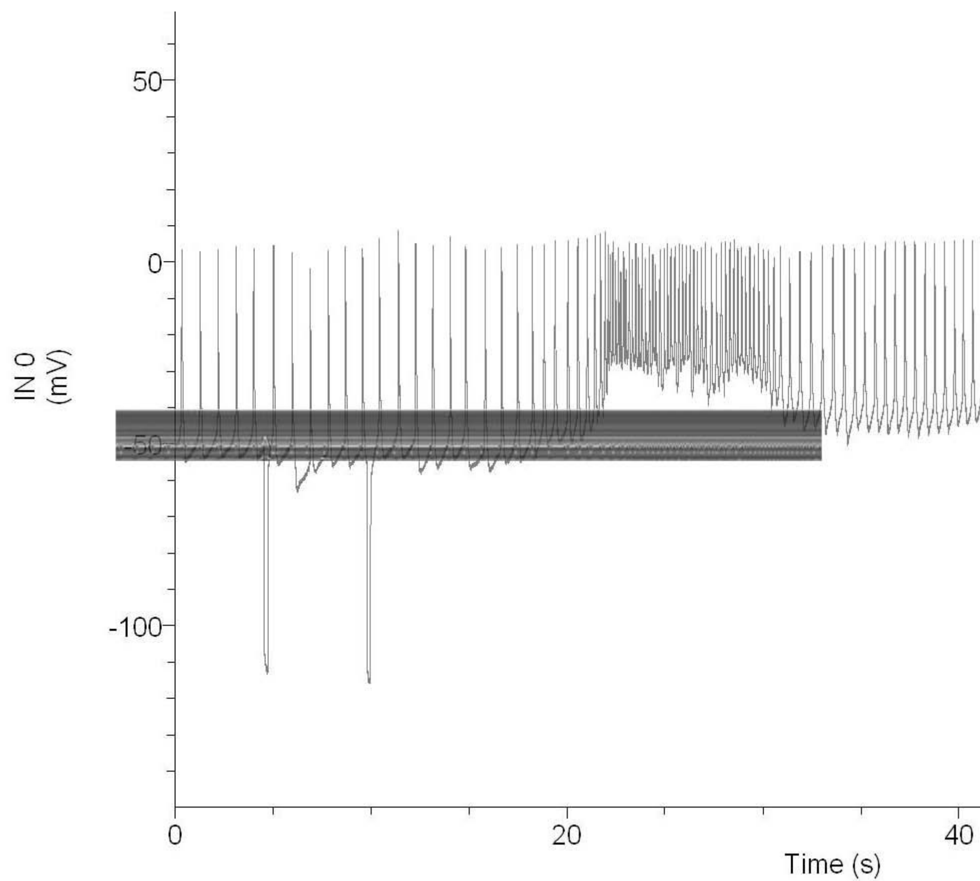



**Figure 3.23 Simultaneous electrical and optical fly recording.** I recorded an optical movie from a beating heart that was synced through a TTL (Transistor-Transistor Logic) pulse to electrical intracellular current clamp recording. Recording was only stable for 20 seconds but shows that electrical recording can match optical recording.

# CHAPTER 4 KCNMA1 A CONTRIBUTOR TO ARRHYTHMIA RISK

## 4.1 ABSTRACT

Although the BK channel is important for a host of physiological functions very little was known about its cardiac function. The *KCNMA1* gene encoding this channel spans an AF locus (Brugada et al., 1997) and a cohort study of an affected family by our collaborators showed a nonsynonymous SNP associated with AF. However, limitations of some common genetic models made it difficult to study. The commonalities between *Drosophila m.* and human in voltage activated $K^+$ channels along with the versatile genetic tools available in the fly made it an attractive genetic model to test the channel mutation. We were able to functionally characterize this mutation using the *Drosophila m.* heart and show an association with poorer cardiac function. However, this mutation mapped to a region in the N-terminus of the protein that was not directly conserved from fly to human. Thus, although this mutation does not relate directly to *Drosophila m.* cardiac physiology, it shows a reflection of possible channel interaction in the heart and drives further interest to model mutations in the endogenous fly gene to elucidate mechanisms of disease and function.

## 4.2 INTRODUCTION

As the most common arrhythmia, atrial fibrillation (AF) affects some 2-6 million Americans annually (LM, 2011). Furthermore, it has strong embolization comorbidity with a 5 fold increase in stroke risk and double the

mortality rate with accompanying heart disease. Yet, for the huge health burden it imparts, there are gaps regarding not only preventive and therapeutic strategies but also its molecular mechanism (Menezes et al., 2013).

AF is a complex and heterogeneous disease whose causes are difficult to ascertain. It is normally thought of as an ectopic beat that when imparted on a vulnerable cardiac tissue evokes high frequency and irregular electrical activity. The cause of the ectopic beat, however, and how the substrate itself becomes vulnerable to paroxysmal and then chronic AF is an ongoing question of cardiac research. There is a long list of risk factors associated with AF. Various metabolic syndromes like diabetes and obesity are associated with AF but other associated factors like sleep apnea, binge alcohol consumption and excessive exercise also contribute to the disease (Gutierrez & Chung, 2016). All of these risk factors, however, feed into types of electrical and structural cardiac remodeling (Benjamin et al., 2009). Along with the various environmental and behavioral factors, there is a strong heritable component to AF. It was this genetic component that lead to genetic studies looking for AF genomic loci. There was some success as linkage analysis for example yielded among others KCNA5 monogenic mutations associated with AF and the first AF locus at 10q22-q24 (Gutierrez & Chung, 2016). Additionally, GWAS studies have also determined experimental loci on Chr 4 associated with the *PITX2* gene, developmental genes on Chr16, the

*KCNN3* encoded SK channel on Chr 1 and a host of other candidates associated with AF (Gutierrez & Chung, 2016).

One of these studies looking at the heritable component of AF was a linkage analysis two decades ago that looked for loci in an affected family. The researchers were able to identify a 10q22-q24 locus, but unfortunately, like many other such identified loci the causative gene of this locus remained unknown (Brugada et al., 1997; Lubitz et al., 2010). One theorized causative gene was *KCNMA1* encoded BK channel but early studies were not able to detect transcripts in the heart and the mouse null genetic model had no overt cardiac phenotype. However, our collaborator Dr. Diane Fatkin sequenced AF affected families and found a novel SNP in the C terminus of the KCNMA1 gene that could be associated with the disease. We continued this work by functionally characterizing this variant using the *Drosophila m.* heart. Although, the mutated portion of the KCNMA1 gene is not conserved in the fly we can still use the fly model to understand its function as the fly heart has similar $K^+$ currents and channels dictating cardiac physiology .In this integrated 3D organ system, our results showed a strong effect of the mutant to decrease cardiac function. These results serve as a basis to build understanding of this channel and move further to model possible disease causing variants in conserved regions from fly to human and test possible mechanisms of dysfunction.

## **4.3 RESULTS**

### *4.3.1 A Human KCNMA1 Mutation is Associated With Atrial Fibrillation and Bradycardia*

The original human *KCNMA1* SNP variant was identified by sequencing the gene of a 118 AF affected proband individuals. They identified 6 different variants, of which four were synonymous variants and one was a 3-nucleotide insertion that did not alter the reading frame. One of the variants had a C-terminal deletion of 4 nucleotides and insertion of 1 nucleotide (Figure 4.1). The variant p.S11_S12delinsG was confirmed by the restriction enzyme deletion of a MspAII site. The loss of the site was confirmed in the affected proband and affected family members while it was still present in non-affected members (Figure 4.1). It was not detected in 150 healthy Australian control subjects and has not been identified in >60,000 unrelated subjects in the ExAC population database. The family proband had previously undergone genetic screening of 14 AF-associated $K^+$ channel genes as well as the *SCN5A*, *NPPA*, *GJA1*, *GJA5*, *GJA7*, *HCN1*, *HCN2*, and *HCN4* genes, with no mutations found. The major clinical features of the affected proband and family members shown consisted of AF and bradycardia. However, the symptoms only showed up with age and had not yet affected young members of the family with the mutation.

### *4.3.2 Functional Characterization of the Human KCNMA1 Mutation in the Drosophila Heart*

We cloned the human wild type and mutant KCNMA1 genes and injected them into $W^{1118}$ flies as UAS transgenes. We used the same transheterozygote (*$Slo^4$/ Df (3R) BSC 397)* as a sensitized background in which we could examine the effect of cardiac specific expression of these human channels in an integrated organ system. Although, this region is not directly conserved in the fly *slowpoke* gene, we can still the effect of the mutation versus the wild-type human channel. At 3 weeks of age flies with heart-specific over expression of the mutant human KCNMA1 gene exhibited an increase in heart period compared to the null background control and the human wild type KCNMA1 overexpression (Figure 4.3). Additionally, the mutant KCNMA1 overexpression genotypes had a higher AI than background controls and were higher than the wild type KCNMA1 overexpressors although this difference did not reach significance (Figure 4.3 B). While human KCNMA1 wild-type gene overexpression did not rescue the null phenotype, it did not worsen it in the same way as the human KCNMA1 mutant gene (Figure 4.3 A, B). This suggests again that the mutant channel is detrimental to cardiomyocyte function. Similarly, action potential characteristics in Heart Gal4>$KCNMA1^{wt}$ in the *slo* transheterozygote mutant background were similar to those in *$Slo^4$/ Df (3R) BSC 397* alone shown in Figure 4.4, quantified as a bar graph for Figure 4.5 and Table 4.1. In contrast, Heart Gal4>$KCNMA1^{mut}$ flies had relatively longer action potential bursts and a greater number of peaks per burst than the wild type overexpression or *$Slo^4$/ Df (3R) BSC 397*

(Figure 4.5, Table 4.1). However, the exact reason why mutant overexpression is worse than the null transheterozygote is unclear. The longer heart period of the mutant in both wild-type and null background suggest that it is causing some dysfunction of not just *slo* but perhaps other channels. However, to test this, we need to know more about the individual properties of the mutant channel. The RMP was within normal range for overexpression genotypes as well as the maximum amplitude, although it did trend towards the higher side (Table 4.1). Finally, the average repolarizing curve for the mutant genotype had a longer repolarizing potential compared to the transheterozygote and wild-type overexpression. While we were able to pick out an average smooth curve for all past genotypes, the mutant overexpression was arrhythmic and irregular curve (Figure 4.5).

Aside from the transheterozygote background we also looked at human gene overexpression in homozygous *Slo*$^{4}$/ *Slo*$^{4}$ genotype. Similar to the transheterozygote background, the human mutant overexpression also worsened cardiac function compared to the human wild-type overexpression and the *Slo*$^{4}$/ *Slo*$^{4}$. There was a significant increase to both the mutant background and wild-type overexpressor in the 3 week systolic interval (Figure 4.6, C). All the other parameters were increased for the human mutant overexpression in the *Slo*$^{4}$/ *Slo*$^{4}$ but did not reach significance (Figure 4.6 A, B, D). Electrically, the human mutant overexpressor in the *Slo*$^{4}$/ *Slo*$^{4}$ background was had a similar outcome to overexpression in the null *Slo*$^{4}$/ *Df (3R) BSC 397*

background with significantly longer event duration, peaks per burst and greater amount of time to reach RMP in the average repolarizing curve (Table 4.1, Figure 4.5).

When we overexpressed either the mutant human *KCNMA1* gene in a normal wild-type background, we saw a significantly higher HP compared to control (Heart Gal4/ RNAi Cont.) and a trend towards increased AI (Figure 4.2 A, B), and although we did not effect a rescue the wild-type overexpression did not worsen cardiac function compared to the transheterozygote. These were increases comparable to the HP and AI of the tranheterozygote *Slo*$^{4}$*/ Df (3R) BSC 397* mutant alone compared to the same control (Figure 4.2). As a comparison, the transheterozygote mutant as shown before (Figure 3.14) had a significantly higher SI, DI, HP, and AI at 3 weeks of age compared to wild-type ($W^{1118}$), and one heterozygote (Df (3R) BSC397/+). While it was higher than the other heterozygote ($slo^{4}$/+) it only reached significance for the SI. When we looked at overexpression in a null *Slo*$^{4}$*/ Df (3R) BSC 397* the mutant overexpressor in the null background had significantly higher HP than any other genotype and although not significant also had the highest AI than any other genotype (Figure 4.2).

### *4.3.3 Cardiac Mutations in the Endogenous Slowpoke Gene*

We have also started examining the effect of other *KCNMA1* cardiac disease associated variants. Other collaborators in Massachusetts's General Hospital were able to find two variants in important ion sensing regions of the

channel. The first was a P1000R mutation in the RCK1 region and the second a N541S mutation in the RCK2 region also called the "$Ca^{2+}$ bowl". While only the P1000R is directly conserved, *Drosophila m.* has an Aspartic Acid versus Asparagine at the 541 site, but both are in strongly conserved regions that bind $Ca^{2+}$ and are important for channel activation (Figure 4.7). We are using CRISPR/Cas9 to induce single base pair mutation in the *Drosophila m. slowpoke* gene. These mutations will allow us to further characterize the channel's role in the cardiac physiology of the fly with possible application to mechanism's of human disease.

## 4.4 DISCUSSION

I attempted to rescue the loss of *slo*/BK channel function in the fly heart by overexpression of the human *KCNMA1* gene.I did not see the same reversion of the bradyarrhythmic phenotype as when we used the extra endogenous copy of *slow* (PBac Slo). However, we did see a significant worsening of function when over expressing the human mutant *KCNMA1* versus the wild-type in the null background. Overexpression of the human KCNMA1 mutation increased the HP, and AI compared to both background transheterozygote controls, although only the HP reached significance (Figure 4.2). Additionally, in the wild-type background both the human wild-type and the mutant *KCNMA1* gene had deleterious effect on cardiac function (Figure 4.2). Overexpression of the mutant gene also worsened electrical cardiac function and significantly increased the event duration and peaks per burst compared

to all other genotypes (Figure 4.3, 4.4, 4.5 Table 4.1). The loss of *slo*/BK function had a greater impact on the electrical phenotype than the functional measurements, suggesting both a greater level of sensitivity for our electrical assay and susceptibility of the mutant hearts to dysfunction under experimental conditions. If the human mutant channel was simply nonfunctional, then we would not expect the worsening function at when we overexpress it in the null background compared to the null mutation by itself. Perhaps the human mutation encodes a dominant negative effect on existing $K^+$ ion channels. The human wild type overexpression had an effect on the wild-type background but none in a null mutant.

From the current work of our collaborators and others, it seems like the channel is heaviest expressed in the pacemaker regions of the heart. The Gal4 drivers that we used did not attenuate the expression to the pacemaker region as what could maybe be expected physiologically. However, there is no pacemaker Gal4 to efficiently test this hypothesis. One way that this could be avoided, would be to genetically tag the endogenous gene in order to localize it better within the heart, but we were not able to complete this experiment.

In flies, we found that extra copies of the *slo* locus increased the heart period. Similarly, there is higher expression of *KCNMA1* in older subjects with chronic AF, when compared to young subjects with AF and age-matched individuals without AF. Increased $K_{Ca}1.1$ activity may contribute to the substantial reductions (~70%) in atrial $I_{CaL}$ that occur with persistent AF (Van

Wagoner et al., 1999) and would represent an adaptive response to mitigate against intracellular $Ca^{2+}$ overload. Paradoxically, this electrical remodeling would shorten the atrial action potential duration and increase the propensity for initiation and maintenance of AF via re-entry mechanisms. In a transheterozygote *slo* mutant we saw a decrease in the major $Ca^{2+}$ channel (Figure 3.4). However, we would need to quantify $Ca^{2+}$ flow using genetically encoded $Ca^{2+}$ sensors and match it to the arrhythmic activity we see in order to determine if this is occurring in *Drosophila m.* heart.

The second and third mutations, of which we are final stages of generating, are in highly conserved regions. Additionally, the mutations are carried out on the endogenous gene instead of the using expression systems. There remains the possibility of a developmental effect as we observed some early lethality and flies aged to 5 weeks. Regardless, these experiments along with the associated human mutation, would highlight the important physiological function of *KCNMA1* not just *Drosophila m.* but also human physiology.

**4.5 ACKNOWLEDGEMENTS**

Parts of Chapter 3 and Chapter 4 are being preparedfor joint publication with coauthors: Santiago Pineda, BA; Vesna Nikolova-Krstevski, PhD; Christiana Leimena, PhD; Andrew J. Atkinson, MPhil; Arie Jacoby, PhD; Inken G. Huttner, MD; Yue-Kun Ju, MD, PhD;Magdalena Soka, BSc(Hons); Gunjan Trivedi BSc(Hons); Renee Johnson, PhD, MGC; Dennis Kuchar, MD;

Jamie I. Vandenberg, MB BS, PhD; David G. Allen, PhD; Halina Dobrzynski, PhD; Karen Ocorr, PhD; Rolf Bodmer, PhD[1]; Diane Fatkin, MD. The working title of the manuscript is: “The Large Conductance Calcium-Activated Potassium Channel, $K_{Ca}1.1$, is a Novel Contributor to Sinus Node Function and Arrhythmia Risk”

Dr. Vesna Nikolova-Krstevski, did immunofluorescence and electron microscopy in Chapter 3. Various members from Dr. Diane Fatkin’s lab helped in developing the mutation used in Chapter 4. The dissertation author performed all other experiments.

## 4.5 Figures

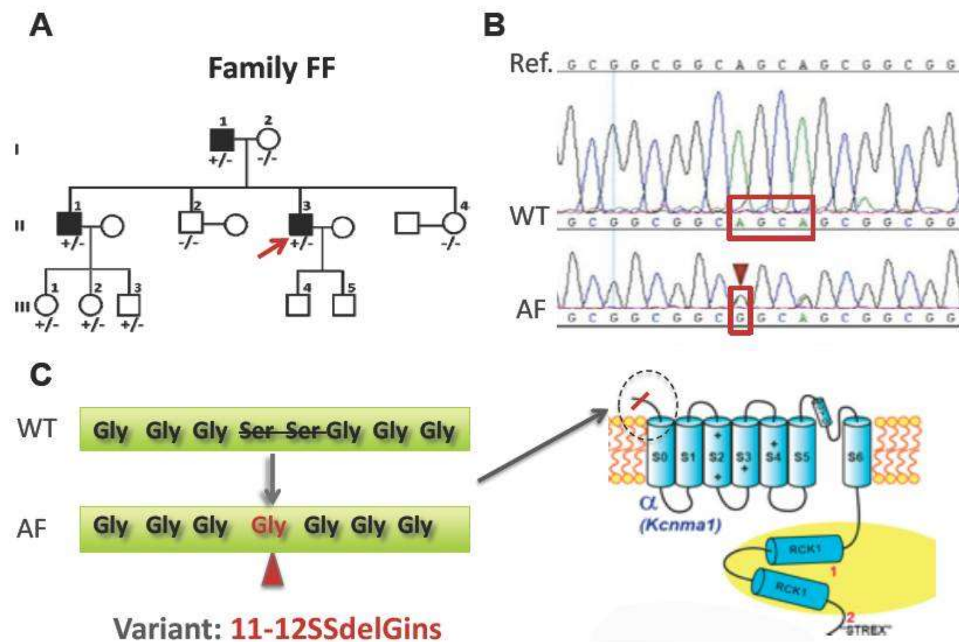



**Figure 4.1** ***KCNMA1*** **sequence variant identified in a family with atrial fibrillation**. **(A)** *KCNMA1* sequence traces showing variation identified in the Family FF proband, FF-II-3. (**B**) This sequence change (AGCA deletion, G insertion) results in loss of two serine residues at amino acid positions 11 and 12, with the addition of a glycine residue. (**C**) Pedigree of Family FF. Phenotypes are denoted as follows: AF (solid symbols), palpitations with no documented AF (hashed symbols), no symptoms or signs of AF (open symbols). The presence (+) and absence (-) of the p.S11_S12delinsG variant are indicated and the family proband is shown (arrow). (**D**) Schematic showing KCa1.1 (-subunit, blue) with its pore and two RCK (regulator of potassium conductance) domains and a regulatory -subunit (pink). The location of the p.S11_S12delinsG variant in the short extracellular amino terminus of the -subunit is indica

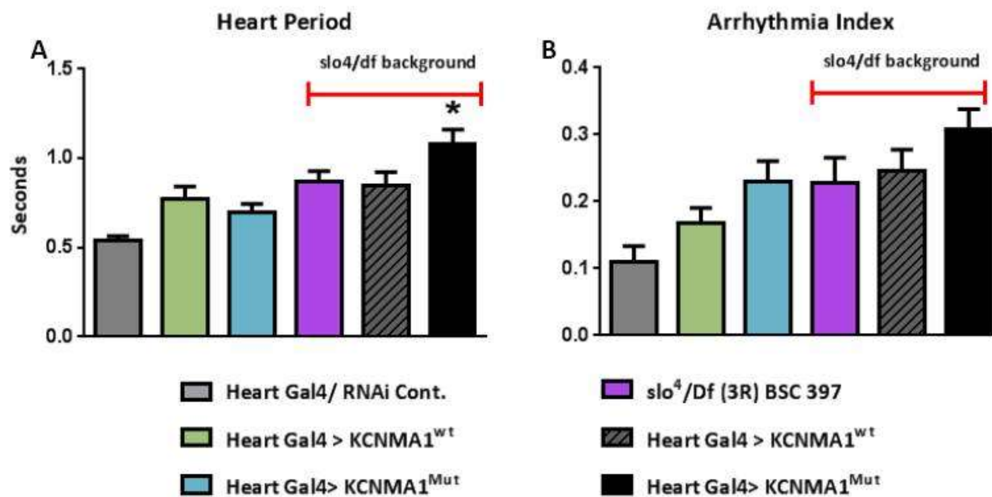



**Figure 4.2 *KCNMA1* human mutation negatively impacts cardiac function in the fly heart.** Cardiac expression of the human wildtype KCNMA1 ortholog or a mutant KCNMA1 variant found in a human pedigree associated with cardiac arrhythmias (see Fig. 1) was carried out in a *slowpoke* null background. The mutant, but not the wildtype form, worsened cardiac function by exhibiting a significantly higher heart period (**A**) and trending towards a higher arrhythmia index (**B**). Statisticss were one-way ANOVA and post-hoc tests as indicated in the methods section.

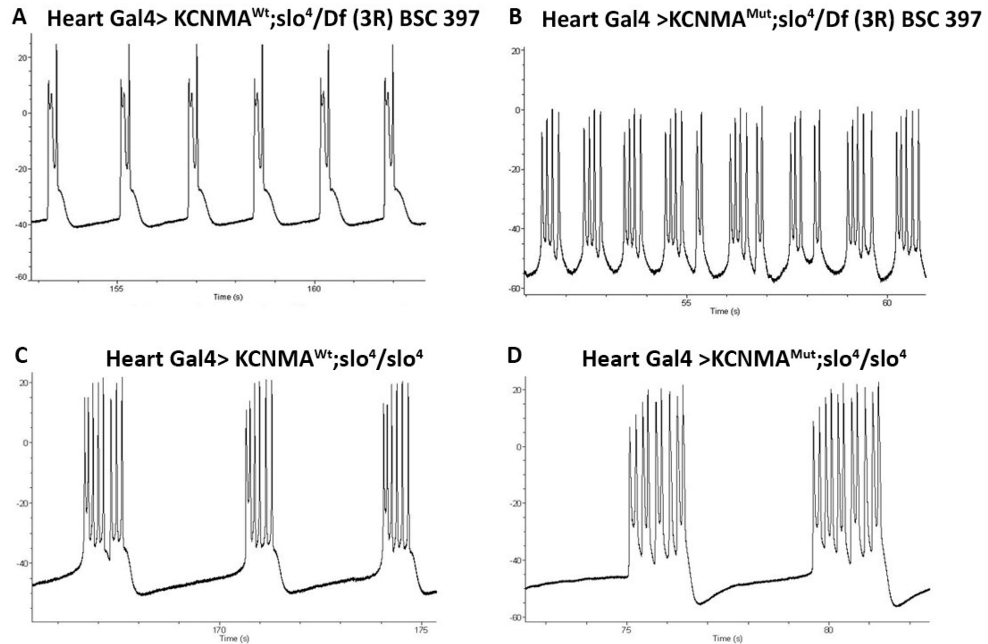



**Figure 4.3 Current clamp traces of human KCNMA1 mutant and wild-type overexpressed in a null *slowpoke* background.** Electrophysiological evaluation of *Drosophila slowpoke* mutants with cardiac specific expression of human *KCNMA1* mutant and wild tyep. Ten second representative traces of the electrical activity with time on the X axis in milliseconds and the Y axis as voltage in millivolts.We saw increased peaks per event and significantly higher event duration for the human cardiac *KCNMA1* overexpression as compared to the wild-type.

**Table 4.1 Quantitative data from intracellular recording human *KCNMA1* overexpression**. The cardiac specific expression of human KCNMA1 in a *slo* null background (Heart Gal4>KCNMA1$^{WT}$) had longer event duration and peaks per burst than wild-type controls but shorter than mutant KCNMA1 variant cardiac specific expression in a *slo* null background (Heart Gal4>KCNMA1$^{mut}$). Asterisk denotes significantly higher event duration compared to wild-type and null transheterozygote as shonw in Figure 4.4. Statisticss were one-way ANOVA and post-hoc tests as indicated in the methods section.

| Table 4.1 | N | Resting $V_m$ (mV) | Maximum Amplitude (mV) | Peaks / Burst | Event Duration (ms) | Interevent interval (ms) |
|---|---|---|---|---|---|---|
| Heart Gal4> KCNMA$^{wt}$;Slo$^4$/ Df (3R) BSC 397 | 4 | -43.9±1.2 | 55.3±.8 | 4.9±.5 | 902.0± 47.4 | 1886.5 |
| Heart Gal4> KCNMA$^{mut}$;Slo$^4$/ Df (3R) BSC 397 | 4 | -50.4±.6 | 65.3±.5 | 5.7±.3 | *1108.3± 74.8 | 2030 |
| Heart Gal4> KCNMA$^{mut}$;Slo$^4$/ Slo$^4$ | 4 | -46.6 ± | 66.5 ± | 14.7 ±3.9 | 1617 ± 223.6 | 2977.1 |
| Heart Gal4> KCNMA$^{wt}$;Slo$^4$/ Slo$^4$ | 3 | -62.1 ± | 72.6 ± | 5.6 ±.7 | 1179 ± 76.5 | 2896.2 |

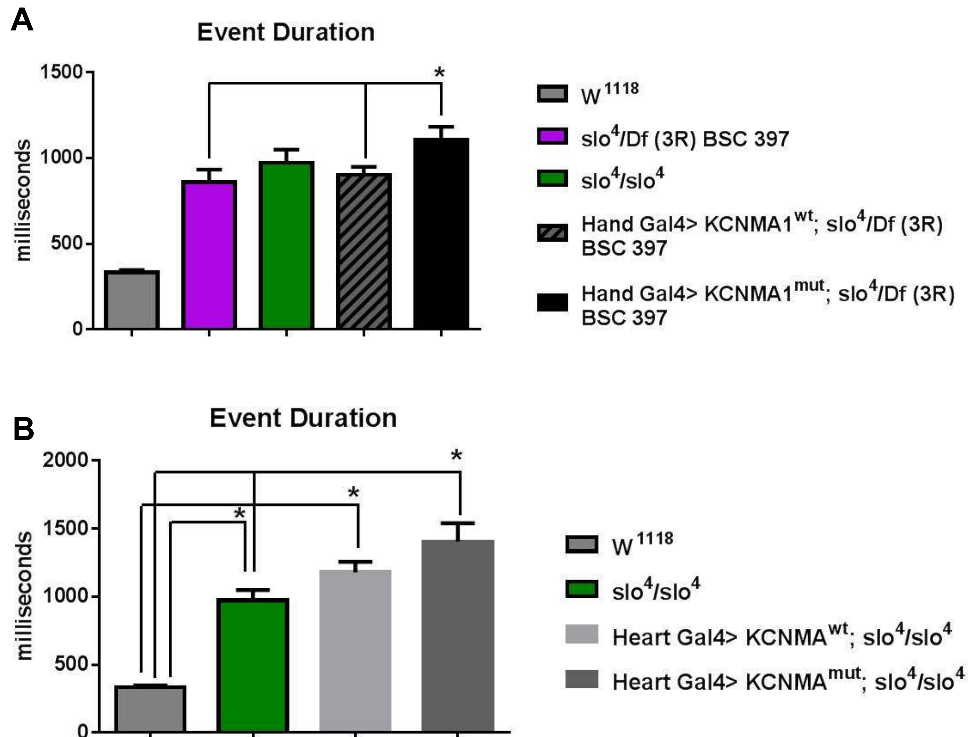



**Figure 4.4 Cardiac specific overexpression of mutant human KCNMA1 in a null mutant causes significantly higher event duration. (A)**. Cardiac specific overexpression of the human KCNMA1 mutant gene in a null transheterozygote background has a significantly longer event duration compared to human KCNMA1 wild type overexpression in the same background, the transheterozygote null mutant by itself and a control $W^{1118}$. **(B)** Overexpression of the same human KCNMA1 mutation in another genomic background ($slo^4/slo^4$) also caused a significantly higher event duration compared to the $slo^4/slo^4$ genomic control and wild-type $W^{1118}$. However, while it had a higher event duration compared to the human wild-type KCNMA1 overexpression in the same mutant background, this difference was not significant.

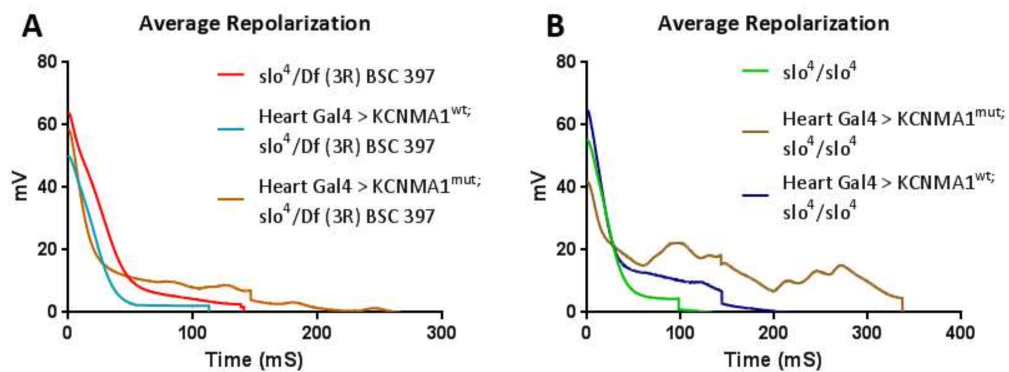



**Figure 4.5 KCNMA1 human cardiac specific overexpression in null background average repolarization curves**. The mutant overexpressors (Heart Gal4> $KCNMA^{mut}$;$Slo^4$/ Df (3R) BSC 397and Heart Gal4> $KCNMA^{mut}$;$Slo^4$/ $Slo^4$) had longer repolarization decay of the average trace compared to human wild type overexpression (Heart Gal4> $KCNMA^{wt}$;$Slo^4$/ Df (3R) BSC 397 and Heart Gal4> $KCNMA^{wt}$;$Slo^4$/ $Slo^4$) and genomic mutants ($Slo^4$/ Df (3R) BSC 397 and $Slo^4$/ $Slo^4$). The wild type overexpressors also had a longer repolarization curve compared to the $Slo^4$/ $Slo^4$ mutant background (B) but not the $Slo^4$/ Df (3R) BSC 397 background (A).

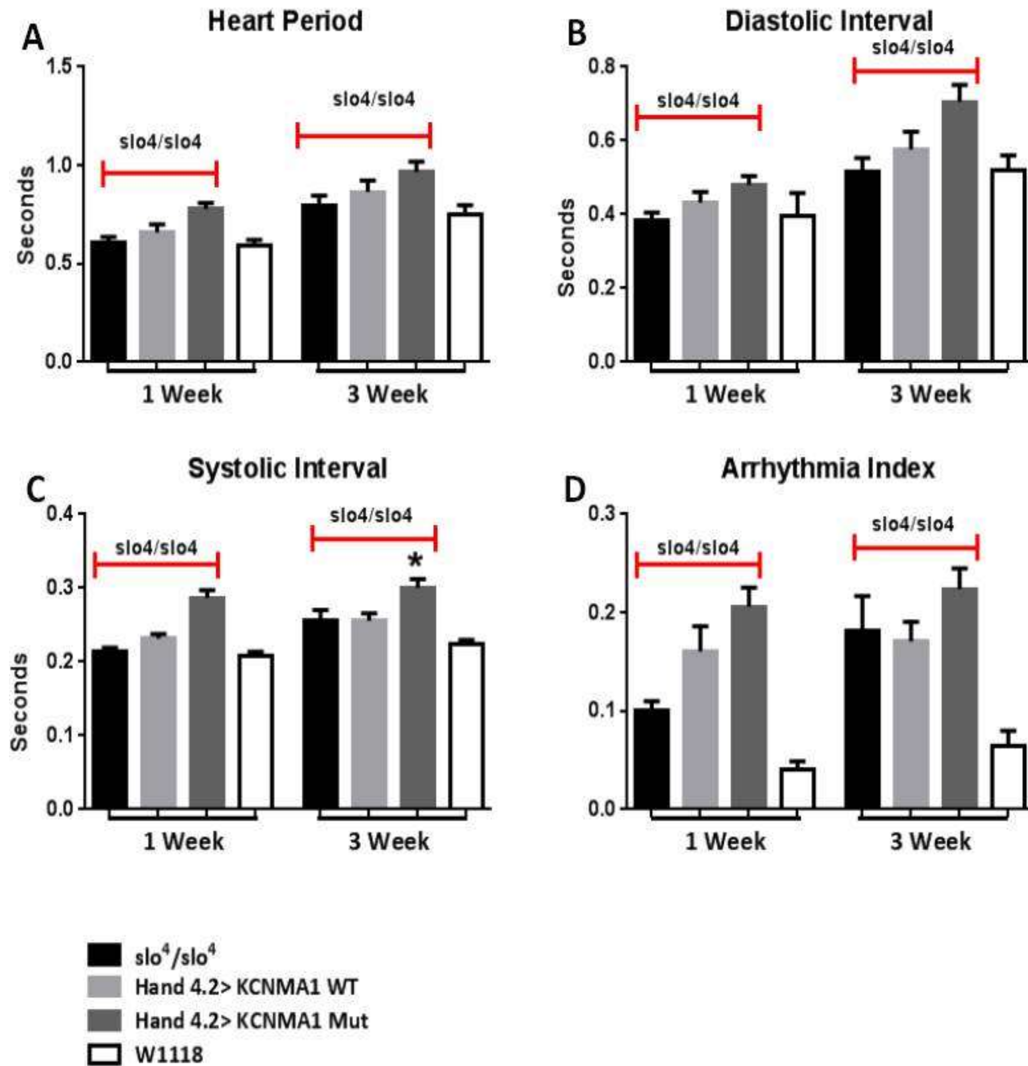



**Figure 4.6 Expressing a human mutation in a different mutant background.** We carried out the same experiment in a different null *slowpoke* background and found again that overexpression did not ablate the cardiac phenotype. However, again we saw that overexpression of the human *KCNMA1* mutation caused poorer cardiac function as measured by a significant increase in the systolic interval (C) and a higher trend towards a higher heart period (A), diastolic interval (B) and arrhythmia index (D).

```
Q03720 SLO_DROME          964SATAGSPI------VLQRRGSVYGANVPMITELVNDSNVQFLDQDDDDDPDTELYLTQPF
Q12791 KCMA1_HUMAN        973GMDRSSPDNSPVHGMLRQPSITTGVNIPIITELVNDTNVQFLDQDDDDDPDTELYLTQPF
                             .  .**       :*:: . .  *.*:*:*******:***********************
```

**P1000R: A novel AF variant, located in the RCK2 domain (Calcium Bowl) in the C-terminal tail.**
**It is in a highly conserved residue and is predicted pathogenic by Proven, Sift and PolyPhen2**
**P985R in the fly**

```
Q03720 SLO_DROME          489WDWKQGDDVICLAELKLGFIAQSCLAPGFSTMMANLFAMRSFKTSPDMQSWTNDYLRGTG
Q12791 KCMA1_HUMAN        540WNWKEGDDAICLAELKLGFIAQSCLAQGLSTMLANLFSMRSFIKI-EEDTWQKYYLEGVS
                             *:**:***.***************** *:***:****:**** .  : ::* : **.*..
```

**N541S: A novel AF variant located in the RCK1 domain in the C-terminal tail.**
**D490S in the fly**

**Figure 4.7 Novel AF variants in conserved ion binding regions.** The first mutation P1000R is directly conserved from fly to human and is located in the $Ca^{2+}$ bowl, which is one of two regions important for $Ca^{2+}$ mediated activation of the channel. The second mutation N541S is located in the second $Ca^{2+}$ sensing region of the channel. It is not directly conserved but the residues share strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix.

## APPENDIX 1 *Slowpoke* Interactions with Other Ion Channels

### A.1 ABSTRACT

Within the context of ion channel physiology the *slowpoke* channel is unique in its large $K^+$ conductance and synergistic voltage and $Ca^{2+}$ activation. Previously we identified a possible new role for the channel in cardiac function, particularly as a $Ca^{2+}$ feedback regulator. We looked at how this could interact with other cardiac $K^+$ channels and the effect on *Drosophila m.* cardiac physiology when different channel deficiencies are combined. Double heterozygote mutant combination of different putative cardiac voltage-activated $K^+$ channels had poorer cardiac function than heterozygotes of either mutant alone. The combinatorial effect underscores the importance of these homologs in adult *Drosophila m.* cardiac function and how the *Drosophila m.* cardiac model can inform future studies of cardiac ion channels.

### A.2 INTRODUCTION

While we know the effect of *slowpoke* knockdown on the *Drosophila m.* cardiac physiology, we still do not know exactly where the channel could function in the action potential. It could perhaps function similarly to the theorized SK channel mechanism in the phase 4 of the pacemaker action potential (Weisbrod et al., 2016). There it would function to fine tune the $I_f$ current and modulate the depolarizing pacemaker current. Then, as a decrease in local $Ca^{2+}$ concentration closes the channel, voltage-dependent

inward currents finally take over until opening of L and T-type $Ca^{2+}$ channels along with depolarization opens them again. Some of the evidence from our work here suggests that it works in the repolarization phase of the cardiac action potential. In the context of this possible function we wanted to see how the channel behaved with the voltage-activated $K^+$ channels *seizure* and *KCNQ1* that underlie the major repolarizing currents, $I_{ks}$ and $I_{kr}$, of the human heart. A previous study in our lab identified the *KCNQ1 Drosophila* homolog *KCNQ* as important for cardiac function; its knockdown resulted in increased AI and electical EADs similar but more severe to *slowpoke* knockdown (Ocorr et al., 2007). Another studying in our lab approaching the finishing stages looked at looking at the hERG *Drosophila* homolog *seizure* in *Drosophila* cardiac function. It found that *seizure* knock down increased AI and resulted in structural cardiac defects (Work Unpublished). Work in our lab shows that these voltage-activated $K^+$ channels are important for *Drosophila* cardiac function. We know that the homologs hERG and KCNQ1 are important for human cardiac physiology and my collaborator Dr. Diane Fatkin's work suggests it could be important for human cardiac physiology. With this in mind, I wanted to see if interactions between the channels could help determine if they functionally interact. The ultimate goal to build from this type of work, which this appendix would just be the onset for, is to find if they can compensate for each other during times of failure. I took the approach of combining $K^+$ channel heterozygotes and found that some of the cardiac

phenotypes of individual channel knockdown are recapitulated within these transheterozygotes. These phenotypes ranged from the bradyarrhythmia we saw in the *slo* mutations, to sustained fibrillatory events and even decreased contracitility via FS. Some of the transheterozygotes even had a stronger cardiac phenotype than individual channel mutants. A combinatorial approach for when several channels in conjunction are non functional can show the importance of all these $K^+$ channels and lay a foundation for future studies regarding how these could compensate for each other in aging and disease.

## A.3 RESULTS

### *A.3.1 The Cardiac Effects of Seizure and Slowpoke Knockdown*

Knockout of the *hERG Drosophila* homolog *seizure,* significantly increases the AI and the DI (Unpublished data not shown). Additionally, there is a significant decrease in the FS of these mutant flies. All of this data suggest that much like in the human, the channel underlies an important cardiac repolarizing current. We combined a heterozygote genomic *seizure* and *slowpoke* ($sei^{ts1}$/+; $slo^{4}$/+) to look for possible synergistic phenotypes and found that this transheterozygote combination recapitulated the increased HP (Figure 5.1 A) and DI (B) phenotype of the *seizure* and *slowpoke* homolog mutants. However, we did not have the heterozygote $sei^{ts1}$/+ that would bear out the exact contribution of each mutant allele and the relative contribution of each channel to the cardiac phenotype. Regardless, the increase was higher than the DI associated with the *slowpoke* mutants but lower than that of the

*seizure* mutants. However, there was not a significant increase in the AI or decrease in the FS. These results do, however, show an additive phenotype with a larger DI but no effect on the FS or AI in the transheterozygote.

***A.3.2 The Cardiac Effects of KCNQ and Slowpoke Knockdown***

Our past study with the KCNQ homolog in the fly (Ocorr et al., 2007), showed that it is an important channel for *Drosophila* cardiac function and its dysfunction can lead to EADs reminiscent of LQT (Long QT Syndrome). The hallmark of this phenotype was long SIs that encompassed a rapid fibrillatory phenotype and a significantly higher AI. We carried out a similar experiment from the previous section and combined heterozygote mutant alleles of *KCNQ* and *slowpoke* to see if we could discern a synergestic phenotype (*$KCNQ^{370}$*/+*;* *$Slo^{4}$*/+). We also had the heterozygote *KCNQ* mutant genotype to effectively test for genetic interactions. Missing one copy of KCNQ370 seemed to elicit a cardiac phenotype with increased increase HP, DI and AI (Figure 5.2 A, B, C, D), which was greater than missing a single copy of *slowpoke* (*$slo^{4}$*/+). Although the KCNQ370 homozygous mutant was higher in the HP and DI values  than the heterozygote (Figure 5.2 A, B) this was not significant. The $KCNQ^{370}$/+; *$Slo^{4}$*/+ transheterozygote in turn had a higher HP and DI than both the homozygous and heterozygous mutants, but again this value was not significant. The huge increase in the heterozygote $KCNQ^{370}$ in comparison to the phenotype oand probable huge contribution to the cardiac phenotype from lack of $KCNQ^{370}$ makes it difficult to differentiate in the to any genetic

interaction between KCNQ and slowpoke. The AI was also the highest of the transheterozygote combinations that we tried although smaller than that of the *slowpoke* and *seizure* genomic mutants (Figure 5.2). It is interesting to note that we did not see concomitant significant increases in the SI compared to control as previously reported. Perhaps I need a larger N for the study and a different to mutation to verify our results.

### *A.3.3 The Cardiac Effects of KCNQ and Seizure Knockdown*

After looking at the combination of the *slowpoke* mutant with both *seizure* and *KCNQ* mutations, we also looked at heterozygote combinations of *seizure* and *KCNQ*. Like the previous heterozygote combinations, the combination of KCNQ and seizure heterozygote mutants when compared to the control W1118 and the $slo^{4}$*/Df (3R) BSC397* had a significantly increased DI and HP but not SI or AI. (Figure 5.3 A, B, C, D). However, this combination did not have significantly larger values for these parameters compared to the $KCNQ^{370}$ heterozygote or other transheterozygote combinations. It did, however, recapitulate the significantly decreased FS *seizure* mutant phenotype that we did not see in the $sei^{ts1}$/+; $slo^{4}$/+ combination (Figure 5.3).

### *A.3.4 KCNQ can partially rescue seizure deficiency*

The different voltage activated $K^{+}$ channels all seem to have a deep impact on cardiac physiology that reflects slightly different on particular cardiac phenotypes. However, I wanted to see if regardless of the different cardiac phenotype deficiencies each elicit, if some amount of functional redundancy

existed between some of these channels. In a pilot experiment I used a weaker cardiac specific Gal4 driver (Tin-Gal4 (Lo & Frasch, 2001)) to overexpress the *KCNQ* channel in a homozygous *seizure* mutant shown in figure 5.5, we found a decrease in the FS D) and AI (C) compared to outcross controls. We did not see a decrease in the SI (B) although there was a partial decrease in the DI (A) in the overexpressor compared to one outcross alone. Altogether, we did not see a rescue of the normal *seizure* FS or DI phenotype; although the AI was significantly decreased. Overexpressing *KCNQ* in the heart partially compensated for the *seizure* mutant phenotype.

## A.4 DISCUSSION

The repolarizing phase of the action potential is made up primarily of the rapid delayed rectifier current ($I_{kr}$) and slow delayed rectifying current ($I_{ks}$), which are in turn driven by the flow of $K^+$ ions across the the *hERG* and the *KCNQ* channel respectively. Their importance in this major action potential phase means that their dysfunction is a major cause of cardiac arrhythmias. However, they are only a part of many different $K^+$ channels acting in concert during every phase of the action potential. Thus the initial simplicity associated with having two major $K^+$ channels driving repolarization belies the complexity and interconnectedness between all the channels. This is a robust system that can accommodate a wide variety of stresses, yet the aging process can finally elicit these types of diseases as evidenced by the incidence of AF in older populations. We used the *Drosophila* model to probe how heterozygote $K^+$

channel genomic mutant could have a synergistic phenotype when combined with a heterozygote $K^+$ mutant. Of the combinations tested the *sei$^{ts1}$/+; slo$^4$/+* transheterozygote had a significantly longer DI and HP compared to controls but I was missing seiz$^{ts1}$/+ control that would help determine how much of the worsening of cardiac function was due to the *seizure* channel mutation. It was also interesting that *seizure* mutant decreased FS was not present in the *slo* and *seizure* mutant transheterozygotes (Figure 5.1 E). When we looked at *KCNQ* in combination with the *slo* heterozygote mutants, we saw a significant increase in HP over the genomic *slo* mutant but it only trended towards a DI and HP increase over the homozygous *KCNQ* mutant and the *KCNQ* heterozygote control (Figure 5.2 A, B). Although, these results should be replicated since we did not see the SI increase normally associated with *KCNQ* homozygous mutant. Finally, the effects of combining the *seizure* and *KCNQ* heterozygote mutants again caused a significant increase in the HP and DI compared to wild-type but not to other genomic mutant combinations (Figure 5.4 A, B). However, this combination did replicate the significantly decreased FS homozygous *seizure* phenotype that the combination with *slo* genomic mutant was not able to replicate (Figure 5.3 E). Perhaps, with more the *seizure* heterozygote, we could be able to start to determine what kind of genetic perturbations create the environment for certain phenotypes to emerge. The longer HP and DI seemed universal to most genetic perturbations but differentiating how different mutants could elicit *slowpoke*

mutant's *i*ncreased SI or the decreased FS of *seizure* mutants could give clues to how these channels function in the heart.

The connection between *seizure* and *KCNQ* was evident when we tried to rescue the *seizure* homozygous phenotype with cardiac specific K*CNQ* overexpression. This elicited as significant decrease in the AI (Figure 5.5 C) but did not bring the FS back to wild-type levels (Figure 5.5 D). It could be that overexpression of the *KCNQ* leads to decreased FS but this effect was not noted in earlier studies looking at *KCNQ* overexpression (Ocorr et al., 2007). The connection could ultimately be that the *seizure* phenotype of decrease FS is only rescued by a specific function of the *seizure* channel. Thus, compensating for the decrease in repolarizing potential by overexpressing *KCNQ* causes a concomitant decrease in the AI (Figure 5.5 C) but not in the FS.

The combinatorial experiments are another example of the importance of the *slowpoke* channel to cardiac function. Heterozygote mutants of this channel in combination with other important established $K^+$ channel mutants lead to strong cardiac phenotypes. Additionally, decreasing the repolarizing potential with *seizure* and *KCNQ* heterozygote mutants uncovered the FS cardiac phenotype associated with homozygous *seizure* mutants. This combinatorial approach could uncover not just interaction between channels but a process by which aging and stress can uncover primed cardiac tissue containing channel deficiencies. We need to replicate this experiment with

more mutants, cardiac specific knockdown, rescue lines, and other putative $K^+$ channels in the cardiac action potential. However, these experiments function as a first step to show the interconnectedness of $K^+$ channels and a model system to test how they work.

## Appendix Figures:

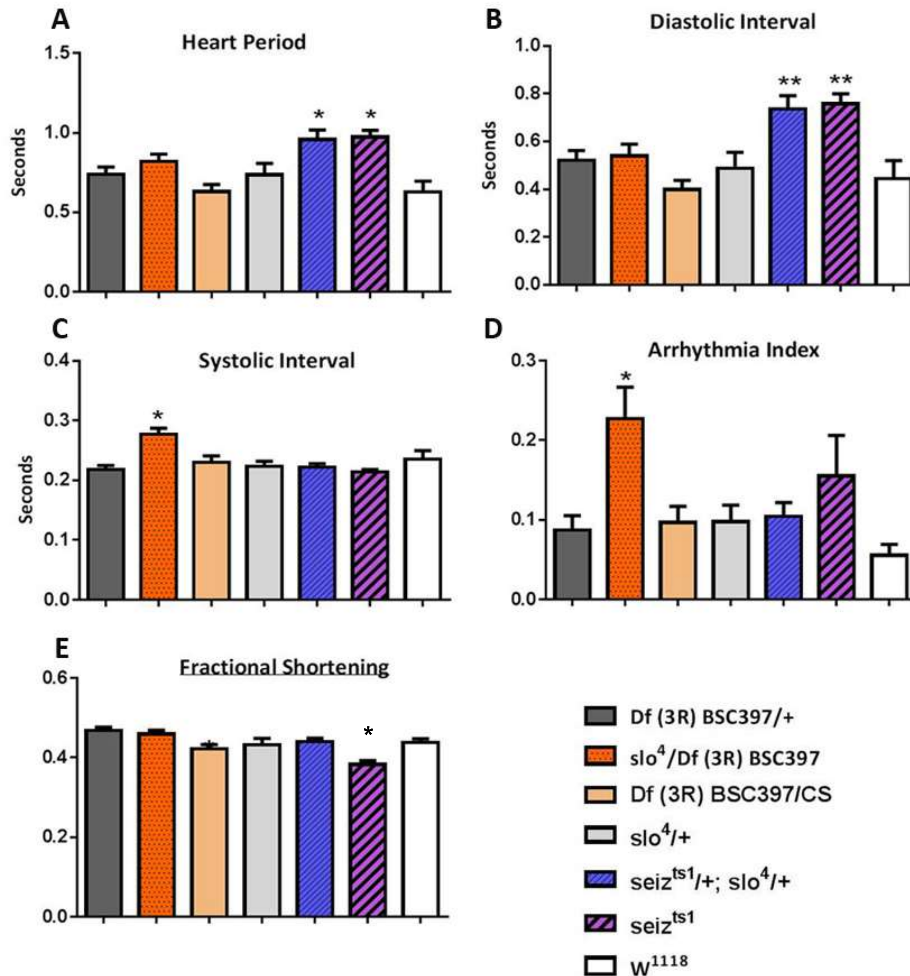



**Figure A.1 Double heterozygote mutants of *slo* and *seizure* recapitulate some cardiac phenotypes of each individually.** When we combine double heterozygote mutants of *slowpoke* and *seizure,* we see a significantly increased diastolic interval (B) and heart period (A). However, the transheterozygote did not have a smaller fractional shortening (E), larger systolic interval (C) or arrhythmia index (D). Note that we did not have $seiz^{ts1}$ to determine if the transheterozygote effect was primarily due to *seizure* mutation alone.

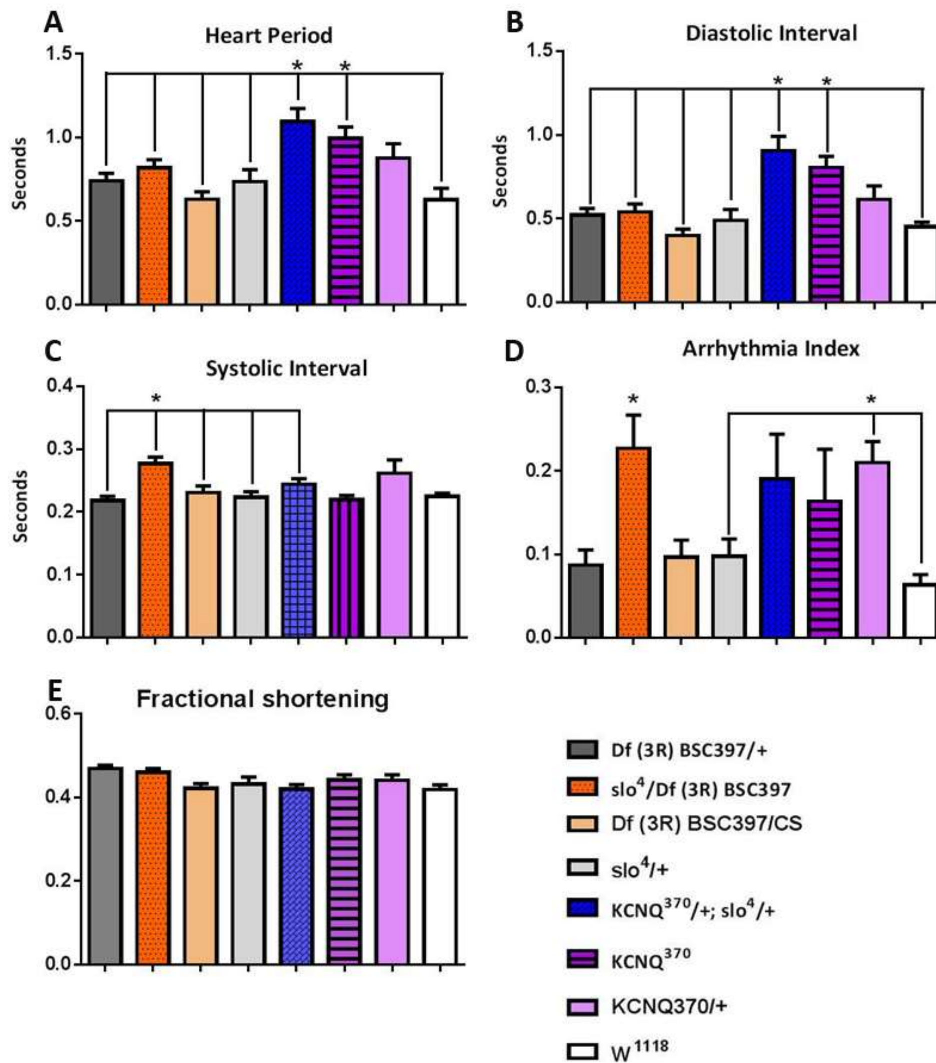



**Figure A.2 Double heterozygote mutants of *KCNQ* and *slo* worsen cardiac function.** The double *KCNQ* and *slowpoke* transheterozygotes had a significantly longer heart period (A), diastolic interval (B), and arrhythmia index (D) compared to wild-type controls. However, it seems a lot of the effect was due to the effect of the *KCNQ* allele as the heterozygote did not have a significantly smaller HP, DI, SI or AI compared to either the *slowpoke* transhetorzygote mutant (slo4/Df (3R) BSC397), homozygous *KCNQ* mutant (KCNQ370) or *KCNQ* and *slowpoke* transheterozygote (.

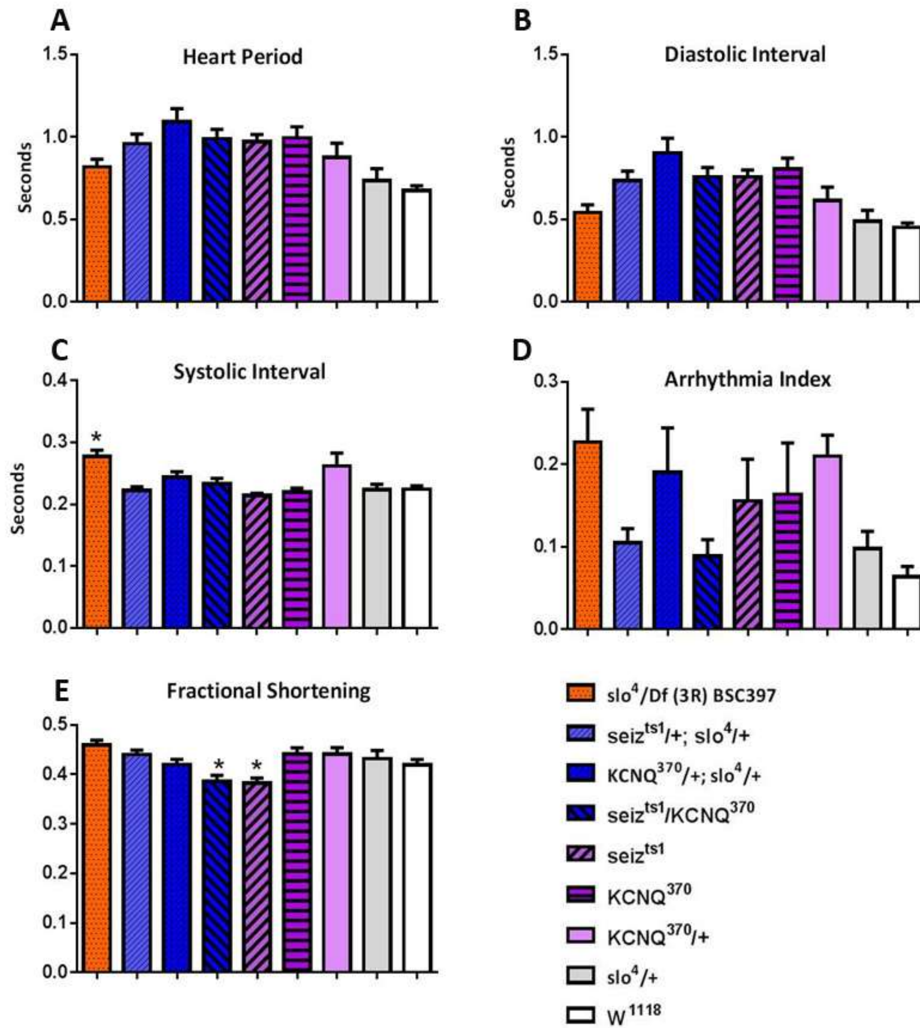



**Figure A.3 The *KCNQ* and *seizure* transheterozygotes recapitulate some cardiac phenotypes of each mutant individually.** The *KCNQ* and *seizure* transheterozygote recapitulates the long diastolic interval (B) and heart period (A) of *seizure* and *KCNQ* mutants. Although, without the *seizure* heterozygote, we are not able to determine which mutation is driving the cardiac phenotype. We did see, however, a decrease in the fractional shortening for the *KCNQ* and *seizure* transheterozygote that was only seen in the *seizure* mutant but not in the *seizure slowpoke* mutant combination (E). We did not, however, see an increase in the arrhythmia index (D).

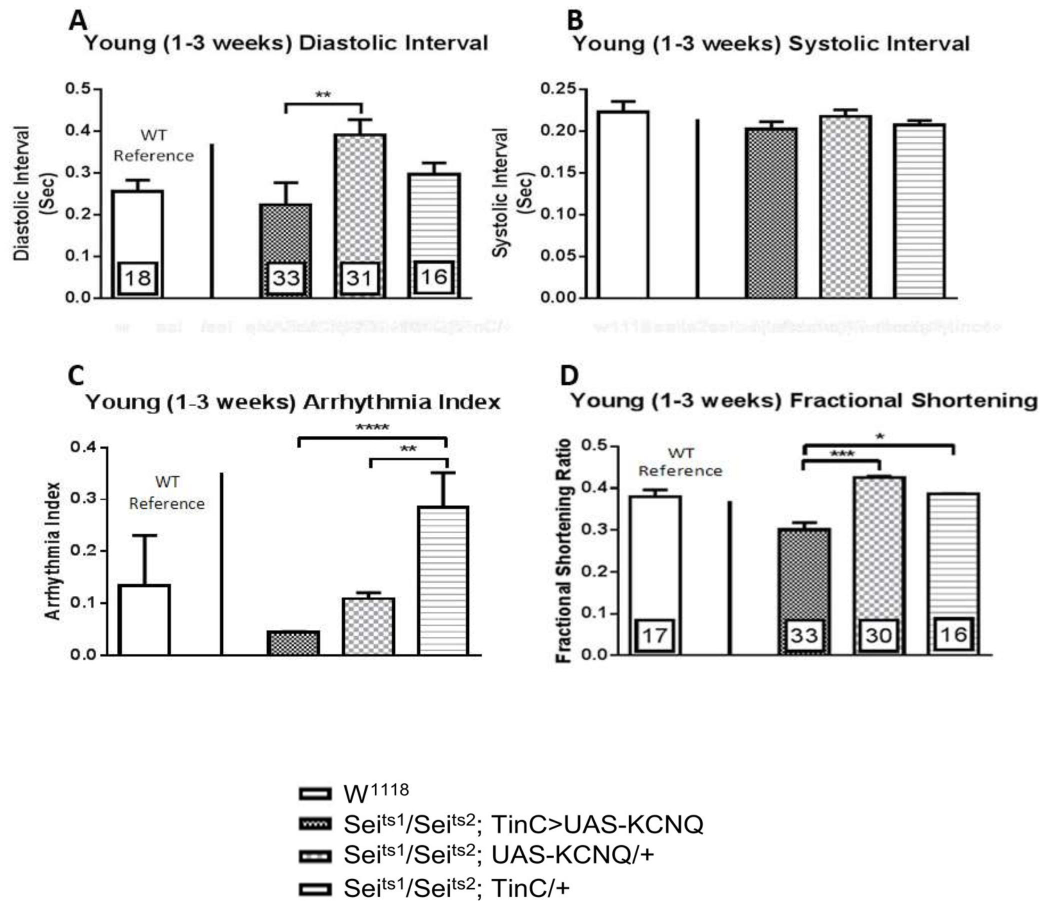



**Figure A.4 *KCNQ* overexpression partially compensates *seizure* deficiency.** Cardiac specific overexpression of *KCNQ* in a *seizure* mutant background significantly decrease the FS (D) and AI (C) compared to outcross controls. The DI (B) also trended towards a decreased value.

**Table A.1 Electrophysiological rescue of *seizure* deficiency by *KCNQ* overexpression.** Compared to the outcross control, overexpressing *KCNQ* in a *seizure* mutant background significantly decreases event duration. The maximum amplitude was also significantly different but a higher N is required.

| | N | Resting $V_m$ (mV) | Maximum Amplitude (mV) | Peaks / Burst | Event Duration (ms) |
|---|---|---|---|---|---|
| $W^{1118}$1WK | 1 | -39.5 | 42.9 | 2 | 412.5 |
| $W^{1118}$2WK | 2 | -32.8 | 43.6 | 2.5 | 343.9 |
| $Sei^{ts1}/Sei^{ts2}$; uaskcnq2/tinc4 | 1 | -54.5 | 74.3 | 3.74 | 564.4 |
| $Sei^{ts1}/Sei^{ts2}$; uaskcnq2 | 1 | -34.0 | 48.4 | 4.2 | 722.0 |

# CHAPTER 5: DISCUSSION

## 5.1 DISCUSSION

The electrical system of the heart is a complex phenomena of which we are still discovering new facets. The dysfunction of this system is an equally complex problem made all the more obtuse by wide assortment of genetic and environmental causes that can synergize to cause disease. In this field cardioversion therapy, pharmacologic or electrical methods of restoring normal rhythm, has been incredibly helpful for patients. However, some of these treatments carry their own risks and decrease in efficacy with time. Getting a deeper understanding of the biology behind electrical dysfunctions can help to discover new methods to help these patients and improve normal cardiac function with age.

It is within this context that we looked at the *KCNMA1* homolog *slowpoke* and its function in the cardiac system. Its $Ca^{2+}$ activation and high single channel conductance make it a prime candidate for modulation of electrically active cells. It was found in the neural system but even there its function was not immediately clear and it took decades of research to flesh out its role in $Ca^{2+}$ feedback homeostasis in particular neural cell types (Brenner et al., 2005). Nevertheless, its absence in the cardiac tissue was not explored since the knockout mouse had no cardiac phenotype due to its normal rapid heartbeat (Meredith et al., 2004). Yet, experiments on cardiac pacemaker cells of the mouse showed a decrease in the RMP (Imlach et al., 2010). This result

was replicated in human embryonic stem cell derived pacemaker-like cells but with the SK channel rather than *KCNMA1 (Weisbrod et al., 2016)*. Both of these, however, do not address how the channel's dysfunction affects cardiac physiology. In our study, we found initially that the channel was important for *Drosophila m.* cardiac function. The longer heart period, increased arrhythmia, and electrical dysfunction were evidence of its importance (Figure 3.5, 3.9). Yet, the type of dysfunction and mechanism were tougher to discern. The increased average repolarization time from mutant to wild-type does point to its importance for cardiac repolarization (Figure 3.12). However, the aforementioned complexity of the systems involved with heart rate do not preclude a variety of other explanations or identify exactly where in the repolarization curve this channel could function. Previous research has shown that this channel is localized to mammalian mitochondria and is important for hypoxic preconditioning during cardiac arrest. Its activity there could affect metabolic function and thus affect mechano-electrical function. Yet, the recordings from human iPS and mouse pacemaker cells suggest that at least this is not the only effect. In both studies knockout of a $Ca^{2+}$ sensitive $K^{+}$ current resulted in a lengthening of diastolic depolarization (DD) represented as a shallower DD slope and subsequent decreased RMP (Weisbrod et al., 2016). The channel cell specificity and regulation of firing rate through the RMP is also present in the neural system. A recent study showed not only that the BK channel activity increased the afterhyperpolarization but importantly

this change was functionally coupled to Ryanodine receptor (RyRy) (Brenner et al., 2005). Channel function was previously coupled to L-type $Ca^{2+}$ channels during repolarization but the afterhyperpolarization similar to the effect in pacemaker cells was now coupled to RyRy. An effect which suggests that there could be sets of BK channels coupled to different $Ca^{2+}$ pools. These studies open new questions for probing mechanism of BK channel action in the heart. We could try to identify and record from pacemaker cells in the fly heart and check RMP differences. This would correlate a strong cardiac phenotype with a mechanistic process of $Ca^{2+}$ feedback in heartbeat. Phemonologically we see this effect on the whole heart as slower heartbeat and electrically longer interevent interval frequency (Table 3.1). However, the mechanism behind this effect was uncertain in the studies looking at either cadiac pacemaker or neural cells. A general idea is that a shallower DD does not prime for activation of funny current channels that are needed to hit the threshold action potential activation. Functionally, we could test this effect in the fly by genetic epistasis of BK channels with both RyRy and HCN. These experiments could solidify the mechanism of action for the BK channel in the heart and inform future mammalian studies.

The human mutation associated with AF seemed to worsen cardiac function. There are yet many venues to explore with this channel and its impact on cardiac physiology. Voltage clamping of these channel under different pharmacological conditions could further tease out its contribution to

the cardiac action potential. We know in neurons that a synergistic $Ca^{2+}$ and voltage change are the drivers of channel activation. If this channel is truly a regulator of $Ca^{2+}$ homeostasis then genetically encoded sensors could paradoxically show a decrease in the $Ca^{2+}$ wave as the system tries to compensate for the decrease in this negative feedback regulator.

Our preliminary data showing a decrease in the $Ca^{2+}$ channel expression in *slo* mutant hearts suggested this could be a compensatory mechanism (Figure 3.4). My preliminary combinatorial mutation showed a process of how certain cardiac phenotypes could arise from partial deficiencies of two different channels. In particular the combination of the *KCNQ* and *seizure* mutant showed a significantly decreased FS that I did not see with *slowpoke* and *seizure* mutant combination (Figure A.3 E). However, I did not have the seizure heterozygote mutant that could help us determine if this effect arose from an interaction with *KCNQ* or solely by *seizure* mutation. With more $K^{+}$ channel mutant combinations we could see if a pattern of cardiac phenotypes emerges that could tells us more about how these channels function together.

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