Aus dem Institut Max-Delbrück-Centrum für Molekulare Medizin
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DISSERTATION

Defining the molecular cause of an altered response to anti-arrhythmic drugs in Pitx2-dependent atrial fibrillation

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Preamble:

This thesis is a result of the cooperation of the Max-Delbrück-Centrum für Molekulare Medizin and the Institute of Cardiovascular Sciences, University of Birmingham, UK. Parts of the results were published in the papers below:

1. Syeda F, Holmes AP, Yu TY, Tull S, Kuhlman SM, Pavlovic D, Betney D, Riley G, Kucera JP, Jousset F, de Groot JR, Rohr S, Brown NA, Fabritz L, and Kirchhof P, PITX2 Modulates Atrial Membrane Potential and the Antiarrhythmic Effects of Sodium-Channel Blockers. J Am Coll Cardiol, 2016. 68(17): p. 1881-1894.

2. Holmes AP, Yu TY, Tull S, Syeda F, Kuhlmann SM, O'Brien SM, Patel P, Brain KL, Pavlovic D, Brown NA, Fabritz L, and Kirchhof P, A Regional Reduction in Ito and IKACh in the Murine Posterior Left Atrial Myocardium Is Associated with Action Potential Prolongation and Increased Ectopic Activity. PLoS One, 2016. 11(5): p. e0154077.

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Index of abbreviations:

AAD	antiarrhythmic drugs
AF	atrial fibrillation
APA	action potential amplitude
APD	action potential duration
Ca^{2+}	calcium
CL	cycle length
ERP	effective refractory period
GWAS	Genome-wide association studies
HEK-hNa_V1.5	human embryonic kidney cells, stably transfected with the SCN5A gene and thus expressing the human Na_V1.5 sodium-channel
$ICa,_L$	long-lasting voltage-dependent calcium channel
I_{Na}	sodium current
I_{K1}	inward rectifying potassium current
I_{KACh}	acetylcholine-sensitive G-protein-coupled inward rectifier potassium current
I_{Kpeak}	$I_{Ksteadystate}$ + I_{to}
$I_{Ksteadystate}$	delayed rectifier (consisting of I_{Kur}, I_{Kr} and I_{Ks})
I_{Kr}	rapidly-activating potassium current
I_{Ks}	slow-activating potassium current
I_{Kur}	ultra-rapidly activating potassium current
I_{to}	transient outward K^+ current
K^+	potassium
LA	left atrial / left atrium
microRNA	micro ribonucleic acid
mRNA	messenger ribonucleic acid
Na^+	sodium
Na_V1.5	ion channel for Na^+
NppaCrePitx2$^{-/-}$	atria-specific deletion of Pitx2
Pitx2 CKO	deletion of Pitx2 in the postnatal atria
PITX2	PITX2 mRNA in humans
Pitx2	Pitx2 mRNA in mice
Pitx2c$^{+/-}$	heterozygous Pitx2c knockout mice

RA	right atrium
RMP	resting membrane potential
Ryr_2	ryanodine receptor 2
SAN	sinoatrial node
Scn5a	mRNA coding for the $Na_V1.5$ protein
SNP	single nucleotide polymorphism
SR	sarcoplasmic reticulum
WT	wild-type

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1. Abstracts:

1.1 Abstract (English):

Purpose: Atrial fibrillation (AF) is the most prevalent cardiac arrhythmia in humans and is associated with stroke, heart failure, hospitalisation and dementia. Genome-wide association studies (GWAS) indicate that single nucleotide polymorphisms (SNPs) on chromosome 4q25, close to the PITX2 gene, are strongly associated with AF. Heterozygous Pitx2c knock-out mice ($Pitx2c^{+/-}$) have a shorter action potential duration (APD), a more positive resting membrane potential (RMP) and an enhanced sensitivity to flecainide. In this thesis, the causes of the modification in atrial electrophysiology and response to antiarrhythmic drug (AAD) therapy in $Pitx2c^{+/-}$ mice were studied.

Methods: Sodium currents (I_{Na}), potassium currents (I_{Kpeak}, $I_{Ksteadystate}$, I_{to}, I_{K1}, I_{KACh}) and the effect of flecainide on I_{Na} were investigated using the patch-clamp technique in isolated left atrial (LA) cardiomyocytes from wildtype (WT) and $Pitx2c^{+/-}$ mice. Furthermore, the effect of flecainide on human embryonic kidney cells stably expressing the human $Na_v1.5$ sodium-channel (HEK293-h$Na_v1.5$) was assessed at different RMPs. Additionally, calcium (Ca^{2+}) transients and the impact of flecainide were investigated in WT and $Pitx2c^{+/-}$ LA by high spatial resolution optical mapping.

Results: There was no difference between WT and $Pitx2c^{+/-}$ LA cardiomyocyte cell size (measured by cell capacitance), I_{Na} density and the inhibiting effect of flecainide across a range of fixed RMPs. However, a more positive RMP increased the I_{Na}-inhibiting effect of flecainide in WT and $Pitx2c^{+/-}$ LA cardiomyocytes and in HEK293 cells expressing the human $Na_v1.5$ channel. Neither Ca^{2+} transient duration, amplitude, nor the effect of flecainide were significantly different between genotypes. Flecainide shortened Ca^{2+} relaxation times and reduced the amplitude in both genotypes. There was no significant difference in I_{Kpeak}, $I_{Ksteadystate}$, I_{to}, I_{K1} and I_{KACh}, between genotypes. A more positive RMP decreased I_{Kpeak}, $I_{Ksteadystate}$ and I_{to}. I_{K1} remained unaffected by a more depolarised RMP. A modulated RMP had a dual effect on I_{KACh} (more K^+ inflow at potentials more negative to -80mV and smaller outward K^+ outflouw at potentials above -70mV).

Conclusion: Whilst the cause of the shortened APD still remains elusive, the cause of the greater antiarrhythmic effect of flecainide in $Pitx2c^{+/-}$ LA seems to be apparent now: The more positive RMP in LA with reduced $Pitx2c^{+/-}$ increases flecainide's I_{Na}-inhibiting

effect, leading to a greater antiarrhythmic protection. Thus, it is proposed that flecainide may provide more effective treatment of AF in patients with reduced PITX2.

1.2 Abstract (German):

Einleitung: Vorhofflimmern ist die häufigste kardiale Arrhythmie des Menschen und ist assoziiert mit Schlaganfällen, Herzinsuffizienz, Hospitalisierung und Demenz. Genomweite Assoziationsstudien (GWAS) weisen darauf hin, dass Einzelnukleotid-Polymorphismen (SNPs) auf Chromosom 4q25 in der Nähe des PITX2 Gens stark mit Vorhofflimmern assoziiert sind. Heterozygote Pitx2c knockout Mäuse ($Pitx2c^{+/-}$) präsentieren eine kürzere Aktionspotentialdauer (APD), ein positiveres Ruhemembranpotential (RMP) und eine erhöhte Sensitivität gegenüber Flecainid. In dieser Arbeit wurden die Ursachen der modifizierten atrialen Elektrophysiologie und der veränderten Reaktion auf medikamentöse antiarrhythmische (AAD) Therapie in $Pitx2c^{+/-}$ Mäusen untersucht.

Methoden: Natriumstrom (I_{Na}), Kaliumströme (I_{Kpeak}, $I_{Ksteadystate}$, I_{to}, I_{K1}, I_{KACh}) und der Effekt von Flecainid auf I_{Na} wurden mittels Patch-Clamp Technik in isolierten links atrialen (LA) Kardiomyozyten von Wildtyp (WT) und $Pitx2c^{+/-}$ Mäusen untersucht. Außerdem wurde der Effekt von Flecainid auf humane embryonische Nierenzellen, die den humanen $Na_v1.5$ Natriumkanal (HEK293-h$Na_v1.5$) stabil exprimieren, bei unterschiedlichen RMPs untersucht. Zusätzlich wurden Kalzium (Ca^{2+}) Ströme und der Effekt von Flecainid auf diese in WT und $Pitx2c^{+/-}$ Mäusen mittels hochauflösender Optical Mapping Technik untersucht.

Ergebnisse: Zwischen WT und $Pitx2c^{+/-}$ LA Kardiomyozyten zeigte sich kein Unterschied in Zellgröße (gemessen anhand der Zellkapazität), I_{Na} Dichte und dem hemmenden Effekt von Flecainid über mehrere RMPs. Ein positiveres RMP erhöhte den I_{Na} hemmenden Effekt von Flecainid in WT und $Pitx2c^{+/-}$ LA Kardiomyozyten und HEK293-h$Na_v1.5$. Weder die Dauer, noch die Höhe, noch der Effekt von Flecainid auf Ca^{2+} Ströme unterschieden sich signifikant zwischen den Genotypen. Flecainid verkürzte die Ca^{2+} Erholungszeiten und verringerte die Amplitude in beiden Genotypen. Es zeigte sich kein signifikanter Unterschied in I_{Kpeak}, $I_{Ksteadystate}$, I_{to}, I_{K1} und I_{KACh} zwischen den Genotypen. Ein moduliertes RMP hatten einen dualen Effekt auf I_{KACh} (mehr K^+ Einfluss bei Potentialen, die negativer als -80mV sind, und weniger K^+ Ausfluss bei Potentialen, die positiver als -70mV sind).

Zusammenfassung: Während die Ursache für eine verkürzte Aktionspotentialdauer noch unklar bleibt, scheint der Grund für einen höheren antiarrhythmischen Effekt von Flecainid in $Pitx2c^{+/-}$ LA nun offen zu liegen: Das positivere RMP in LA Kardiomyozyten mit reduzierter Pitx2c Expression erhöht Flecainids I_{Na} hemmenden Effekt, was zu einem

größeren antiarrhythmischen Schutz führt. Daher wird vorgeschlagen, dass Flecainid bei Patienten mit reduziertem PITX2 eine effektivere Therapie darstellt.

2. Introduction:

2.1 Atrial fibrillation: epidemiology, risk factors and consequences

Atrial fibrillation (AF) is the most prevalent cardiac arrhythmia in humans [1, 2]. Its prevalence increases with age from 2.4% in people 65 years old to 10% in those older than 80 years [3]. AF increases mortality, morbidity [4] and the risk of stroke, heart failure, hospitalisation and dementia [5, 6]. Antiarrhythmic drug therapy (AAD), alone or combined with cardioversion or catheter ablation, is the most common treatment to restore and maintain sinus rhythm in patients with AF ("rhythm control therapy").

Parental AF is a risk factor for AF in the offspring generation [7]. This finding led to the proposal that genes could play a crucial role in the predisposition to AF. Genome-wide association studies (GWAS) indicate that common gene variants adjacent to the PITX2 gene, measured as single nucleotide polymorphisms (SNPs), correlate more strongly with AF than any other genetic modification [1, 8-10]. The first SNPs, which were found to correlate with the occurrence of AF, were SNP rs2200733 and rs2220427 [1, 11]. The most significant SNP was rs2200733 on chromosome 4q25, approximately 150 kb upstream of the PITX2 gene [12]. In addition, Rs2200733 also increases the risk of AF recurrence after direct current cardioversion [13] and after catheter ablation in humans [14]. Latest research added several novel SNPs to the list of independent SNPs associated with AF [10].This novel data strongly supports the hypothesis that inherited factors predisposing to AF are clustered in a small genomic region on chromosome 4q25, close to the PITX2 gene.

2.2 The role of PITX2 in atrial fibrillation

Whether or not these SNPs act to reduce PITX2 expression remains controversial. In a small population-based study, Chinchilla et al compared right (RA) and left atrial (LA) appendage biopsies of humans with AF vs. sinus rhythm and found that the PITX2 expression was significantly decreased in RA and LA of AF patients [8]. Furthermore, using human stem-cell-derived cardiac myocytes, it has now been proposed that the key SNPs lie in an enhancer region that functions to modify PITX2 transcription [15]. However, this has been challenged by the findings that show no correlation between SNPs and PITX2 expression [16]. There is even some evidence that PITX2 expression may increase in the RA of AF patients [17]. Recently, it has been shown that in a larger patient cohort with AF undergoing catheter ablation, PITX2 expression is variable [18]. These authors identified a distinct population of patients with low PITX2 mRNA expression [19]. This may not only be a consequence of SNP status but also due to other factors that regulate PITX2 transcription.

2.2.1 The physiological role of PITX2

Pitx2 is an important transcription factor that is highly expressed in the developing embryo [20]. Pitx2 is part of the bicoid group of paired type homeobox genes and serves as a mediator between nodal signaling in the left lateral plate mesoderm and the forming organs [21]. Leftness is modulated by activation of nodal [22]. Pitx2 is asymmetrically expressed in the left lateral plate mesoderm and plays a determinant role in left-right signaling [21]. The concentration of Pitx2 is important for normal organogenesis [23], especially the heart seems to be very sensitive to varying Pitx2 levels [23]. The Pitx2 expression extends into the left side of the developing heart [24, 25], where it suppresses the sinoatrial node (SAN) formation on the left side of the venous pole [26].
More recent evidence has shown that PITX2 continues to be expressed in both the human and murine LA at levels 100-fold higher than all other cardiac chambers [27]. Indeed, PITX2 is the most differentially expressed gene between the left and right atria, making it a very good marker for left atrial tissue [28]. In the adult LA, Pitx2 regulates the expression of a host of ion channels, transcription factors, microRNAs and Ca^{2+} handling proteins (see figure 1) [8, 27, 29-33].

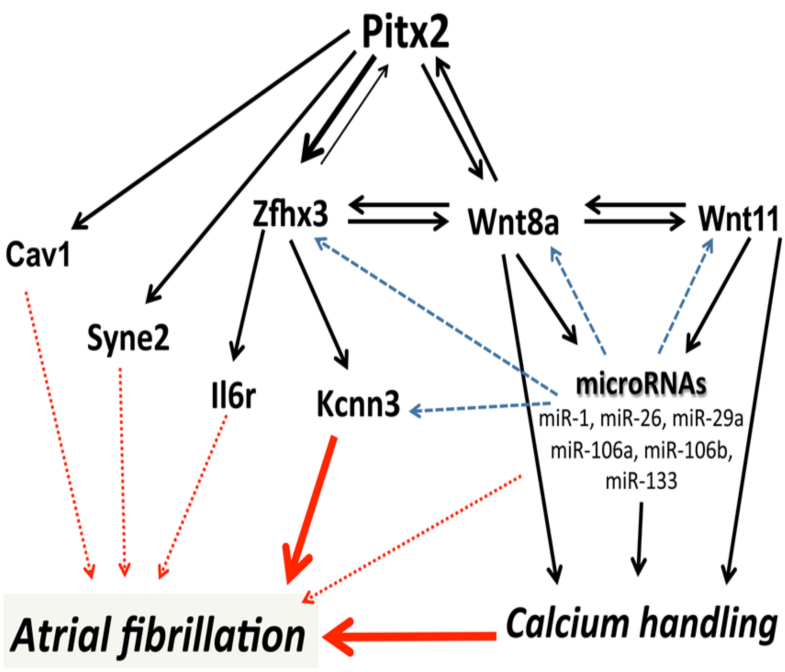


Figure 1: Pitx2 interactome in the context of AF. The transcription factor Pitx2 plays a crucial role in regulating the translation of transcription factors, microRNAs, ion-channel and Ca^{2+}-handling genes. Adapted from: Lozano-Velasco E, Hernandez-Torres F, Daimi H, Serra SA, Herraiz A, Hove-Madsen L, Aranega A, and Franco D, *Pitx2 impairs calcium handling in a dose-dependent manner by modulating Wnt signalling.* Cardiovasc Res, 2016. **109**(1): p. 55-66.

2.2.2 Pitx2-deficient mouse models

For investigating the effect of a lack of Pitx2c on molecular and electrophysiological basis, several mouse models have been generated. The C isoform of Pitx2 is expressed in the left atrium and $Pitx2c^{+/-}$ mouse hearts are predisposed to AF [27]. The $Pitx2c^{+/-}$ mouse model is a heterozygous knockout of Pitx2 isoform C in every cell. The $NppaCrePitx2^{-/-}$ mouse model is a homozygous knockout of both Pitx2 alleles just in the atria and Pitx2 CKO (Pitx2 conditional loss of function) is a mouse model with a homozygous knockout of Pitx2 after birth. A homozygous knockout of Pitx2c before birth is lethal.

This thesis is focused on the $Pitx2c^{+/-}$ mouse model, which is predisposed to AF and models the assumed reduction in atrial PITX2 that could be associated with the common gene variants.

2.2.3 Pathophysiology of Pitx2c deficiency

All murine models deficient in Pitx2 exhibit an increased vulnerability to inducible AF [8, 27]. However, the mechanisms underlying this increased AF susceptibility remain to be fully identified. On a molecular basis, Pitx2c$^{+/-}$ LA cardiomyocytes have an increased expression of Kcna3 and a reduced expression of Kcna6, Kcnc4, Kcnk5 and Cacna1c [27, 32] (see figure 2). Similar mouse models lacking Pitx2 just in the atria (NppaCrePitx2$^{-/-}$) or in the postnatal atria (Pitx2 CKO) display a variety of changes in ion channel expression [8, 34, 35] (see figure 2).

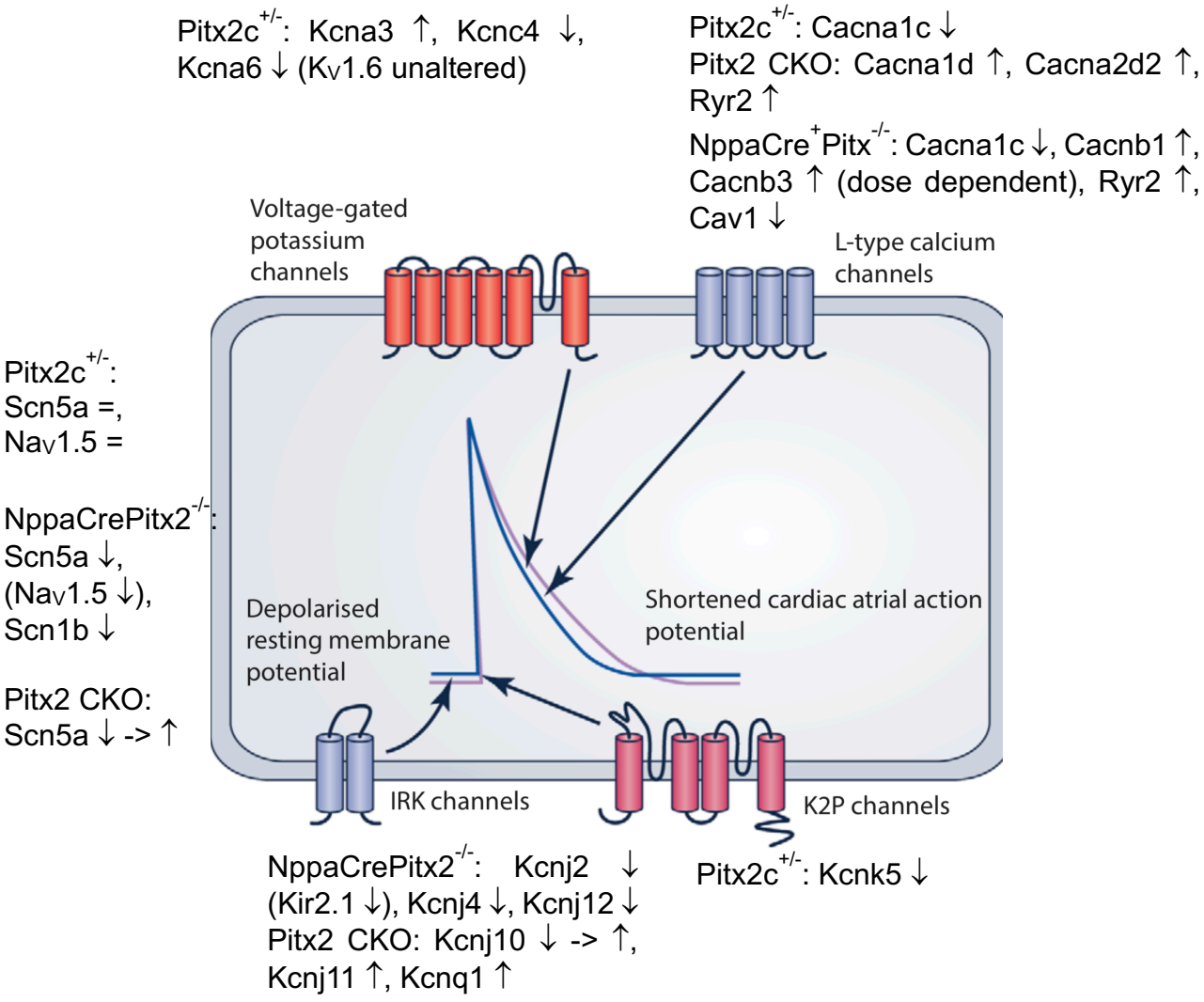


Figure 2: A lack of Pitx2 leads to an impaired ion channel expression. Voltage-gated, inwardly rectifying and two-pore domain potassium currents, sodium currents and calcium currents are severely impaired in different mouse models lacking Pitx2. = no change in expression, ↓ reduced expression, ↑ enhanced expression, -> changed expression after birth; adapted from: Syeda F, Kirchhof P, and Fabritz L, PITX2-dependent gene regulation in atrial fibrillation and rhythm control. J Physiol, 2017. 595(12): p. 4019-4026.

Additionally, three major electrophysiological differences in Pitx2c$^{+/-}$ LA cardiomyocytes have been described:

1. The action potential amplitude (APA) is reduced [8, 32]
2. The action potential duration (APD) is shortened [8, 32]
3. The resting membrane potential (RMP) is more positive [8, 32]

Pitx2c$^{+/-}$ LA cardiomyocytes present a reduced APA [8, 32]. APA is governed by Na^+ inward current (I_{Na}). However, sodium channel *Scn5a* mRNA expression and $Na_V1.5$ channel protein, which are the most important components in determining the magnitude of human I_{Na}, were found not to be significantly altered in Pitx2c$^{+/-}$ LA cardiomyocytes [32]. Still, in a mouse model with atria-specific homozygous deletion of Pitx2 (NppaCre^{+}Pitx$^{-/-}$), a reduction of Scn5a and Scn1b has been reported [8] and in a mouse model with a deletion of Pitx2 in the postnatal atria, a decreased expression prenatal and an increased expression postnatal has been described [34]. Though there is a difference in Scn5a expression between Pitx2c$^{+/-}$ and NppaCre^{+}Pitx$^{-/-}$, they still show the same electrophysiological phenotype (see the three points above), leading us to the hypothesis that the smaller APA is instead due to a change in $Na_V1.5$ protein function and not only a reduced Scn5a expression. In this thesis patch-clamp experiments were performed to directly compare I_{Na} between WT and Pitx2c$^{+/-}$ LA cardiomyocytes and to evaluate whether a change in ion channel function might be the cause of the smaller APA in Pitx2c$^{+/-}$.

Additionally, Pitx2c$^{+/-}$ hearts have been found to be more sensitive to flecainide, displaying a greater protection against inducible atrial arrhythmias [32]. This is supported by the finding that flecainide had a greater impact on suppressing AF in patients with SNP variants adjacent to the PITX2 locus [18]. The mechanism of the increased antiarrhythmic effect of flecainide in hearts with reduced Pitx2c is elusive and could be related to an increased action on either the $Na_v1.5$ channel or the ryanodine receptor 2 (RyR2; an additional target of flecainide), which was found to be up-regulated in NppaCre^{+}Pitx$^{-/-}$ and Pitx2 CKO LA cardiomyocytes. Therefore, in this thesis, the impact of flecainide on both I_{Na} and Ca^{2+} transients was studied to more clearly define how Pitx2 deficiency augments the antiarrhythmic action of fleciainde.

Wakili et al. hypothesise, that a lack of Pitx2 in the pulmonary vein region could lead to AF by either shortening APD, which would promote microreentry, or by causing ectopic activity related to early-after depolarisation mechanisms [36]. In the Pitx2c$^{+/-}$ LA, it has been suggested that there is a shortening of APD and effective refractory period (ERP); effects that are more apparent at higher pacing frequencies [37]. Shortened APD and ERP will predispose to re-entry and provide a substrate for arrhythmia in the presence of ectopic activity. The shortening in APD could be explained by a reduction in either inward Ca^{2+} [33] and/or an increase in outward K^{+} currents.

It is known that AF is associated with a reduced Cacna1c expression, which codes for the alpha 1C subunit of the voltage-dependent, L-type Ca^{2+} channel, and indeed, it has been shown that a reduction in Pitx2 mRNA in Pitx2c$^{+/-}$ and NppaCre^{+}Pitx$^{-/-}$ causes a depletion in the level of Cacna1c [27, 33]. Contrarily, in NppaCre^{+}Pitx$^{-/-}$ LA cardiomyocytes Cacnb1, Cacnb3 and ryanodine receptor 2 expression were increased [33]. Tao et al. saw the increased expression of RyR2 in their Pitx2 CKO mouse model and an increase in Cacna1d and Cacna2d2 [34]. Still, Lozano-Velaso et al. identified that LA with lower Pitx2 (NppaCre^{+}Pitx$^{-/-}$) have a blunted $I_{Ca,L}$ and an excessive sarcoplasmic reticulum (SR) Ca^{2+} load [33]. Collectively, these data, although not conclusive, suggest that a disruption in LA Ca^{2+} homeostasis could underpin increased AF susceptibility. By using microscopic Ca^{2+} imaging of the whole LA, this thesis investigates if Ca^{2+} transients in Pitx2c$^{+/-}$ are disrupted across the LA. In addition, it is examined whether LA with reduced Pitx2c exhibit alterations in Ca^{2+} responses to flecainide. Another reason for investigating Ca^{2+} handling in Pitx2c-deficient mice is, that a disruption of receptor-mediated Ca^{2+} release from the SR leads to a change in heart laterality [38] and Pitx2c plays a determinant role in left-right signaling in organogenesis.

Currently, it is not known if repolarising K^+ currents are up-regulated in the Pitx2c$^{+/-}$ LA, thereby contributing to the shorter APD. Repolarising K^+ currents consist of the transient outward current I_{to}, and the delayed rectifiers (in this thesis referred to as $I_{Ksteadystate}$), which consist of ultra-rapidly activating (I_{Kur}), the rapid activating (I_{Kr}) and the slow activating (I_{Ks}) K^+ channel. Furthermore, there are inward rectifier currents: I_{K1}, which is reported to have an impact on RMP, and acetylcholine-sensitive potassium current (I_{KACh}), which is reported to have an impact on APD and RMP. Both of these currents were examined in this study. In Pitx2c$^{+/-}$ mice Kirchhof et al. found an increase in Kcna3 a voltage-gated, shaker-related K^+ channel ($K_V1.3$), which is known to play an important role in lymphocyte signaling [39], and a reduction in Kcnc4, a voltage-gated, shaw-related K^+ channel ($K_V3.4$), which forms a subunit of I_{to} (figure 2) [27, 40]. Additionally, Kcna6 a voltage-gated, shaker-related K^+ channel ($K_V1.6$), which is expressed more in the atrium than in ventricles in guinea-pigs [41] and is expressed exspecially in the sinus node and the atrioventricular node [42], was reduced in Pitx2c$^{+/-}$. In NppaCre^{+}Pitx$^{-/-}$ LA cardiomyocytes, Kcnj2, Kcnj4 and Kcnj12 expression was decreased [8]. In Pitx2 CKO LA cardiomyocytes, Kcnj10 expression was decreased prenatally and increased postnatally, following Pitx2 deletion, whilst Kcnj11 and Kcnq1 expression was up-regulated (figure 2) [34]. In this thesis, experiments were performed to identify any potential upregulation in voltage-gated K^+ currents in Pitx2c-deficient LA cardiomyocytes, including I_{Kpeak}, $I_{Ksteadystate}$, and I_{to}. In addition, measurements of inward rectifier currents, I_{K1}, and I_{KACh}, that could account for alterations in RMP or APD previously reported in Pitx2c$^{+/-}$ LA cardiomyocytes, were performed.

2.3 Aims

Summarised, the aim of this thesis is to investigate the reason

- for a reduced APA in Pitx2c$^{+/-}$, by comparing I_{Na} density in WT vs. Pitx2c$^{+/-}$ LA cardiomyocytes at different holding potentials
- for the shorter APD in Pitx2c$^{+/-}$, by comparing Ca^{2+} transients and repolarising K^+ currents in WT vs. Pitx2c$^{+/-}$ LA cardiomyocytes
- for the more depolarised RMP in in Pitx2c$^{+/-}$, by comparing inward rectifier K^+ currents in WT vs. Pitx2c$^{+/-}$ LA cardiomyocytes
- why flecainide is more effective in suppressing AF in Pitx2c$^{+/-}$ LA.

3. Methods:

3.1 Information about the mice used

For all experiments paired, age- and weight-matched wild-type mice (WT) and heterozygous Pitx2c knockout (Pitx2c$^{+/-}$) littermates of both genders were used.
Pitx2c$^{+/-}$ mice were generated on a MF1 background by replacing the isoform-c-specific exon 4 by a neoselectin cassette [27, 43]. The protocols were performed according to the Animals (Scientific Procedures) Act, 1986 and were approved by the Home Office (PPL number 30/2967) and the review board of the University of Birmingham. All procedures and analysis were performed blinded to the genotype.

3.2 Isolation of murine atrial cardiomyocytes

Mouse hearts were excised by thoracotomy under deep terminal anesthesia with isoflurane (2.5-4% in O_2, 1.5L/min; Abbott Laboratories Limited, UK) using Fluovac and Fluosorber of Harvard Apparatus (Harvard Apparatus, Cambridge, UK) and immediately placed in ice-cold calcium free HEPES Krebs solution (containing [in mM]: NaCl 145, KCl 5.4, $MgSO_4$ $7H_2O$ 0.83, NaH_2PO_4 0.33, HEPES 5 and Glucose 11; pH 7.3 (NaOH).

The heart was cannulated and attached to a vertical Langendorff setup (HSE easy cell extraction system; Hugo Sachs Elektronik, March, Germany) and retrogradely perfused at a flow rate of 4 ml/min at 37˚C for 5 minutes with standard HEPES Krebs solution (containing [in mM]: NaCl 145, KCl 5.4, $MgSO_4$ $7H_2O$ 0.83, NaH_2PO_4 0.33, HEPES 5, $CaCl_2$ 1.8, and glucose 11.1; pH 7.3 was maintained by adding NaOH), for 3 minutes with calcium free HEPES Krebs solution and for 25 minutes with low calcium and enzyme solution (containing [in mM]: NaCl 145, KCl 5.4, $MgSO_4$ $7H_2O$ 0.83, NaH_2PO_4 0.33, HEPES 5, $CaCl_2$ 0.003, glucose 11.1, Taurine 20 (Alfa Aesar, Heysham, UK), Liberase™ (Liberase TM Research Grade (Roche) 20µg/mL (Roche, Indianapolis, IN)); pH adjusted to 7.3 by adding NaOH.

After digestion of the extracellular matrix and connective tissue by enzymatic digestion the heart was removed from the Langendorff setup, placed in a petri dish with modified Kraftbrühe solution (containing [in Mm]: KCl 25, KH_2PO_4 10, $MgSO_4$ H_2O 2, HEPES 5,

Glucose 20, aspartic acid potassium salt 10, albumine bovine serum 0,1%, L-glutamic acid potassium salt 100, Taurine 20, EGTA 0.5, and Creatine 5; pH 7.2 (KOH)) and retrogradely perfused with modified Kraftbrühe in order to inactivate the liberase and stop the digestion. The left atrium was dissected and transferred into a separate petri dish containing 1 ml modified Kraftbrühe. To isolate single cardiomyocytes, the tissue was gently broken down by mechanical agitation using forceps and trituration with fire-polished glass pipettes (200-100µm).

The suspended cardiomyocytes were filtered through a 100µm nylon mesh (Fisher Scientific, Loughborough, UK); separating the isolated cells. To eliminate the enzyme leftovers, the suspension was centrifuged for 5 minutes at 500 revolutions per minute. The supernatant was transferred into another tube and spun for 5 minutes at 1,000 revolutions per minute. This time the supernatant was discarded, the first pellet was re-suspended in 2ml Kraftbrühe. The suspension was added to the second tube in order to re-suspend the second pellet as well.

Left atrial cardiac myocytes were readapted to physiological extracellular calcium concentrations by adding varying quantities of a 2mM calcium Kraftbrühe (containing [in Mm]: KCl 25, $KH2PO_4$ 10, $MgSO_4$ H_2O 2, HEPES 5, Glucose 20, aspartic acid potassium salt 10, albumine bovine serum 0,1%, L-glutamic acid potassium salt 100, Taurine 20, EGTA 0.5, and Creatine 5, $CaCl_2$ 2; pH 7.2 was maintained by adding KOH) at 15-minute intervals to achieve concentrations of 100µM, 200µM, and 500µM Ca^{2+} and 30 minute-intervals to achieve concentrations of 800µM and finally 1000µM Ca^{2+}. During the process, the suspension was kept at 4˚C.

3.3 Cellular Electrophysiology

3.3.1 Sodium channel patch-clamp

For sodium channel patch-clamp experiments, seven wild-type mice (WT) and ten heterozygous Pitx2c knockout mice (Pitx2c$^{+/-}$) of both genders were used. The animals were age-matched (WT 12.92 ± 0.3580 weeks vs. Pitx2c$^{+/-}$ 13.67 ± 0.3202 weeks, $P > 0.05$ un-paired t-test) and weight-matched (WT 34.46 ± 1.891 grams vs. Pitx2c$^{+/-}$ 31.00 ± 1.375 grams, $P > 0.05$ un-paired t-test).

Whole-cell patch-clamp was performed on freshly isolated cardiomyocytes from the LA at room temperature using Axopatch-1D patch-clamp amplifier (Axon Instruments, Sunnyvale, USA) and Signal software version 6 (Cambridge Electronic Design, Cambridge, UK). 6mm diameter coverslips (VWR International, Leicestershire, UK) were coated with laminin (Engelbreth-Holm-Swarm, Sigma Aldrich, UK) before cardiomyocytes were added and allowed to attach. A coverslip with the attached cardiomyocytes was transferred into the perfusion chamber, which was mounted on the stage of a Zeiss microscope (Axiovert 25, Carl Zeiss, Germany). Cardiomyocytes were superfused with external solution (containing [in mM]: NaCl 130, CsCl 5 (VWR International, Leicestershire, UK), HEPES 10, $CdCl_2$ 0.3, $CaCl_2$ 1.8, MgCl 1.2, and Glucose 10; pH 7.4 was maintained by adding CsOH (Arcos Organics, Geel, Belgium)) and left for 5 minutes to equilibrate. Patch-clamp pipettes with a resistance between 1.5 to 3.5 mΩ were prepared using borosilicate glass (Harvard Apparatus UK, Cambridge, UK) pulled with a P-97 pipette puller (Sutter Instruments, California, USA). The intracellular solution contained [in mM]: CsCl 115, NaCl 5, HEPES 10, EGTA 10, MgATP 5, $MgCl_2$ 0.5 and Tetraethylammonium 10; pH 7.2 was maintained by adding CsOH.

To evoke whole-cell voltage-gated sodium currents, cardiomyocytes were held at -100mV, -80mV, -75mV and -70mV, and -65mV before being stepped to a test potential of -10mV for 100ms. The maximum downward deflection was recorded as the peak sodium current (I_{Na}). This protocol allows for observing the effect of holding potential (mimicking varying RMPs) on the magnitude of the peak sodium current. For flecainide experiments, cardiomyocytes were superfused with 1μM Flecainide for 5 minutes, while being stimulated at 1Hz, before recording. After this time, a steady-state reduction in sodium

current was achieved. The percentage inhibition of the sodium current caused by flecainide was recorded at each holding potential.

The capacitance of each cardiomyocyte was measured by integrating the capacitance current evoked by 10mV depolarising steps from a holding potential of -80mV. Pipette capacitance was offset and series resistance compensated for between 80 and 100%. Experiments were terminated if series resistance increased to above 10mΩ. Data was only analysed from cells where the membrane resistance remained above 500MΩ throughout. Leak currents were not subtracted and experiments were included only if maintained at less than 150pA. The liquid junction potential varied between 3 and 5mV and was not compensated for. Currents were low pass filtered at 20kHz. All currents were normalised to cell capacitance and expressed as current density.

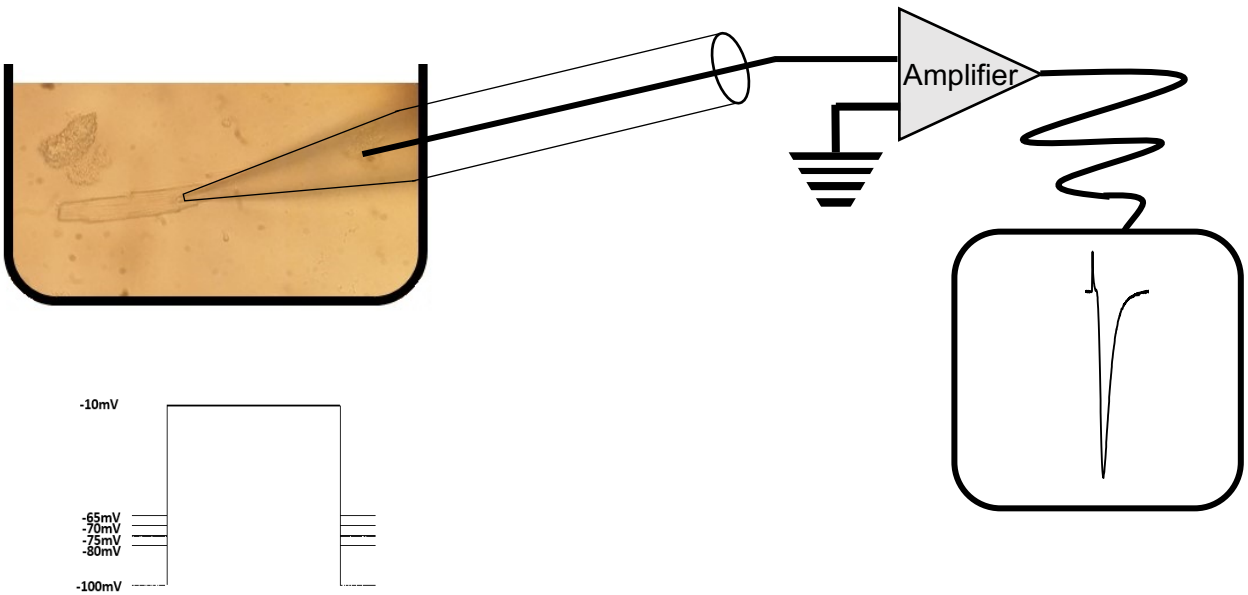


Figure 3: Scheme of sodium current patch-clamp setup. After isolation of left atrial cardiomyocytes, patch-clamp experiments were perfomed by forming a gigaΩ-seal between a fire-polished glass pipette and the cardiomyocyte. After forming a gigaΩ-seal, the cardiomyocyte membrane in the seal was ruptured by suction in order to have a connection to the intracellular space. Then the stimulation protocol was applied and sodium currents were elicited. Stimulation protocol of I_{Na}. Cardiomyocytes were held at different RMPs and stepped up to -10mV to elicit peak I_{Na}

3.3.2 Sodium-channel patch-clamp of HEK-hNa$_v$1.5

In order to investigate the voltage dependency of the effect of flecainide on human sodium channels, human embryonic kidney cells, stably transfected with the SCN5A gene and thus expressing the human Na$_V$1.5 sodium-channel, were obtained from SB Drug Discovery (HEK-hNa$_v$1.5 stable cell line, SB Drug Discovery, Glasgow). Cells were cultured and split according to the SB Drug discovery confidential standard operating procedures. Experiments were conducted at passage 10 – 15.

Sodium channel and flecainide patch-clamp experiments were conducted as described above. Briefly, HEK-hNa$_v$1.5 were plated on laminin-coated cover slips and placed in the perfusion chamber mounted on the stage of a Zeiss Microscope (Axiovert 25, Carl Zeiss, Germany). Cells were superfused with external solution (composition see above). Cells were held at -100mV, -80mV, -75mV, -70mV and -65mV and stepped to -10mV for 100ms, to evoke peak sodium currents. This protocol was performed in the presence and absence of 1µM flecainide, to evaluate percentage inhibition at each holding potential.

3.3.3 Potassium patch-clamp:

After the cell-isolation of LA cardiomyocytes and calcium reintroduction, the laminin coated coverslips with the attached cardiomyocytes were superfused with Tyrodes solution (containing [in mM]: NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 10, and Glucose 11.1; pH 7.4 was maintained by adding NaOH). Cells were held at -60mV, -65mV and -70mV before being stepped from -120mV to +60mV in 10mV increments for 500ms. After baseline recordings, the cardiomyocytes were superfused with 0.05mM $BaCl_2$ for one minute in order to isolate IK_1 currents. The barium-sensitive (difference) current was considered to represent I_{K1} [44, 45].

A similar voltage protocol was used to measure I_{KACh}. In the presence of 0.05mM $BaCl_2$ (to block I_{K1}), cells were held at -60mV, -65mV and -70mV before being stepped from -120mV to +60mV in 10mV steps for 500ms. This was performed in the presence and absence of 10µM carbachol (Sigma-Aldrich, Dorset, UK). I_{KACh} was determined as the magnitude of the current evoked by carbachol. Current signals were low pass filtered at 5kHz and leak currents were always smaller than 100pA. Data was analysed only from

cells which membrane resistance remained above 500MΩ throughout. Series resistance was not compensated; however, voltage errors were assumed to be low, given that the measured K^+ currents were usually less than 2nA.

3.3.4 High spatial resolution optical mapping for measurements of Ca^{2+} transients

Following isolation, hearts were placed in ice-cold bicarbonate Krebs solution (containing [in mM]: NaCl 118, KCl 3.52, $MgSO_4$ 7 H_2O 0.83, KH_2PO_4 1.18, $NaHCO_3$ 24.9, $CaCl_2$ 1.8, and glucose 11); pH was maintained by ventilation with 95% O_2 and 5% CO_2). To avoid hypoxia of the heart, the aorta was mounted on a cannula within 45 seconds to allow early retrograde perfusion with bicarbonate Krebs and heparin.

For dye loading, the cannula with the heart was attached to a vertical Langendorff setup (Hugo Sachs, March, Germany) and retrogradely perfused at a flow rate of 4ml/min at 37˚C for 3 minutes with bicarbonate-buffered Krebs solution. Under ongoing perfusion, the Ca^{2+} sensitive, Rhod-2 acetoxymethyl ester derivative (5μM; Life Technologies, Paisley, UK) was added to the perfusate and loaded into the LA via a port in the Langendorff system over a period of 7 minutes.

The left atrium was dissected and gently pinned down in a superfusion chamber while being superfused at 37˚C with bicarbonate Krebs containing the excitation-contraction uncoupler blebbistatin (10μM; Cayman Chemical, Michigan, USA) and probenecid (2mM; Sigma-Aldrich, Gillingham, UK), to inhibit dye leakage. A stimulating electrode (Digitimer, UK), which was driven by an analogue to digital converter with spike2 software (Cambridge Electronic Design, UK), was placed on the septal region and the atria were paced with twice the diastolic voltage threshold at 2ms pulse width. Two twin LEDS (Cairn Research, UK) excited the dye at 530 nm and a lens with high numerical aperture (Schneider Kreuznach Xenon 0.95/25, NA = 0.52) was used to capture fluorescence. Emitted fluorescence (585 ± 20nm) was captured using a high-resolution (2048 by 2048 pixels), high-speed camera (ORCA flash 4.0; Hamamatsu, Japan) at a sampling frequency of 1kHz. For image extraction and data processing, WinFluor V3.4.9 was used (Dr John Dempster, University of Strathclyde, UK). For analysis custom made algorithm produced in MATLAB were used [46].

Preparations were stimulated incrementally over a range of 300 to 80ms. At least 50 Ca^{2+} transients were triggered to ensure sufficient rate adaptation. The pacing frequency was performed in the presence and absence of 1µM flecainide. Recordings in the presence of flecainide were made after 15 minutes for equilibration. 25 calcium transients were averaged and used for analysis at each cycle length. Calcium transient relaxation time at 30, 50 and 70% and the Ca^{2+} transient amplitude were measured in a fixed region of interest (8x8 pixels) before and after flecainide using MATLAB algorithms developed and described by YU et al [46].

3.4 Statistical analysis

Data were compared by unpaired t-test or repeated measures single or two-factor analysis of variance (ANOVA) followed by Bonferroni post-hoc test, if ANOVA were significant. P values < 0.05 were considered significantly different. For statistical analysis and composition of graphs GraphPad Prism v6 was used.

4. Results

4.1 Investigating the effectiveness of sodium current inhibition by flecainide at depolarised holding potentials in WT and Pitx2c-deficient left atrial cardiomyocytes.

Flecainide is reported to be more effective in reducing atrial arrhythmia in hearts deficient in Pitx2c [18]. This might be due to changes in sodium channel number or function that is related to a reduction in Pitx2c. In this section, weight- and age-matched mice of both genders were examined.

For I_{Na} experiments, 7 WT, 4 males and 3 females, and 10 Pitx2c$^{+/-}$, 2 males and 8 females, mice were used (figure 4.A). They were weight-matched (WT: 34.46 ± 1.891g; Pitx2c$^{+/-}$: 31.00 ± 1.375g (p = 0,1499) figure 4.B) and age-matched (WT: 12.92 ± 0.358 weeks; Pitx2c$^{+/-}$: 13.67 ± 0.3202 weeks (p = 0.1424, figure 4.C)).

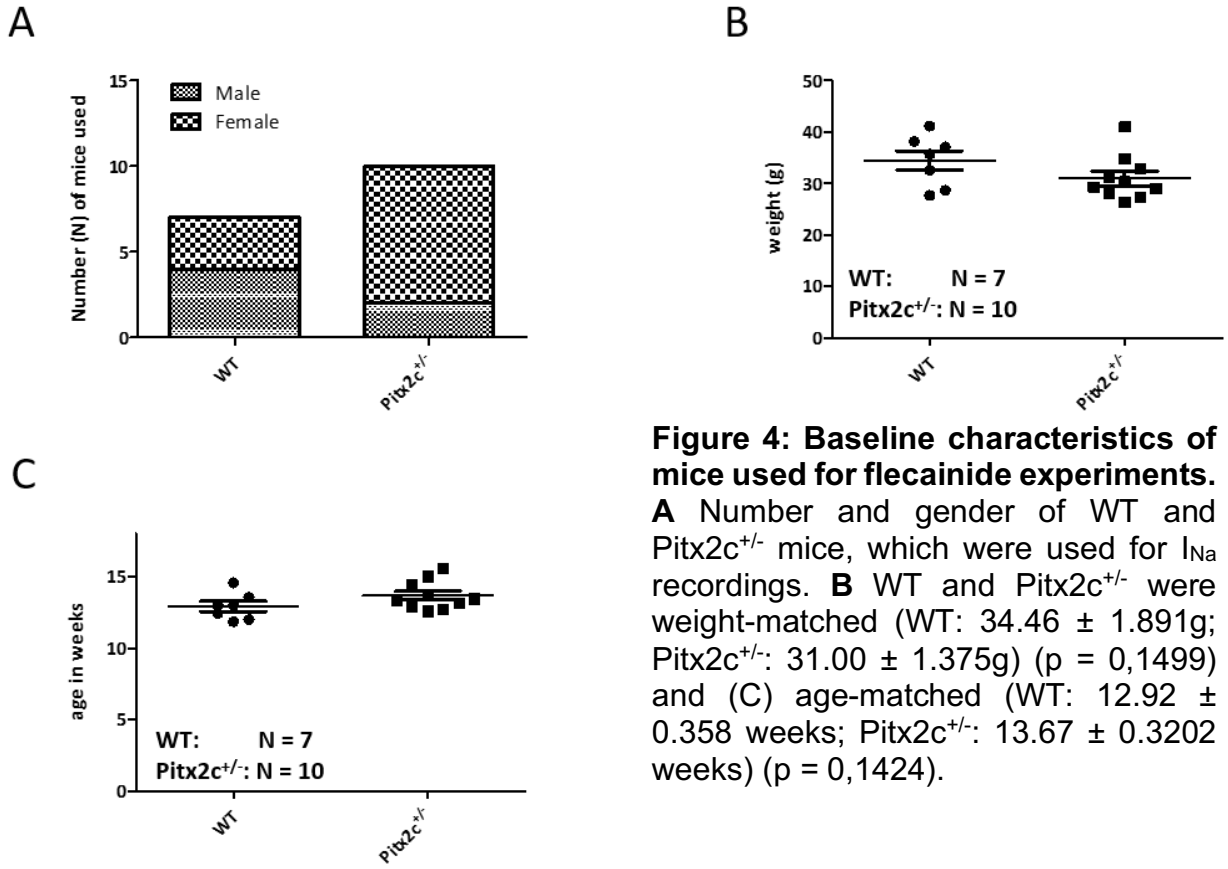


Figure 4: Baseline characteristics of mice used for flecainide experiments. **A** Number and gender of WT and Pitx2c$^{+/-}$ mice, which were used for I_{Na} recordings. **B** WT and Pitx2c$^{+/-}$ were weight-matched (WT: 34.46 ± 1.891g; Pitx2c$^{+/-}$: 31.00 ± 1.375g) (p = 0,1499) and (C) age-matched (WT: 12.92 ± 0.358 weeks; Pitx2c$^{+/-}$: 13.67 ± 0.3202 weeks) (p = 0,1424).

As cell capacitance is directly related to cell surface area, it can be used as a measure of cell size. When comparing the capacitance of 40 left atrial WT and 46 left atrial Pitx2c$^{+/-}$ cardiomyocytes, there was no difference in magnitude (WT: 51.85 ± 1.835pF, N = 40; Pitx2c$^{+/-}$: 49.65 ± 1.813pF, N = 46 (p = 0.3967) figure 5.A). Thus, there was no significant change in cell size caused by Pitx2c deficiency.

Figure 5.B shows a picture of a left atrial cardiomyocyte with a fire-polished pipette in cell-attached whole-cell voltage-clamp mode. In order to elicit I_{Na} peak current, isolated cardiomyocytes were held at different holding potentials (-100, -80, -75, -70 and -65mV) and stepped up to -10mV (figure 5.C).

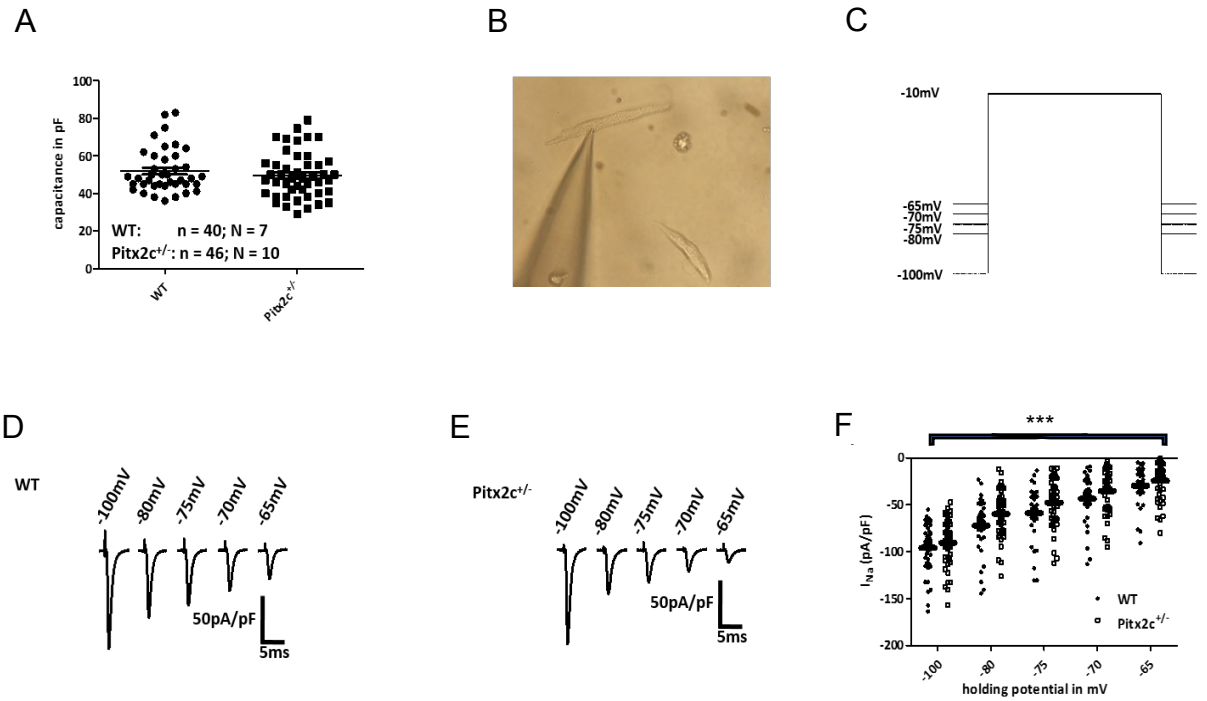


Figure 5: Peak sodium currents are similar in WT and Pitx2c deficient LA cardiomyocytes across a range of holding potentials. A There is no significant difference in cardiomyocyte capacitance (WT: 51.85 ± 1.835pF, N = 40; Pitx2c$^{+/-}$: 49.65 ± 1.813pF, N = 46) (p = 0.3967). **B** Representative left atrial cardiomyocyte in cell attached patch-mode. **C** Stimulation protocol for peak sodium current. **D & E** Representative sodium current (I_{Na}) traces of WT **(D)** and Pitx2c$^{+/-}$ **(E)**. **F** There is no significant difference between WT and Pitx2c$^{+/-}$ in I_{Na} (p = 0.1). A more positive holding potential decreases I_{Na} significantly (p < 0.0001) in both genotypes. ***p < 0.001

Figure 5.D and 5.E present representative traces of a single isolated left atrial WT (5.D) and Pitx2c$^{+/-}$ (5.E) cardiomyocyte, and show a characteristic decrease in peak sodium current at more positive holding potentials. There was no significant difference in peak I_{Na} current density between genotypes when measured at the same holding potentials (WT: -100mV: -95.9 ± 4.46pA/pF; -80mV: -72.14 ± 4.63pA/pF; -75mV: -58.78 ± 4.54pA/pF;

-70mV: -43.36 ± 4.03pA/pF; -65mV: -29.62 ± 3.22pA/pF; Pitx2c$^{+/-}$: -100mV: -90.59 ± 4.00pA/pF; -80mV: -59.80 ± 3.91pA/pF; -75mV: -47.78 ± 3.84pA/pF; -70mV: -35.31 ±3.53pA/pF; -65mV: -24.22 ± 3.08pA/pF; p = 0.1044, figure 5.F). However, in both genotypes, the holding membrane potential had a highly significant effect on I_{Na} ($p < 0.0001$). A more depolarised holding membrane potential caused a smaller magnitude of I_{Na} (figure 5.F). Given that the Pitx2c$^{+/-}$ LA cardiomyocytes have a more depolarised resting membrane potential, these findings could explain why Pitx2c$^{+/-}$ action potential amplitude is reduced without any significant reduction in Scn5a expression [32].

The I_{Na}-blocking effect of flecainide was examined in WT and Pitx2c$^{+/-}$ LA cardiomyocytes at different holding potentials (figure 6). There was no genotype-dependent difference in the effect of flecainide ($p = 0.8$) at any holding potential. However, the efficacy of flecainide was significantly increased at more positive holding potentials (figure 6). This was consistent in both WT and Pitx2c$^{+/-}$ LA cells (WT: -100mV: 47,66% ± 4.70%; -80mV: 55.99% ± 4.82%; -75mV: 60.77% ± 5.39%; -70mV: 66.39% ± 6.91%; -65mV: 72.90% ± 6.89%, $p < 0.0001$, figure 6.C and Pitx2c$^{+/-}$: -100mV: 50.12% ± 5.37%; -80mV: 59.85% ± 6.12%; -75mV: 65.19% ± 6.77%; -70mV: 69.05% ± 7.98%; -65mV: 78.38% ± 8.62%, $p < 0.0001$, figure 6.D). These data suggest that the RMP will have an important effect on the magnitude of sodium current inhibition caused by flecainide. This could be important, given that Pitx2 deficiency has been reported to cause RMP depolarisation and an augmented response to flecainide [32].

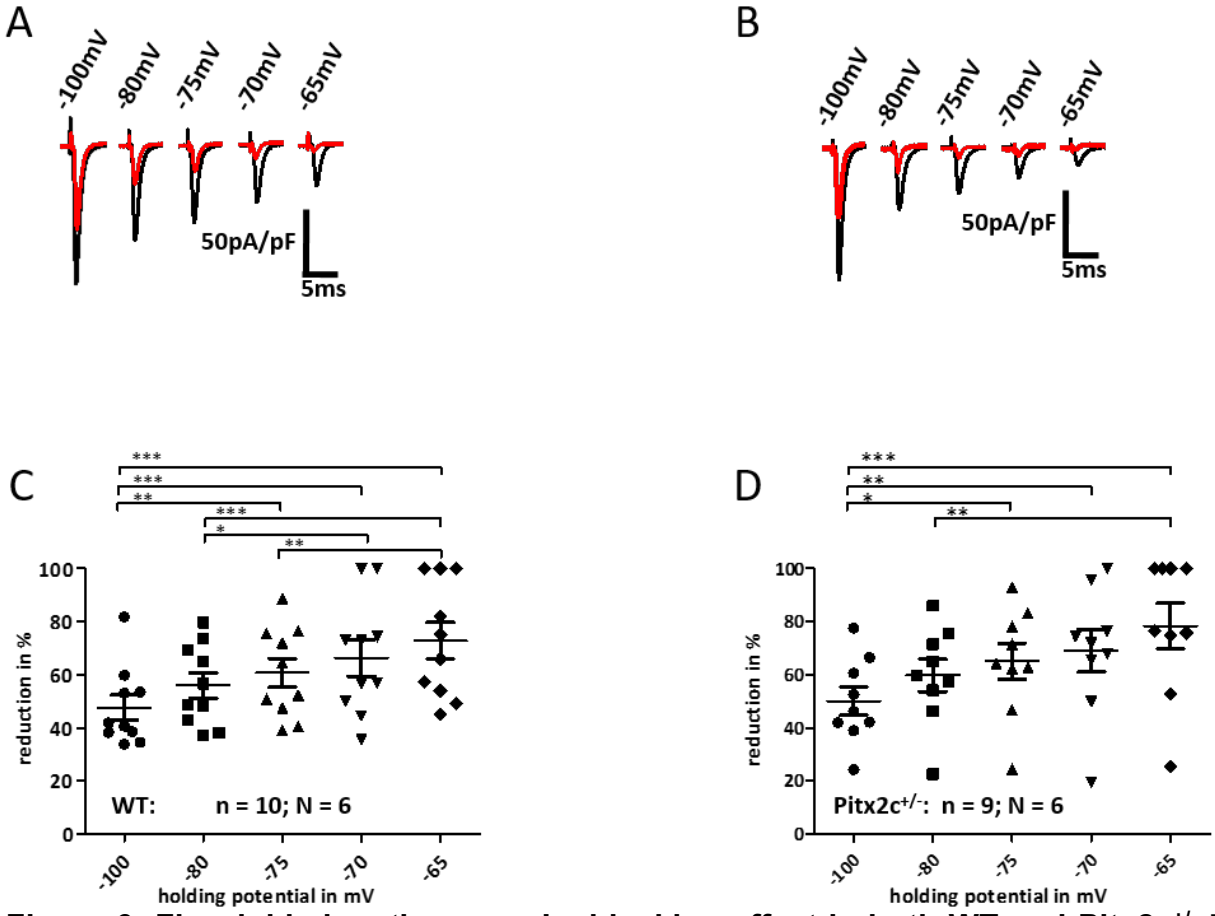


Figure 6: Flecainide has the same I_{Na} blocking effect in both WT and Pitx2c$^{+/-}$ LA cardiomyocytes, however the I_{Na}-blocking effect of flecainide significantly increases at more positive holding potentials. A & B Representative I_{Na} traces of WT **(A)** and Pitx2c$^{+/-}$ **(B)** before (black) and after (red) superfusing for 5 minutes with 1µM flecainide. Left atrial cardiomyocytes were held at -100, -80, -75, -70 and -65mV. Peak Na^+ current was elicited by stepping to -10mV. **C & D** The effect of flecainide is not genotype-dependent ($p = 0.66$). A depolarised holding membrane potential, reduces I_{Na} and increases the I_{Na}-blocking effect of flecainide massively for WT **(C)** and Pitx2c$^{+/-}$ **(D)** (both overall p-values for holding potential < 0.0001).

4.2 A more depolarised holding membrane potential increases the effect of flecainide in human HEK293 cells, stably transfected with the human $Na_V1.5$ channel.

In order to investigate whether flecainide also has a greater I_{Na} blocking effect at more depolarised holding membrane potentials on human cardiac sodium channels, experiments were performed on HEK-cells, stably expressing the human $Na_V1.5$ channel. Cells were held at varying holding potentials (-100mV, -80mV, -75mV, -70mV and -65mV) and then stepped to -10mV in order to elicit I_{Na} peak current. This was done before and after superfusing with 1μM flecainide for 5 minutes. The inhibitory effect of flecainide increased significantly at more depolarised holding membrane potentials (-100mV: 34.50% ± 4.08%; -80mV: 57.87% ± 5,22%; -75mV: 64.63% ± 5.35%; -70mV: 66.39% ± 5.86%; -65mV: 100% ± 0,0%, $p < 0.0001$, figure 7.B).

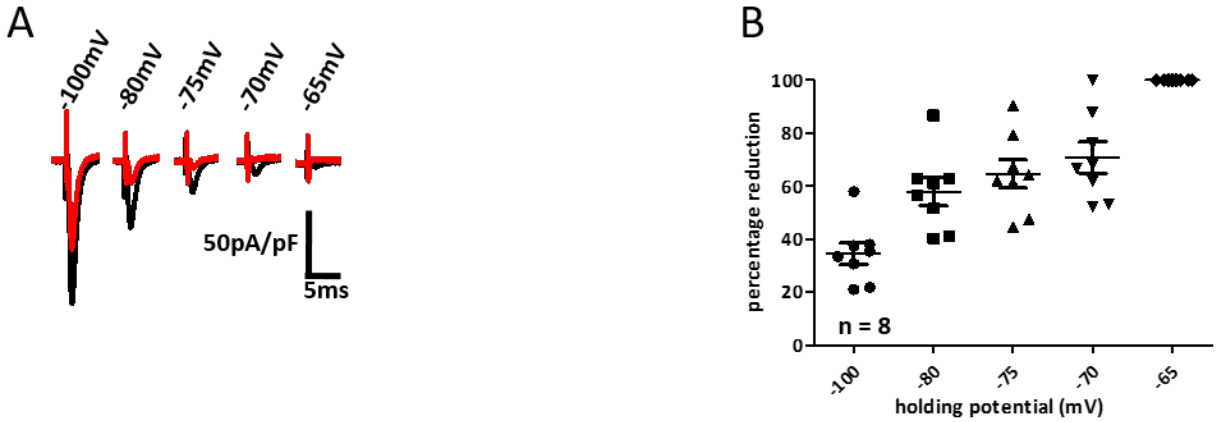


Figure 7: Flecainide has a greater sodium current inhibiting effect on human $Na_V1.5$ sodium channels at more positive holding potentials. A Representative traces of I_{Na} of $hNa_V1.5$ cells before (black) and after (red) flecainide. Cells were held at -100, -80, -75, -70 and -65mV and peak I_{Na} was elicited by stepping up to -10mV. **B** Percentage reduction of I_{Na} by flecainide. A more depolarised holding membrane potential increases the effect of flecainde.

To summarise this section, a more positive holding potential increased the I_{Na} blocking effect of flecainide in murine WT and $Pitx2c^{+/-}$ left atrial cardiomyocytes (figure 6) and also in HEK-cells, expressing the human $Na_V1.5$ channel (figure 7). Thus, the sensitivity to flecainide therapy is likely to be dependent on the RMP.

4.3 Investigating the inhibitory effects of flecainide on Ca^{2+} transients in WT and Pitx2c$^{+/-}$ left atria

In addition to blocking sodium channels, flecainide also has an inhibitory effect on other channels including the RyR2 on the sarcoplasmic reticulum. The heightened antiarrhythmic response to flecainide detected in Pitx2c$^{+/-}$ LA may thus be related with varying actions on the fluctuations in cellular Ca^{2+}. Ca^{2+} measurements were performed on 7 WT and 7 Pitx2c$^{+/-}$ LA. The stimulation protocol is presented in Figure 8.A. Figure 8.B displays a fluorescent left atrium in the superfusion chamber when paced and illuminated with LEDs. The atria were stimulated at 300ms, 120ms, 100ms and 80ms cycle lengths. Baseline measurements of transient 30 (WT: 300ms: 21.26 ± 1.50; 120ms: 20.56 ± 0.72; 100ms: 19.78 ± 1.04; 80ms: 19.02 ± 0.75; Pitx2c$^{+/-}$: 300ms: 20.37 ± 0,61; 120ms: 21 ± 0.39; 100ms: 20.16 ± 0.5; 80ms: 18.84 ± 0,52, p = 0.95 , figure 8.E), transient 50 (WT: 300ms: 26.87 ± 1.34; 100ms: 24.91 ± 0.73; 100ms: 24.91 ± 0,73; 80ms: 23.73 ± 0.84; Pitx2c$^{+/-}$: 300ms: 26.05 ± 0.7; 120ms: 26.03 ± 0.44; 100ms: 25.20 ± 0.53; 80ms: 23.7 ± 0,55, p = 0.93 figure 8.F), transient 70% Ca^{2+} relaxation times (WT: 300ms: 35.02 ± 1.39; 120ms: 32.13 ± 0.69; 100ms: 31.08 ± 0.7; 80ms: 29.23 ± 0.73; Pitx2c$^{+/-}$: 300ms: 32.79 ± 0.76; 120ms: 32.27 ± 0.50; 100ms: 31.01 ± 0.55; 80ms: 29.11 ± 0.66, p = 0.57 figure 8.G) and the Ca^{2+} transient amplitude (WT: 300ms 31.84 ± 7.0; 120ms: 20.99 ± 4.13; 100ms: 19.02 ± 2.35; 80ms: 11.32 ± 1.56; Pitx2c$^{+/-}$: 300ms: 29.89 ± 6.87; 120ms: 22.85 ± 4.19; 100ms: 15.51 ± 1.71; 80ms: 12.08 ± 1.34, p = 0.88 , figure 8.H) were not significantly different between genotypes (all p-values > 0.5). A faster stimulation rate (shorter cycle length) shortened all transients significantly and reduced the amplitude in both genotypes (all p-values < 0.0001; figure 8.E, 8.F, 8.G & 8.H).

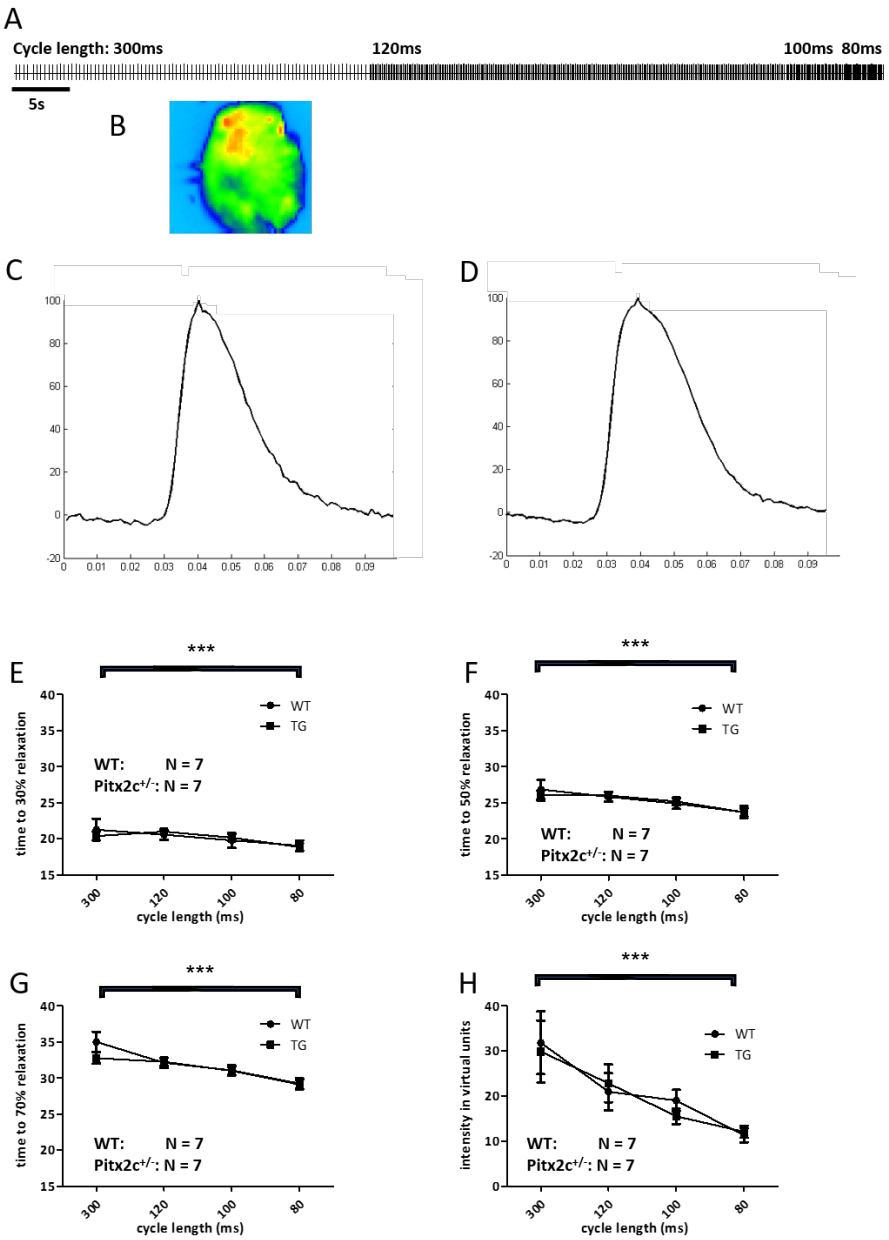


Figure 8: Baseline Ca^{2+} transients are similar in WT and Pitx2c$^{+/-}$ left atria. A Stimulation protocol. Dissected left atria were paced at 300ms and left for 3 minutes to equilibrate. Ca^{2+} transients were recorded for 10 seconds at 300ms cycle length and then switched to an incremental ramp protocol, consisting of 300 stimuli at 120ms, 50 stimuli at 100ms and 50 stimuli at 80ms cycle lengths. **B** Image of baseline Ca^{2+} fluorescent intensity signal from a dissected left atrium in the recording chamber. **C & D** Representative optical calcium transient consisted of 25 averaged calcium transients of WT **(C)** and Pitx2c$^{+/-}$ **(D)** when paced with 100ms cycle length. **E, F & G** Mean calcium relaxation times at 30% **(E)**, 50% **(F)** and 70% **(G)** measured in WT and Pitx2c$^{+/-}$ LA. **H** Mean Ca^{2+} amplitude normalised to baseline in WT and Pitx2c$^{+/-}$. Faster pacing significantly shortened all transients and decreased amplitude (***p < 0.0001 for all overall p-values).

Next, it was investigated whether flecainide had a genotype specific impact on calcium transients. Panel A for WT and panel B for Pitx2c$^{+/-}$ in figure 9 show representative traces of calcium transients before (black) and after (red) superfusing with 1µM flecainide for 15 minutes. For WT, at 300ms CL, flecainide significantly shortened the transient 30 by 15% (overall $p < 0.0001$; figure 9.C), transient 50 by 10% (overall $p < 0.0001$; figure 9.E), and transient 70 by 7% (overall $p = 0.0006$; figure 9.G). Flecainide also decreased the amplitude by 55% (overall $p < 0.0001$; figure 9.I). Similarly, in Pitx2c$^{+/-}$ left atria flecainide shortened transient 30 by 9% (overall $p < 0.0001$; figure 9.D), transient 50 by 4% (overall $p < 0.0001$; figure 9.F) and transient 70 by 1% (overall $p = 0.0001$; figure 9.H). The amplitude was decreased by 51% (overall $p < 0.0001$; figure 9.J). Relaxation times and amplitude were not significantly different between genotypes after flecainide.

In the presence of flecainide, there was also a significant shortening of Ca^{2+} transients at 80ms cycle length pacing frequency in the Pitx2c$^{+/-}$ LA: transient 30 ($p = 0.003$), transient 50 ($p = 0.0008$) and transient 70 ($p < 0.0001$). A similar trend was observed in WT LA, though not statistically significant: transient 30 ($p = 0.46$), transient 50 ($p = 0.2$), transient 70 ($p = 0.09$). Furthermore, in the presence of flecainide, there was a significant reduction in Ca^{2+} transient amplitude at higher pacing frequencies (80ms cycle length) in both Pitx2c$^{+/-}$ ($p = 0.05$) and WT LA ($p = 0.0005$) (figure 9.I).

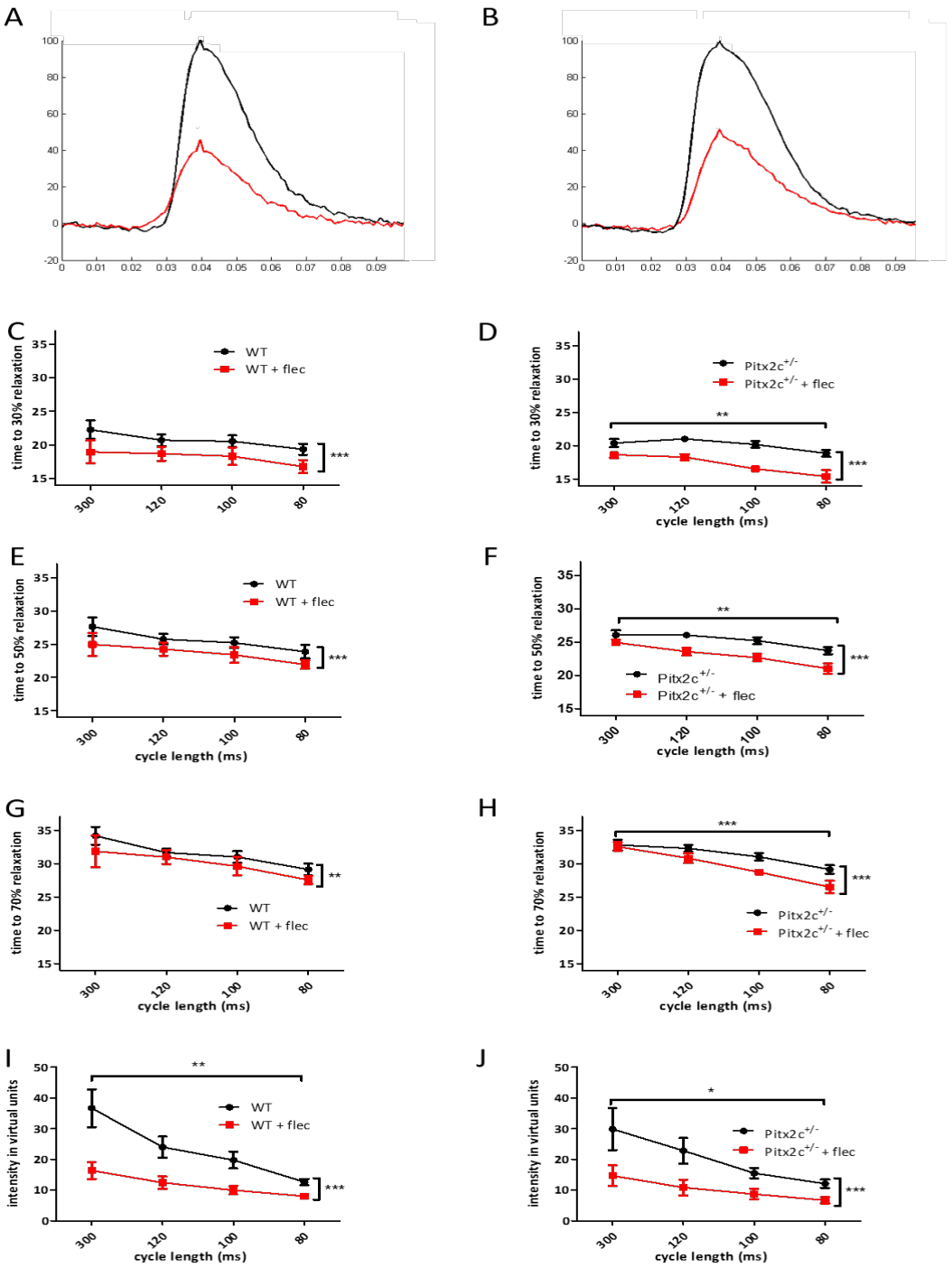


Figure 9: Flecainide shortens Ca^{2+} relaxation time and reduces transient amplitude in both WT and Pitx2c$^{+/-}$ LA. Faster pacing shortens transients and reduces amplitude in both genotypes with and without flecainide. A & B Representative traces of 25 averaged optical calcium transients before (black) and after (red) superfusing with flecainide, when stimulated at 100ms cycle length. **C, D, E, F, G & H** Transient 30 **(C & D)**, Transient 50 **(E & F)**, Transient 70 **(G & H)** and amplitude **(I & J)** for WT **(C, E & G)** and Pitx2c$^{+/-}$ **(F, H & J)**. Flecainide shortened all transients significantly and decreased the transient amplitude significantly. In Pitx2c$^{+/-}$ left atria lower cycle length led to shorter transients and a lower amplitude. The same trend was observed in WT, though it was not as significant. *p < 0.05, **p < 0.01 ***p < 0.0001

The percentage reduction of transient relaxation time and amplitude was compared between WT and Pitx2c$^{+/-}$ LA (figure 10). Percentage reduction was calculated as follows:

((transient$_x$ [before flecainide]- transient$_x$ [after flecainide])/ transient$_x$ [before flecainide]) * 100

The shortening of transient 30 (p = 0.2, figure 10.A), transient 50 (p = 0.2; figure 10.B) and transient 70% relaxation times (p = 0.29, figure 10.C) and the reduction of transient amplitude (p = 0.43; figure 10.D) by flecainide were similar for both genotypes. Additionally, the cycle length did not have a significant impact on the effect of flecainide: transient 30 (figure 10.A, p = 0.18), transient 50 (figure 10.B p = 0.71), transient 70 (figure 10.C p = 0.09) and amplitude (figure 10.D, p = 0.19). This suggests that the effect of flecainide on Ca^{2+} kinetics is use-independent.

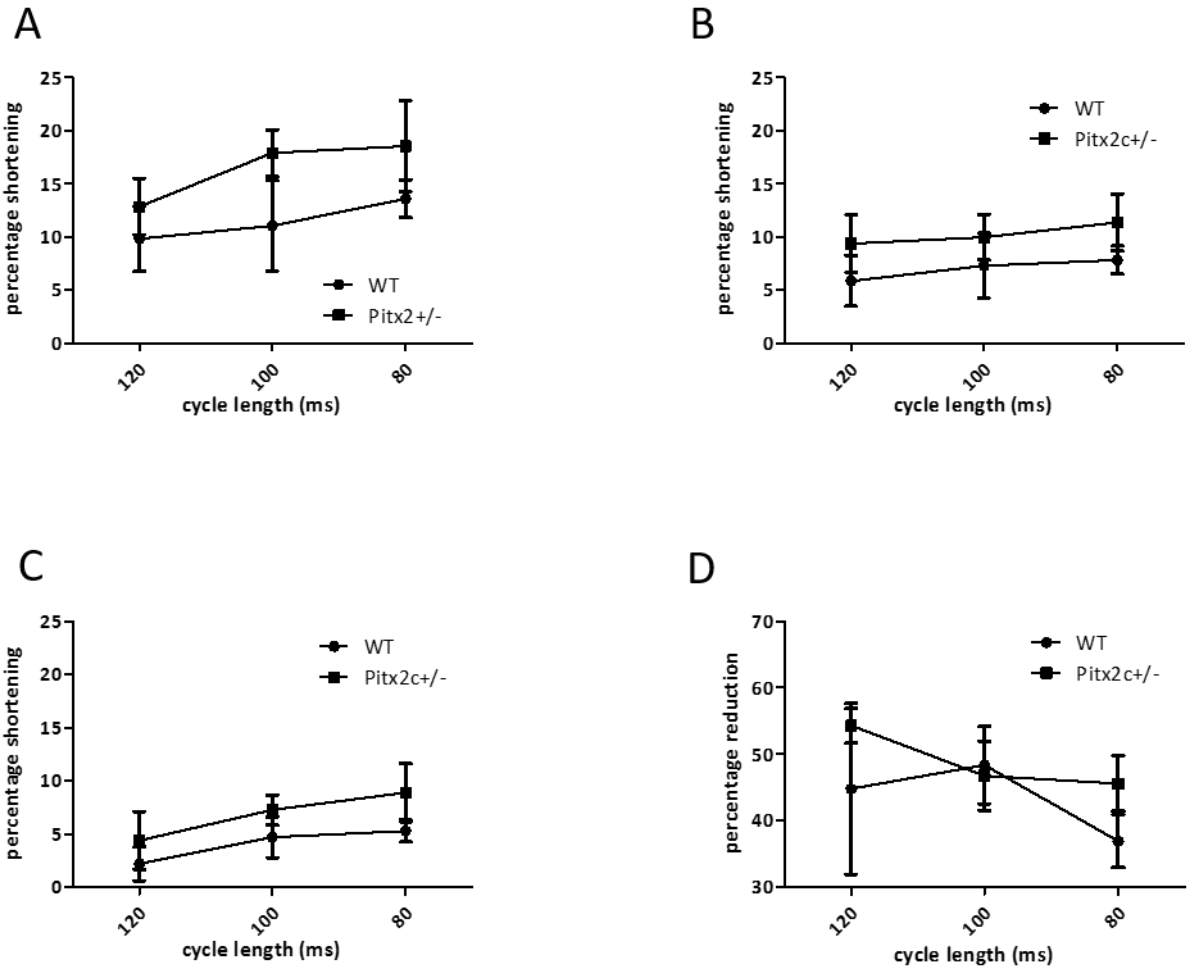


Figure 10: Effect of flecainide is not genotype-dependent and the cycle length does not have an impact on the effect of flecainide. A, B, C & D Percentage shortening of the calcium transient at 30% **(A)**, 50% **(B)** and 70% **(C)** relaxation & percentage reduction of amplitude **(D)** caused by 1µM flecainide in WT and Pitx2c$^{+/-}$ left atria. Genotypes responded similarly (all p-values > 0.2). The effect of flecainide is not changed by pacing frequency.

Collectively, these data identified important changes in Ca^{2+} transients caused by flecainide in the intact atria. Flecainide shortened Ca^{2+} relaxation times and reduced Ca^{2+} transient amplitude independent of stimulation frequency. However, the magnitude of the changes induced by flecainide was remarkably similar in WT and Pitx2c$^{+/-}$ LA. It is unlikely therefore that any potentiation of the antiarrhythmic action of flecainide in atria with reduced Pitx2c is due to differential effects on Ca^{2+} transients.

4.4 Evaluating Pitx2c and voltage-dependent changes in K^+ currents in isolated atrial cells

Pitx2c deficiency is reported to cause significant action potential shortening in the LA [32]. Experiments were therefore performed to examine if changes in outward repolarising K^+ currents could explain this shortening. In addition, the Pitx2c deficient cells have a depolarised RMP which could be caused by an alteration in either I_{K1} or I_{KACh}. Furthermore, the impact of holding potential on these currents was explored to artificially mimic the effect of RMP depolarisation in the Pitx2c$^{+/-}$ atria.

4.4.1 There is no genotype-related difference in I_{Kpeak}, $I_{Ksteadystate}$ and I_{to}.

When holding isolated murine WT (n = 34; N = 7) and Pitx2c$^{+/-}$ (n = 39; N = 7) LA cardiomyocytes at -70mV and stepping to +60mV in 10mV increments, there was no genotype-related difference in I_{Kpeak} (p = 0.4, figure 11.C), $I_{Ksteadystate}$ (p = 0.36, figure 11.D) or I_{to} (p = 0.34, Figure 11.E) between WT and Pitx2c$^{+/-}$ LA cells. Thus, the action potential shortening reported previously in Pitx2c$^{+/-}$ cells is unlikely to be caused by any inherent upregulation in these large repolarising currents.

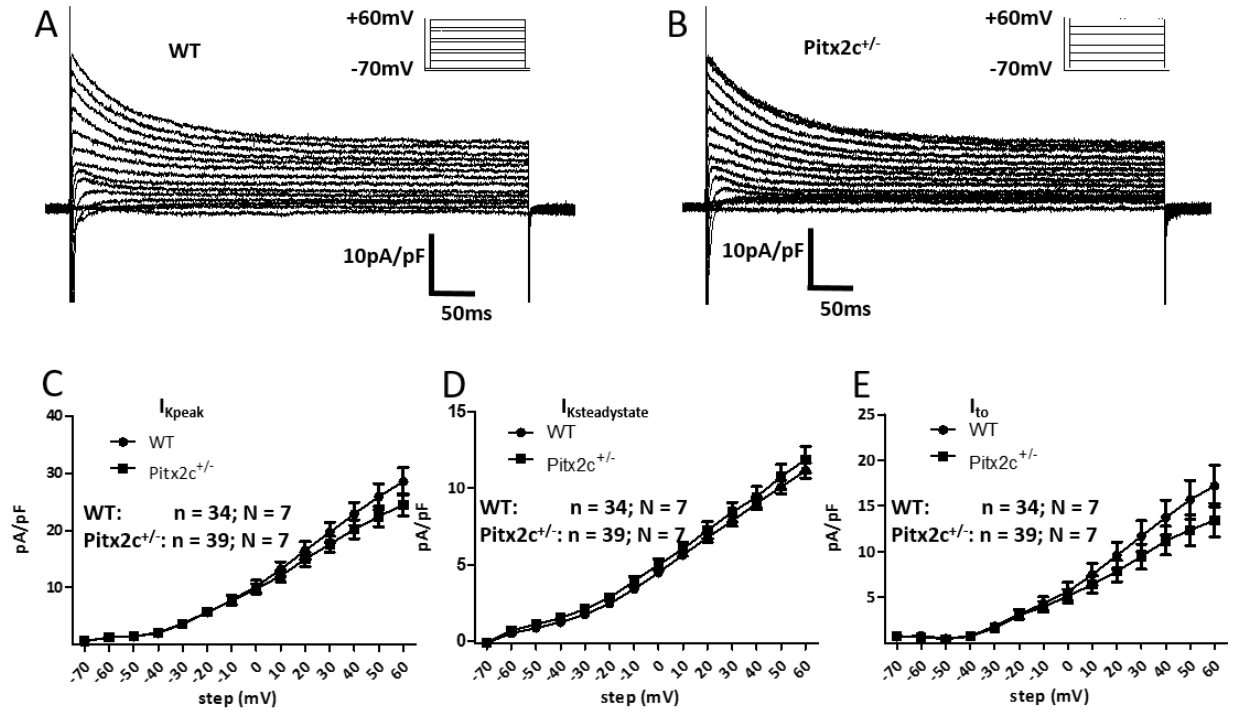


Figure 11: No genotype-related difference in I_{Kpeak}, $I_{Ksteadystate}$ and I_{to} A & B Representative traces of K^+ currents elicited by holding an isolated murine LA WT **(A)** and Pitx2c$^{+/-}$ **(B)** cardiomyocyte at -70mV and stepping up to +60mV in 10mV increments. **C, D & E** There was no significant difference in I_{Kpeak} (p = 0.4), $I_{Ksteadystate}$ (p = 0.36) and I_{to} (p = 0.34) between WT (n = 34; N = 7) and Pitx2c$^{+/-}$ (n = 39; N = 7).

4.4.2 A more depolarised holding potential decreases repolarising K^+ currents.

10mV increment steps elicited significantly smaller K^+ currents in murine LA WT cardiomyocytes when the cardiomyocytes were held at a more positive holding membrane potential (-60mV instead of -70mV) (I_{Kpeak} $p < 0.0001$, figure 12.C; $I_{Ksteadystate}$ $p = 0.0012$, figure 12.D; I_{to} $p < 0.0001$, figure 12.E; -70mV: n = 32; N = 7, -60mV n = 32; N = 7). This seemed to be more apparent for the I_{to} than the $I_{Ksteadystate}$.

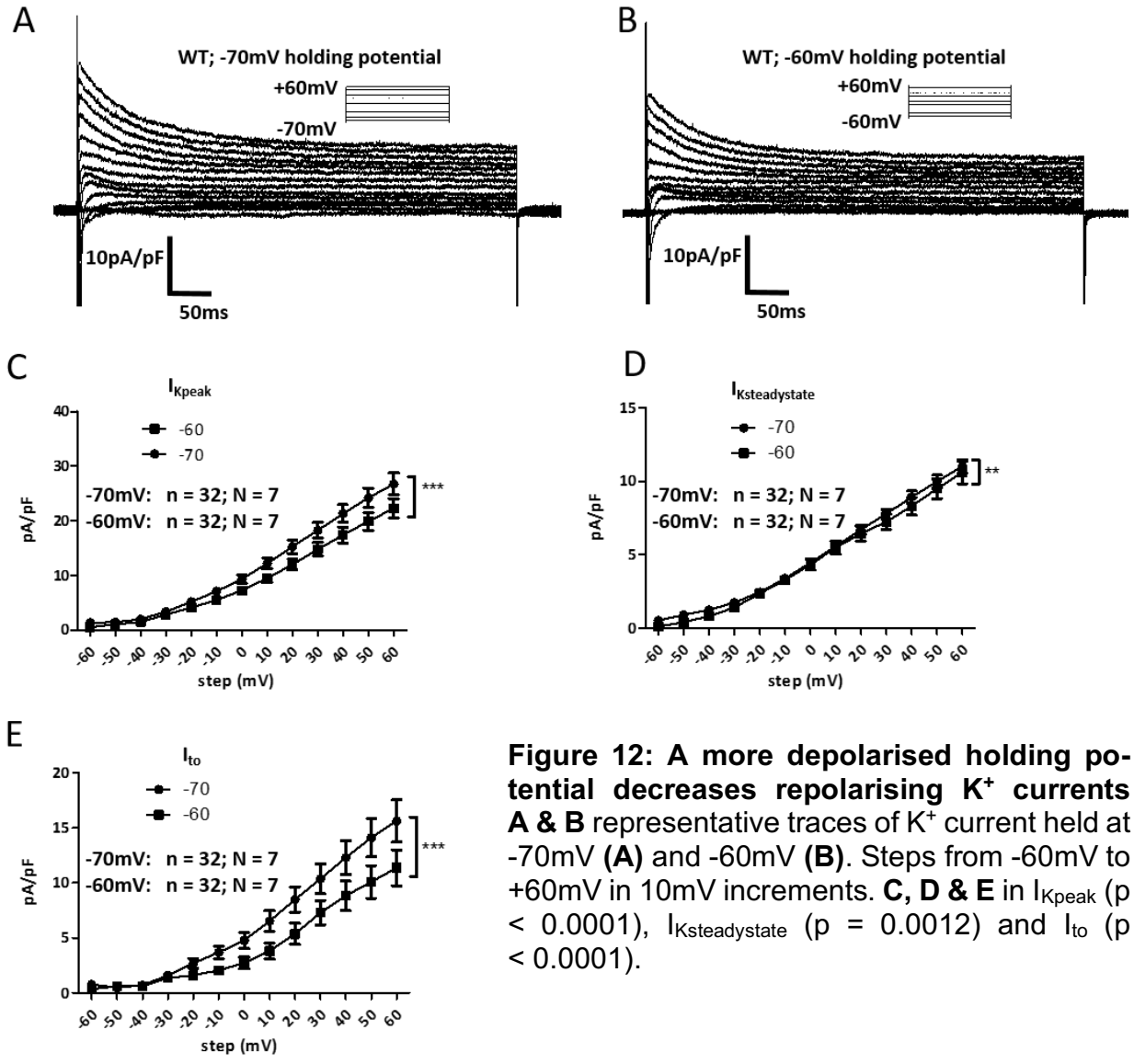


Figure 12: A more depolarised holding potential decreases repolarising K^+ currents **A & B** representative traces of K^+ current held at -70mV **(A)** and -60mV **(B)**. Steps from -60mV to +60mV in 10mV increments. **C, D & E** in I_{Kpeak} ($p < 0.0001$), $I_{Ksteadystate}$ ($p = 0.0012$) and I_{to} ($p < 0.0001$).

4.4.3 There is no significant difference in I_{K1} between WT and Pitx2c$^{+/-}$ LA cardiac myocytes.

The repolarising K^+ currents were recorded by superfusing isolated left atrial cardiomyocytes with standard solution (see methods). Afterwards the LA cardiomyocytes were superfused with $BaCl_2$, which is known to block I_{K1} [44]. I_{K1} was calculated by subtracting K^+ current in $BaCl_2$ from K^+ current in standard solution. This is demonstrated in figure 13, for which all the raw data and graphs of the figure were generated by using only one recording of a single WT LA cardiomyocyte.

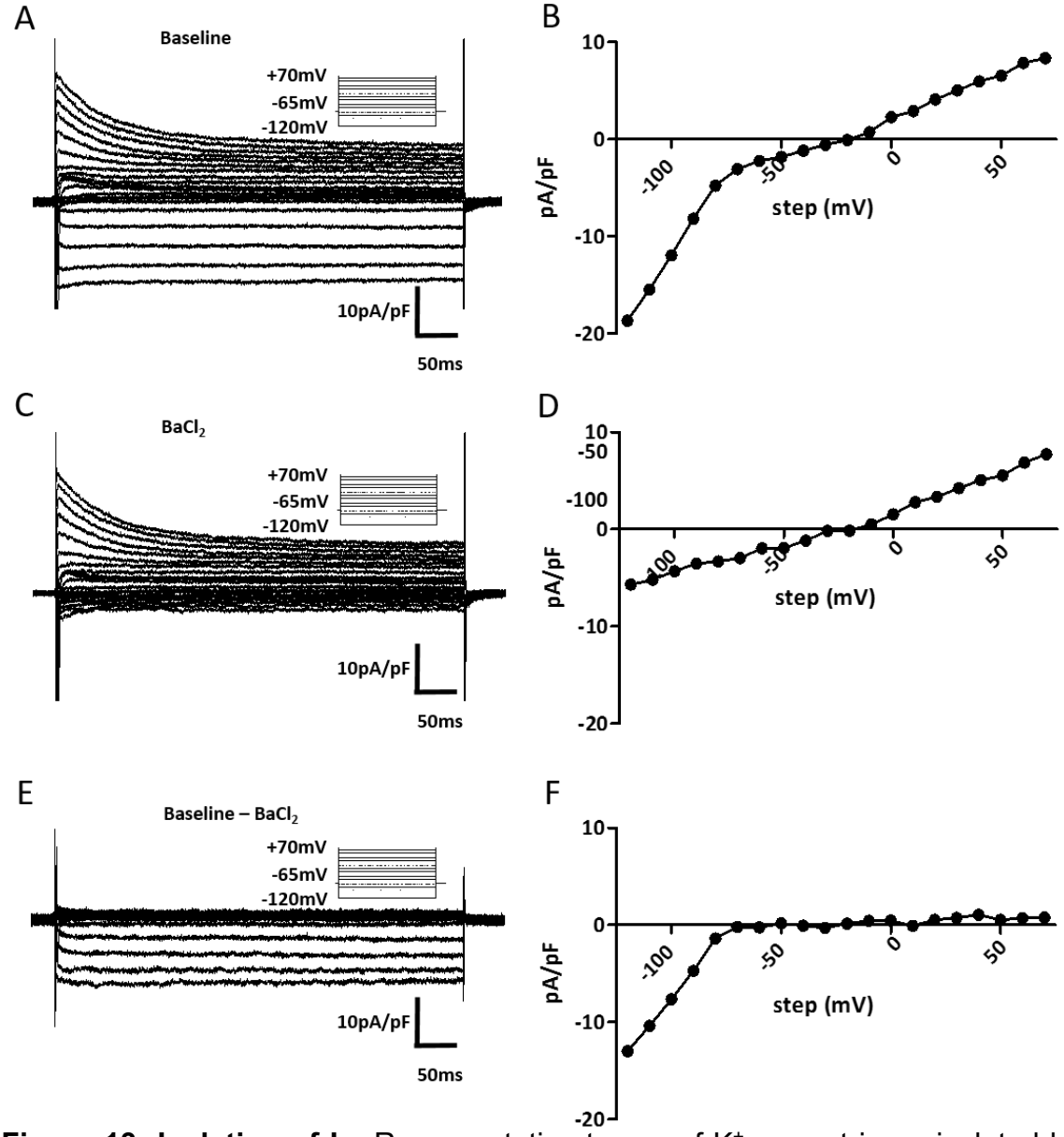
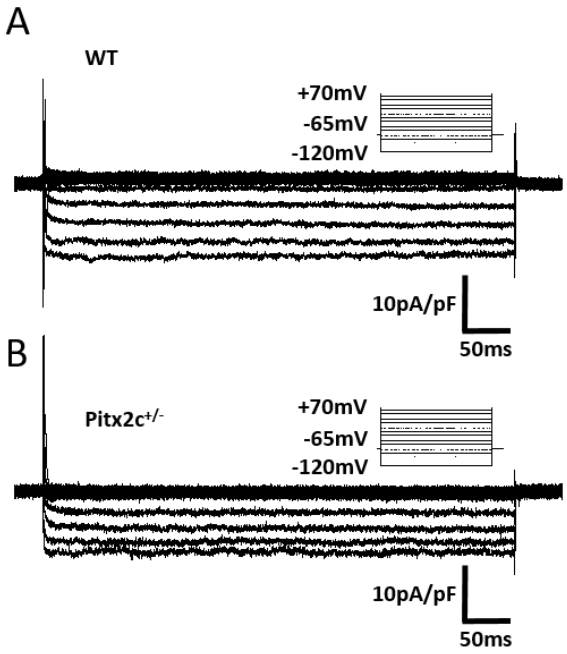


Figure 13: Isolation of I_{K1} Representative traces of K^+ current in an isolated left atrial WT cardiomyocyte in standard solution **(A & B)**, after superfusing with $BaCl_2$ **(C & D)** and the difference of $BaCl_2$ current digitally subtracted from baseline indicating the $BaCl_2$ sensitive current (I_{K1}) **(E & F)**. The Ba^{2+} sensitive current was considered as I_{K1}.

WT and Pitx2c$^{+/-}$ left atrial cardiomyocytes were held at -65mV and stepped from -120mV to +70mV in 10mV increments. There was no significant difference in I_{K1} ($p = 0.36$, figure 14.C) between genotypes (WT: n = 25; N = 7, Pitx2c$^{+/-}$: n = 21; N = 7). This implies that the RMP depolarisation in atrial cardiac myocytes with reduced Pitx2c$^{+/-}$ is not dependent on any reduction in I_{K1}.



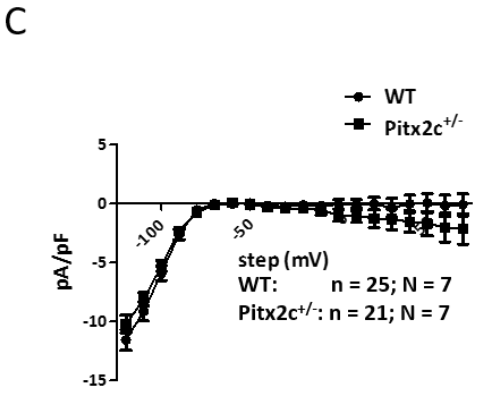


Figure 14: No significant genotype-related difference in I_{K1} A & B representative traces of barium-sensitive (I_{K1}) current in WT **(A)** and Pitx2c$^{+/-}$ **(B)** of left atrial cardiomyocytes when they were held at -65mV and stepped from -120mV to +70mV in 10mV increments. **C** There is no significant difference between genotypes in I_{K1} ($p = 0.36$).

4.4.4 A more depolarised resting membrane potential does not affect I_{K1}.

Left atrial WT cardiomyocytes were held at -65mV (n = 24; N = 7) or -60mV (n = 24; N = 7) and stepped with 10mV increment from -120mV to +70mV. However, the magnitude of I_{K1} – unlike I_{Kpeak}, $I_{Ksteadystate}$ and I_{to} (figure 12) – was not affected by a more positive holding membrane potential (p = 0,25, figure 15).

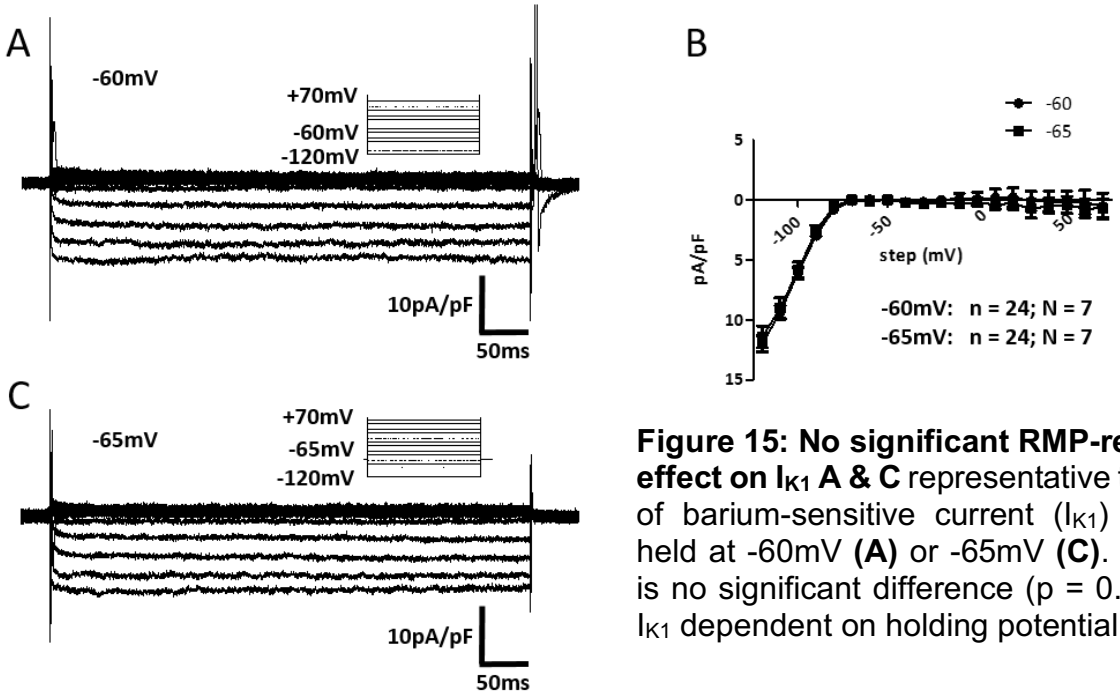


Figure 15: No significant RMP-related effect on I_{K1} A & C representative traces of barium-sensitive current (I_{K1}) when held at -60mV **(A)** or -65mV **(C)**. There is no significant difference (p = 0.25) in I_{K1} dependent on holding potential **(B)**.

4.4.5 Pitx2c$^{+/-}$ does not have an impact on CCh-sensitive current (I_{KACh}).

I_{KACh} is not constitutively active in healthy atrial cardiac myocytes. In these experiments, I_{KACh} was induced by addition of carbachol (10µM) to the superfusate as demonstrated in Figure 16. I_{K1} was blocked using 0.05mM $BaCl_2$.

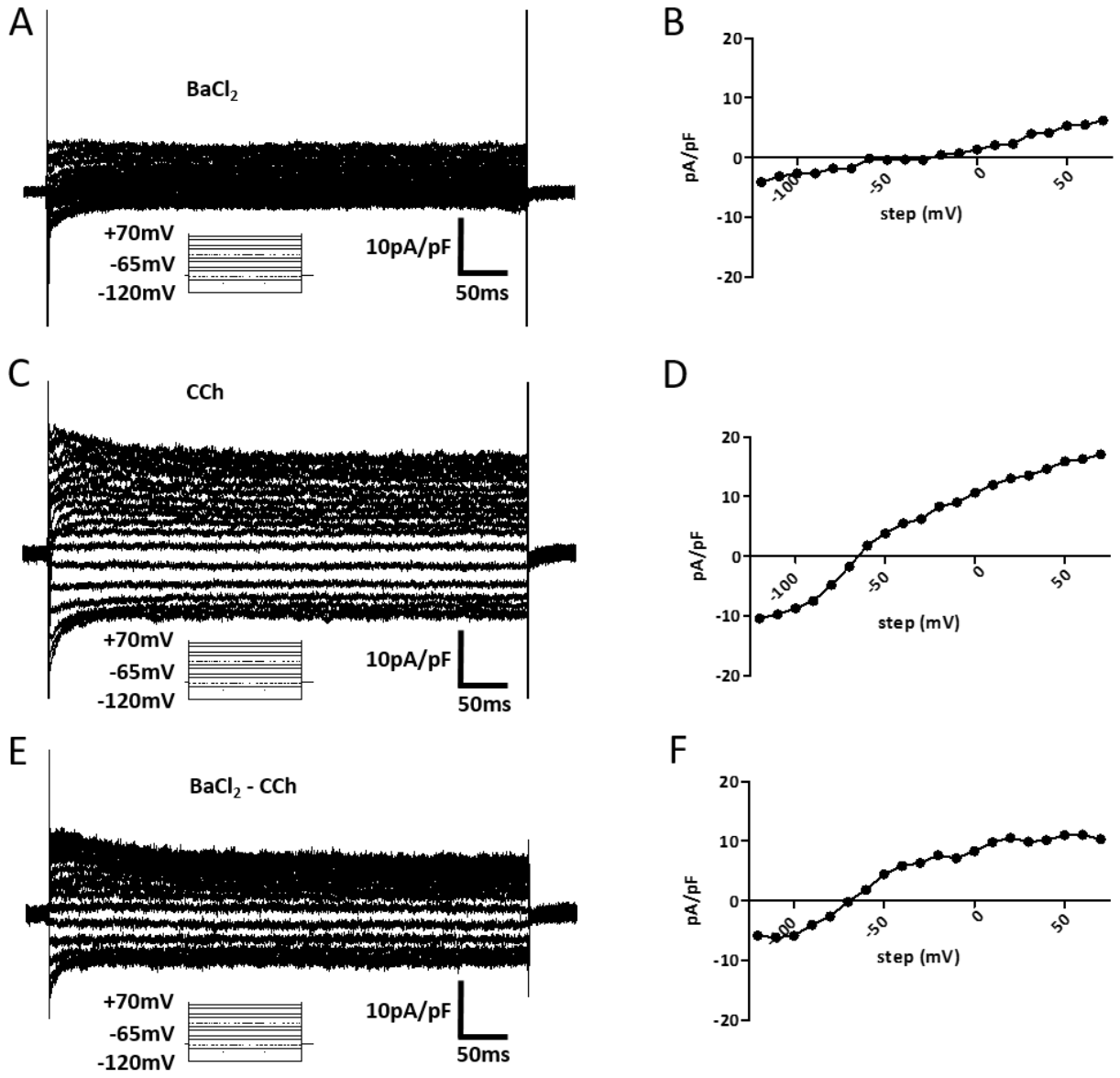
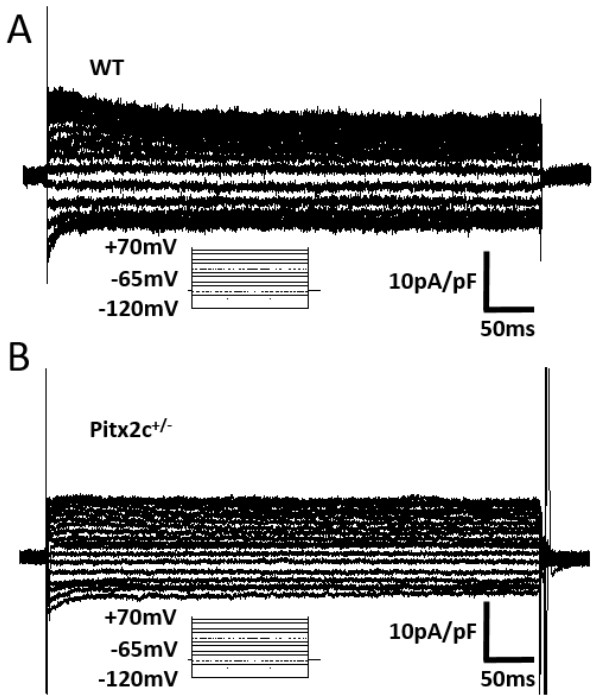


Figure 16: Determination of I_{KACh}. Representative traces and I/V plots of K^+ currents from a single atrial cardiomyocyte in the presence of $BaCl_2$ **(A & B)** and after superfusing with CCh **(C & D)**. The CCh-sensitive current (calculated by subtracting K^+ currents with $BaCl_2$ from currents with $BaCl_2$ plus CCh) is shown in **E** and **F**. The cardiomyocyte was held at -65mV and stimulated with 10mV increments starting at -120mV and going to +70mV.

When holding isolated left atrial cardiomyocytes at -65mV and stimulating in 10mV increments, starting at -120mV and going up to +70mV, there is no difference in I_{KACh} between WT and Pitx2c$^{+/-}$ ($p = 0{,}48$, figure 17.C).



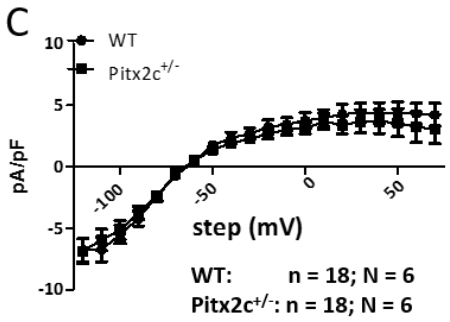


Figure 17: No genotype-dependent differences in I_{KACh} A & B representative traces of CCh-sensitive K^+ current (I_{KACh}) of left atrial isolated cardiomyocytes of WT and Pitx2c$^{+/-}$. Cardiomyocytes were held at -65mV and stimulated with 10mV increments starting at -120mV up to +70mV. **C** No significant difference between WT and Pitx2c$^{+/-}$ in I_{KACh} ($p = 0.48$).

4.4.5 A more positive resting membrane potential has a dual effect on the two different components of I_{KACh}.

I_{KACh} was also measured from a more positive holding potential of -60mV. Interestingly, the more positive holding membrane potential led to more K^+ inflow at step potentials negative to -80mV but also caused a smaller outward K^+ current at potentials above -70mV ($p < 0.0001$, figure 18). These data suggest that whilst there was no intrinsic alteration in I_{KACh} in Pitx2c$^{+/-}$ LA, the physiological K^+ current evoked by activation of acetylcholine receptors in Pitx2c$^{+/-}$ (which have a more depolarised RMP) is likely to be slightly reduced.

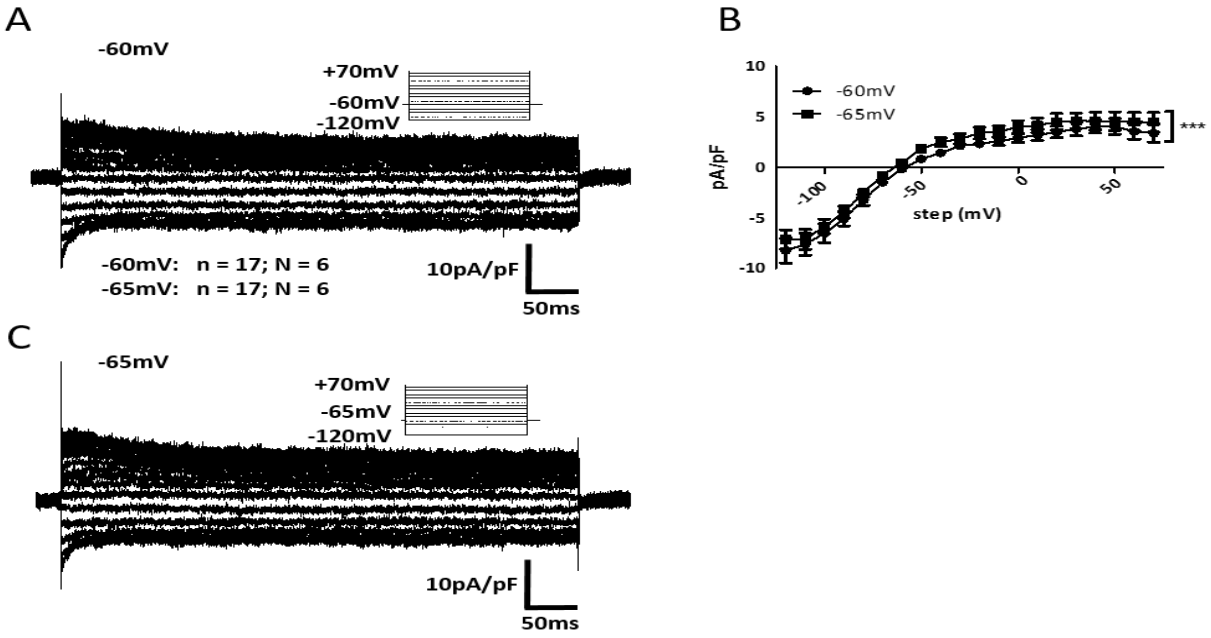


Figure 18: Dual effects of holding potential on the inward and outward components of I_{KACh} A & C representative traces of CCh-sensitive K^+ current (I_{KACh}) in isolated WT cardiomyocytes of the left atrium. Cardiomyocytes were held at -60mV or -65mV to evaluate the effect of holding potential on I_{KACh}. **B** A more depolarised holding membrane potential leads to a bigger I_{KACh} inward current at step potentials more negative than -70mV and a smaller K^+ outflow at holding potentials more positive than -70mV ($p < 0.0001$).

5. Discussion:

5.1 Main findings

The data from this study show that peak I_{Na} is similar in WT and Pitx2c$^{+/-}$ LA cardiac myocytes when measured across a range of physiological holding potentials (figure 5.F). More positive holding potentials induce smaller peak I_{Na} currents (figure 5.F). At equivalent holding potentials, flecainide reduces sodium currents to the same extent in WT and Pitx2c$^{+/-}$ LA cardiomyocytes. Importantly, the efficacy of flecainide is dependent on holding potential as evidenced by flecainide causing more sodium channel block at more positive holding potentials. This is consistent in both primary murine WT and Pitx2c$^{+/-}$ LA cardiomyocytes and in HEK293 cells expressing the human $Na_v1.5$ channel (figure 6.C & D and figure 7.B). Since a reduction in Pitx2c causes RMP depolarisation [8, 32], this finding is likely to explain why flecainide is more effective in prolonging ERP and preventing arrhythmia in Pitx2c$^{+/-}$ mice. If similar reports are found in human atrial cardiac myocytes, then flecainide could be given preferentially to patients with reduced PITX2 mRNA to treat AF. This finding therefore could be an important first step towards personalised therapy for AF (figure 19) [32].

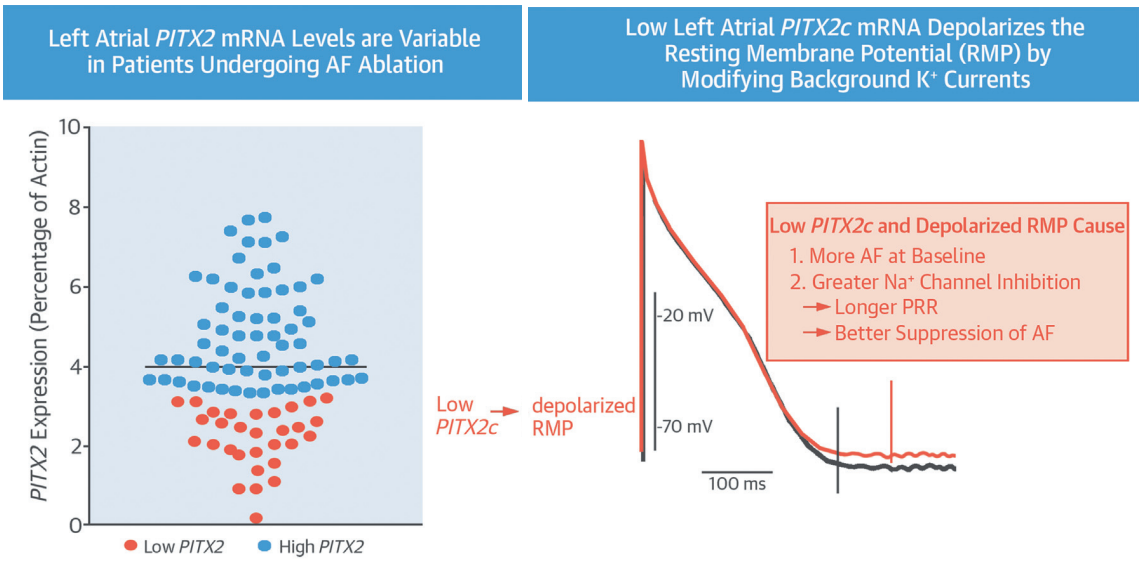


Figure 19: Proposed mechanism of RMP increasing effectiveness of flecainide in human AF patients with reduced PITX2. 1. A reduction in PITX2 causes RMP depolarisation. This increases the amount of sodium channel block by flecainide, thus enhancing antiarrhythmic protection. Adapted from Syeda F, Holmes AP, Yu TY, Tull S, Kuhlmann SM, Pavlovic D, Betney D, Riley G, Kucera JP, Jousset F, de Groot JR, Rohr S, Brown NA, Fabritz L, and Kirchhof P, PITX2 Modulates Atrial Membrane Potential and the Antiarrhythmic Effects of Sodium-Channel Blockers. J Am Coll Cardiol, 2016. 68(17): p. 1881-1894.

Calcium transient relaxation times and amplitude are similar in WT and Pitx2c$^{+/-}$ at cycle lengths 300ms, 120ms, 100ms and 80ms (figure 8). Faster pacing shortens relaxation time and decreases transient amplitude in both genotypes (figure 8). Flecainide shortens calcium transient relaxation times and decreases the amplitude significantly (figure 9). Interestingly, the effect of flecainide on Ca^{2+} is not dependent on cycle length (figure 10). The overall action of flecainide on Ca^{2+} transient morphology is not different between genotypes (figure 9).

Repolarising K^+ currents transient outward K^+ current (I_{to}), and delayed rectifiers ($I_{Ksteadystate}$), I_{Kpeak} (I_{to} + $I_{Ksteadystate}$), and inward rectifier (I_{K1}) and acetylcholine-sensitive G-protein-coupled inward rectifier K^+ current (I_{KACh}) were investigated. All potassium currents are similar in WT and Pitx2c$^{+/-}$ (figures 11, 14 and 17). Thus, shortening of the LA APD in Pitx2c$^{+/-}$ hearts is unlikely to be due to change in any of the investigated repolarising K^+ currents. The cause of APD shortening in Pitx2c$^{+/-}$ LA remains to be identified.

At more positive holding potentials I_{Kpeak}, $I_{Ksteadystate}$ and I_{to} are reduced (figure 12). On the other hand, holding potential does not have an impact on I_{K1} (figure 15). Interestingly, whilst the magnitude of inward I_{KACh} more negative to -80mV is enhanced at more positive holding potentials, I_{KACh} outward current above -70mV is reduced at more positive holding potentials (figure 18).

5.2 A more depolarised RMP accounts for decreased physiological I_{Na} and the reduction in APA in the Pitx2c$^{+/-}$ LA.

Recently published data [8, 27, 32], indicate that left atrial cardiomyocytes of Pitx2c$^{+/-}$ and atrial specific Pitx2 knockout mice (NppaCre^{+}Pitx2$^{-/-}$) mice have a reduced action potential amplitude (APA), a shorter action potential duration (APD) and a more positive resting membrane potential (RMP). The electrical changes are likely to account for increased vulnerability of these hearts to inducible atrial arrhythmia [8, 27, 32]. Atrial-specific Pitx2 knockout mice (NppaCre^{+}Pitx2$^{-/-}$) even display atrial arrhythmias before pacing [8].

One of the aims of this thesis was to investigate causes of the LA action potential differences between WT and Pitx2c$^{+/-}$. First, the difference in APA was investigated by monitoring sodium channel activity. In murine atrial cardiomyocytes, the magnitude of APA and the rate of the depolarisation upstroke is determined by I_{Na}, which itself is determined by the number of membranous sodium channels and their activity. It was hypothesised, that the reduction of APA in Pitx2c$^{+/-}$ cardiomyocytes was caused by a change in I_{Na} due to a change in $Na_V1.5$ expression or function.

There are no changes between WT and Pitx2c$^{+/-}$ in Scn5a mRNA expression, which is the gene coding for the $Na_V1.5$ ion channel, or in $Na_V1.5$ protein expression [32]. Interestingly, Chinchilla et al. used an atrial specific homozygous Pitx2c knockout model (NppaCre^{+}Pitx2$^{-/-}$) and found a reduction in Scn5a expression and $Na_V1.5$ in the LA of murine cardiomyocytes [8]. Even though there is a difference in Scn5a expression, both mouse models have a reduced Pitx2 mRNA expression, a depolarised RMP and a smaller APA - an indication that the reduced APA might not be caused by a reduced $Na_V1.5$ protein expression, but might be rather due to a change in $Na_V1.5$ channel function, resulting in a reduced I_{Na}. Tao et al. strengthen the theory that Pitx2 regulation of Scn5a expression is complex. These authors used a mouse model in which the entire Pitx2 allele was deleted in the postnatal atrium (conditional loss of function, Pitx2 CKO) for studying the effect of Pitx2 after development. They found a reduced expression of Scn5a in the prenatal LA and an increased Scn5a expression in the postnatal LA [34]. Unfortunately, they did measure neither APA, nor I_{Na} before or after birth. This indicates that Pitx2 and its isoform Pitx2c have a different impact during development and maintenance [34].

However, up to now, it is still unknown whether humans with PITX2c deficiency display a reduced expression of Scn5a mRNA and $Na_V1.5$ protein expression. Future research projects need to evaluate this.

Summarised, $Pitx2c^{+/-}$ murine LA cardiomyocytes presented a reduction in APA and a greater antiarrhythmic effect of flecainide without an altered expression of Scn5a or $Na_V1.5$ [32]. Therefore, it was hypothesised that the smaller APA could be caused by a reduced function of $Na_V1.5$ in $Pitx2c^{+/-}$, resulting in a reduced I_{Na}. However, when comparing I_{Na} between genotypes at the same holding potential, there were no genotype-dependent differences (figure 6.C & D). This suggests no apparent alteration in $Na_V1.5$ function caused by reduction in Pitx2c.
It is also demonstrated that a more positive steady-state holding potential (thereby simulating the depolarised RMP in $Pitx2c^{+/-}$ atrial cardiomyocytes, which was observed in $Pitx2c^{+/-}$ and the $NppaCre^{+}Pitx2^{-/-}$ LA cardiomyocytes [8, 32]) results in a smaller peak I_{Na}. It is therefore proposed that the reduction of APA in $Pitx2c^{+/-}$ LA cardiomyocytes is caused rather by the more depolarised RMP (and a subsequent fall in $Na_v1.5$ availability) than by a change in Scn5a mRNA expression or $Na_V1.5$ protein expression. This hypothesis is supported by the fact that $Pitx2c^{+/-}$ and the $NppaCre^{+}Pitx2^{-/-}$ LA cardiomyocytes display similar electrophysiological properties (depolarised RMP and small APA), even though the expression of Scn5a is different [8, 32].

The following mechanism underlying the reduced APA in $Pitx2c^{+/-}$ LA is therefore proposed. The sodium channel has three primary states: activated (A), inactivated (I) and rested/recovered (R) [47]. Initially, $Na_v1.5$ channels are in rested state, depolarisation activates $Na_v1.5$ channels and elicits peak I_{Na} current. $Na_v1.5$ channels then rapidly inactivate and have to switch to rested state again before they can be activated again [47]. The passage between inactivated and rested state is governed by time and membrane potential. Hyperpolarisation increases the transition to rested stage as well as the number of channels that become available for reactivation [48]. $Pitx2c^{+/-}$ and $NppaCre^{+}Pitx2^{-/-}$ left atrial cardiomyocytes have a more depolarised membrane potential [8, 27]. Thus, after activation and a return to a more positive RMP, fewer $Na_v1.5$ channels in $Pitx2c^{+/-}$ – compared to WT – will transition into rested state. Thus, there will be fewer $Na_v1.5$ channels available to be activated (in rested state), eventually resulting in a smaller I_{Na} and APA under physiological conditions.

5.3 A depolarised RMP increases the efficacy of fleciainide in Pitx2c$^{+/-}$ cardiomyocytes

Emerging evidence suggests that class I antiarrhythmic drugs (AADs) – including flecainide – have a greater antiarrhythmic effect in patients with the single nucleotide polymorphism (SNP) rs10033464, adjacent to the PITX2 gene, than class III AADs (potassium channel-blocking drugs) [18]. Accordingly, Syeda et al. found that flecainide was more effective in suppressing induced arrhythmias in Pitx2c$^{+/-}$ murine hearts, than in WT [32]. This was associated with an exaggerated prolongation of the effective refractory period (ERP) and greater reduction in APA in response to flecainide in the Pitx2c$^{+/-}$ LA, pointing towards an enhanced sodium channel blockade. In the present thesis, the mechanism underlying the heightened efficacy of flecainide in Pitx2c$^{+/-}$ was investigated. The inhibitory effect of flecainide on I_{Na} was evaluated at different holding potentials (figure 6) in LA cardiomyocytes, because of the hypothesis that the difference in RMP is responsible for the differences between WT and Pitx2c$^{+/-}$ in Na^+ handling. There was no genotype-dependent difference in the sodium channel-blocking effect of flecainide (figure 6). However, a more positive holding membrane potential increased the inhibitory effect of flecainide significantly (figure 6). This was observed in both WT and Pitx2c$^{+/-}$ LA cardiomyocytes and is consistent with a previous report that showed flecainide decreased V_{max} more at more positive RMPs in guinea pig ventricular cells [48]. This however, is the first indication of such a mechanism existing in atrial cardiac myocytes.

The following mechanism seems to be accountable for the greater effect of flecainide in Pitx2c$^{+/-}$ cardiomyocytes under physiological conditions:
A known binding site of flecainide is in the Na^+ channel pore [49]. Therefore, of the three states of the sodium channel (active, inactive, rested), flecainide has the highest affinity to the activated/open channel – meaning blocking occurs in the activated state [48]. Interestingly, unblocking is highly voltage-dependent: Use-dependent unblocking is enhanced by hyperpolarisation, i.e. depolarisation reduces unblocking and enhances the sodium channel blocking effect of flecainide [48]. In drug-associated channels transition from inactivated state to rested state occurs at more negative potentials [48], so it will take longer in depolarised cells for channels, which are blocked by flecainide, to transition to the rested state and be available for activation and unblocking again. Anno et al. concluded that flecainide would have a greater effect in depolarised ventricular

cardiomyocytes because the availability for unblocking develops less rapidly in depolarised tissue [48]. Burashnikov et al. hypothesise that a more depolarised RMP in the atria may potentiate the effects of I_{Na} blockers such as ranolazine and propafenone by increasing the fraction of channels in the inactivated state, which reduces the availability of sodium channels and prolongs the time needed for the sodium channels to recover from inactivation [50]. Pitx2c$^{+/-}$ atrial cardiomyocytes have a depolarised RMP, thus leading to less unblocking of channels and resulting in a more persistent block. It is proposed that this is the mechanism, which accounts for the exaggerated prolongation of ERP and greater antiarrhythmic effect of flecainide in murine Pitx2c$^{+/-}$ LA. Whether this extends to human patients with SNP risk variants adjacent to the PITX2 gene [18] or in patients with reduced PITX2 due to other factors remains to be confirmed. It must be noted that there may be a greater overall effect of fleciainde in arrhythmic hearts than in normal hearts. This is due to the known use-dependency, which means at a faster heart rate – for example during atrial fibrillation – the antiarrhythmic effect of flecainide increases [48].

To investigate whether depolarisation enhances the sodium channel blocking effect of flecainide in human cells with human $Na_V1.5$ sodium channels, experiments were also performed using HEK cells, which were transfected with the human $Na_V1.5$ channel. A more positive holding potential increased the sodium channel blocking effect of flecainide on human $Na_V1.5$ channels in HEK cells significantly (figure 7). Thus, the RMP modulation of flecainide seems to be consistent in the human cells expressing the human cardiac $Na_V1.5$ channel. It is therefore hypothesised that in patients with reduced PITX2 mRNA expression and a more positive RMP, flecainide will be more effective in terminating atrial arrhythmia [18]. Future studies may aim to directly assess the effectiveness of sodium channel blocking drugs in patients with reduced PITX2 mRNA expression.

The finding that the effect of AADs class Ic – in this case flecainide – is enhanced by depolarisation, might lead to more personalised and novel approaches in antiarrhythmic drug therapy. E.g. Patients with reduced PITX2 may preferentially be given flecainide as opposed to other AADs. Furthermore, in other patient populations, new therapies that target the atrial RMP (e.g. TASK-2 inhibitors) may be given alongside flecainide to enhance the overall AAD potency. Clearly, much future work is required to identify and validate suitable atrial selective compounds that can modulate the atrial RMP. Furthermore, it will be important to evaluate if the RMP dependency of flecainide is also apparent in

other sodium channel blocking AADs. Thus, further validation of how depolarisation of the RMP affects class I AADs (and potentially other AADs) is needed in future studies.

5.4 Does a change in Ca^{2+} handling account for the electrical phenotype of Pitx2c deficient LA cardiac myocytes and sensitivity to flecainide?

To investigate the cause of the shortening in APD in Pitx2c$^{+/-}$ LA cardiomyocytes, [8, 27, 51], further experiments were performed looking at Ca^{2+} and K^{+} handling, because changes in the duration of an atrial action potential can be governed by alterations in calcium and/or potassium currents.

Initially Ca^{2+} handling in the LA of heterozygous Pitx2c-knockout mice (Pitx2c$^{+/-}$) and WT mice was examined. Measurements of 30%, 50% and 70% relaxation times of Ca^{2+} transients and the amplitude of calcium transient were recorded and analysed (figure 8). Interestingly, there was no difference in relaxation time or transient amplitude between genotypes, even though a reduced expression of Cacna1c, the alpha 1C subunit of the voltage-dependent, L-type, Ca^{2+} channel, was described before in Pitx2c$^{+/-}$ [27]. In Nppa-Cre^{+}Pitx2$^{-/-}$ Lozano-Velasco et al. confirmed a reduced expression of Cacna1c and described, on the contrary, an increase in Cacnb1, Cacnb3 and ryanodine receptor 2, resulting all together in a reduction in voltage-dependent L-type calcium channel current ($I_{Ca,L}$) as well as an increased sarcoplasmic reticulum (SR) Ca^{2+} load (figure 2) [33]. Tao et al. found an increased expression of Cacna1d and Cacna2d2 in Pitx2 CKO LA cardiomyocytes (figure 2) [34].

In view of these observations, it is possible that reduction in voltage-dependent L-type calcium channel current ($I_{Ca,L}$) due to a reduced Cacna1c expression is sufficient to account for the shortening of the APD [27, 33]. In the present study however, Ca^{2+} transients were not significantly different (figure 19). A calcium transient consists of two parts. Firstly, Ca^{2+} influx via $I_{Ca,L}$ and secondly Ca^{2+} release from the SR. The increase of RyR_2 expression and the increase in SR load might indicate that the reduced $I_{Ca,L}$ is compensated for by an increase of SR Ca^{2+} release [33], resulting in unchanged Ca^{2+} transients.

It has been reported that $I_{Ca,L}$ is reduced in electrophysiological remodeling in human AF [52, 53], indicating the importance of this current for AF. Furthermore, $I_{Ca,L}$ reduction does not have to be due to reduced expression of channel subunits, and is rather due to decreased channel phosphorylation in AF [52, 54]. Though other studies have found a reduction in L-type calcium channel mRNA [55]. To ensure that the shortening of APD in Pitx2c$^{+/-}$ left atrial cardiomyocytes is not due to changes in $I_{Ca,L}$, patch-clamp experiments investigating $I_{Ca,L}$ need to be performed. As there is still an ongoing debate about whether phosphorylation or mRNA expression is causing $I_{Ca,L}$ reduction, investigation of L-type calcium channel phosphorylation would be recommended, additionally to the $I_{Ca,L}$ measurements in Pitx2c$^{+/-}$. Interestingly, Perez-Hernandez et al. found an augmented PITX2c expression in human right atrial cardiomyocytes in chronic atrial fibrillation patients and as a consequence a decrease in $I_{Ca,L}$ [17]. This is in contrast with published data, which postulate a reduction of Pitx2c to promote atrial fibrillation and decrease $I_{Ca,L}$ [8, 33]. Perez-Hernandez et al. propose that disease duration and sample tissue, which was taken from the right atrial appendage, could be responsible for this difference [17]. However, this needs further investigation, as the reduced $I_{Ca,L}$ might rather be due to AF than PITX2c insufficiency [52, 54].

5.5 Flecainide's impact on Ca^{2+} handling

The results of the present thesis demonstrate that flecainide reduces the Ca^{2+} transient amplitude and also shortens relaxation time. Recently, the effect of flecainide on calcium handling was reported in literature [56-58]. Initially flecainide was reported to have a direct inhibitory impact on RyR_2 channels [59, 60]. Next Lui et al. suggested that flecainide may also have an indirect effect by modifying the transmembrane Na^+ gradient [61].

Sikkel et al. suggested the following mechanism (figure 20), which may explain the additional Ca^{2+} transient shortening caused by flecainide. Under normal conditions, depolarisation opens $Na_v1.5$ ion channels, which leads to Na^+ influx and to further depolarisation, which activates voltage-dependent L-type calcium channels (L-type Ca^{2+}). Consequently, Ca^{2+} gets into the cell and the intracellular calcium concentration is increased. This leads to a Ca^{2+}-triggered Ca^{2+} release – Ca^{2+} activates the ryanodine receptor 2 and Ca^{2+} is released from the sacroplasmatic reticulum (SR) – increasing the intracellular Ca^{2+} concentration. Intracellular Ca^{2+} is now either pumped back into the SR, or – via sodium-calcium-exchanger – pumped out of the cell.

When flecainide binds $Na_V1.5$, I_{Na} is reduced and less Na^+ gets into the cardiomyocyte – resulting in a greater gradient between extracellular Na^+ and intracellular Na^+ concentration. Still, depolarisation activates L-type Ca^{2+} channels – leading to an increase in intracellular Ca^{2+} concentration, which triggers SR Ca^{2+} release. Intracellular Ca^{2+} is pumped back into the SR or outside via sodium-calcium-exchanger. Now the increased gradient of extracellular to intracellular Na^+ becomes apparent. Driven by the larger gradient of Na^+ into the cell, Ca^{2+} is pumped out of the cell more rapidly via sodium-calcium-exchanger, thereby shortening the Ca^{2+} relaxation time. This mechanism is proposed to cause transient shortening and a reduction in amplitude, as Ca^{2+} is pumped out of the cell much more quickly than without flecainide.

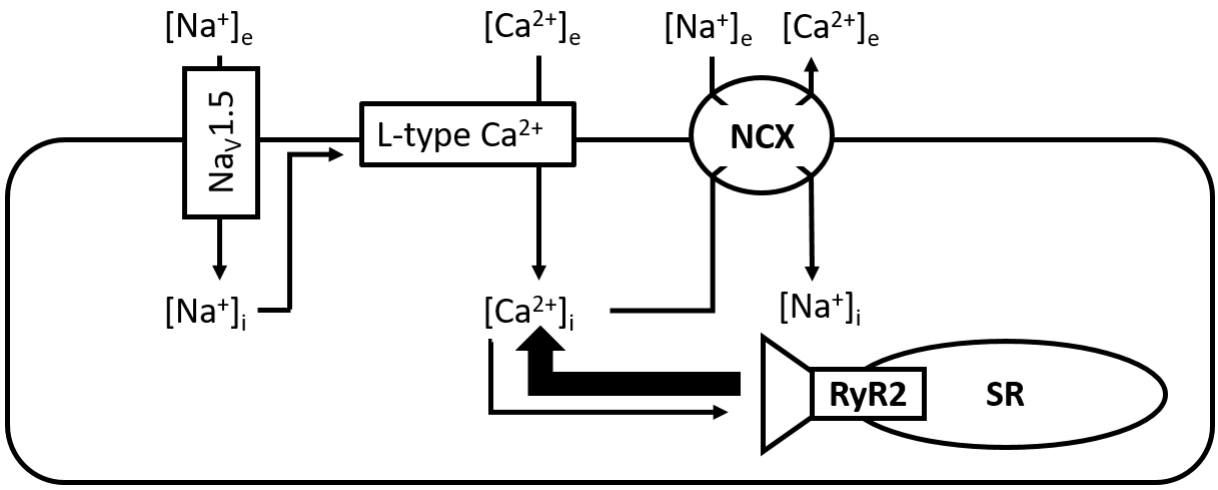


Figure 19: Flecainide modulates Ca^{2+} handling – a model. Flecainide blocks $Na_V1.5$ channels. Thus, I_{Na} is reduced and the gradient of extracellular to intracellular Na^+ is increased. Due to depolarisation, voltage-gated L-type Ca^{2+} channels open, intracellular Ca^{2+} increases and triggers calcium release from sarcoplasmatic reticulum via RyR2. This leads to a high intracellular Ca^{2+} concentration. Because of the increased gradient of extracellular to intracellular Na^+, there is a greater Na^+ influx via NCX resulting in a greater Ca^{2+} outflow, reducing intracellular Ca^{2+} level and resulting in shorter relaxation times and lower amplitude of calcium transients with flecainide.

The data from the present thesis also indicate that flecainide shortens the relaxation time and decreases the amplitude of calcium transients to a similar degree in both WT, and $Pitx2c^{+/-}$ murine left atria. It is therefore unlikely that the enhanced antiarrhythmic response to flecainide in $Pitx2c^{+/-}$ LA is due to differential effects on Ca^{2+} homeostasis. Interestingly, previous research found that flecainide increased the action potential duration in Langendorff experiments (monophasic action potential duration), in microelectrode experiments (transmembrane action potential duration) and in optical mapping experiments (optical action potential duration) [32]. The authors concluded that the APD prolongation is not caused by the effect of flecainide on Ca^{2+} handling, as Ca^{2+} transients themselves are shortened (figure 9), leading to a reduced, not increased NCX current in late phase repolarization. It is more likely that flecainide has some non-selective actions on K^+ channels that account for longer repolarization and APD prolongation.

It would be very interesting to conduct these experiments with $NppaCre^{+}Pitx2^{-/-}$ mice because they have a reduced $I_{Ca,L}$ but an increased SR Ca^{2+} load. Furthermore, they display an increased current via NCX (I_{NCX}) as a result of caffeine application, which leads to an emptying of intracellular Ca^{2+} stores [33]. The increased I_{NCX} could either be a result of

the increased SR Ca^{2+} storage, or it could be enhanced by the mechanism explained above: A reduced Na^{+} influx leads to an increased gradient between intra- and extracellular Na^{+}, which in turn increases Ca^{2+} efflux via NCX.

5.6 Repolarsing K^+ currents are similar in WT and Pitx2c$^{+/-}$ LA

Kirchhof et al. and Chinchilla et al. have reported a shortened APD in Pitx2c$^{+/-}$ and Nppa-Cre^{+}Pitx2$^{-/-}$ left atrial cardiomyocytes [8, 27]. The duration of action potentials is governed by calcium and potassium currents. Because no difference in calcium transients were found (figure 8), repolarising potassium currents in LA cardiomyocytes of WT and Pitx2c$^{+/-}$ were also measured. Repolarising K^+ currents consist of I_{to} – a transient outward K^+ current, and the delayed rectifiers I_{Kur} – an ultrarapidly activating K^+ current, I_{Kr} – a rapid activating K^+ current and I_{Ks} – a slow activating K^+ current (see figure 21). In the mouse I_{Kur}, I_{Kr} and I_{Ks} are less well defined and are often collectively referred to as I_{ss} or $I_{Ksteadystate}$ (figure 11 and 12).

In this study, there were no significant differences between WT and Pitx2c$^{+/-}$ in the magnitude of repolarising voltage-dependent K^+ currents. Thus, it is unlikely that the APD shortening observed in Pitx2c$^{+/-}$ is dependent on the altered activity of the channels responsible for conducting these currents.

Previously, in Pitx2c$^{+/-}$ LA cardiomyocytes Kcna3 mRNA expression was shown to be increased, whereas Kcnc4 and Kcna6 mRNA expression was decreased [27]. Interestingly, K_V1.6 channel, which is encoded by Kcna6, was unaltered [32]. Kcna3 is a voltage-gated, shaker-related K^+ channel (K_V1.3), which is reported to play an important role in lymphocyte signaling [62]. Therefore, is it unlikely to account for any electrophysiological changes in Pitx2c$^{+/-}$ LA cardiomyocytes. Kcna6 is expressed in the heart, especially in the sinoatrial and atrioventricular node, making it an interesting target [42]. However, a decrease of Kcna6 would likely cause a reduced rate of K^+ outflow during repolarisation and, in doing so, prolong the APD. Additional studies have since suggested that K_V1.6 channel protein expression is unaltered [32]. It has also been reported that Kcnc4 mRNA expression was reduced in Pitx2c$^{+/-}$ LA tissue [27]. Kcnc4 codes for K_V3.4, which is important for transient outward (I_{to}) current in the brain [63], but a role in atrial tissue remains to be defined. If physiological, then a reduction would again tend to decrease I_{to} and prolong APD. Thus, it is unlikely that a reduced K_V3.4 expression could cause the APD shortening in LA cardiomyocytes in Pitx2c$^{+/-}$.

Looking at other mouse models lacking Pitx2, it is reported that in Pitx CKO LA cardiomyocytes Kcnq1 mRNA expression is up-regulated [34]. Kcnq1 increases QT-time and forms a channel for the slow component of the delayed rectifier current I_{Ks} [64]. An enhanced I_{Ks} could shorten the APD and a prolongation of QT-time increases the risk of AF [65]. Therefore, measuring I_{Ks} is very interesting. However, this is difficult to perform in murine models, as I_{Ks} is negligible or even totally absent. Future studies in human LA cardiac myocytes with reduced PITX2 are necessary to define a role for I_{Ks} in causing APD shortening.

Perez-Hernandez et al. have investigated right atrial appendages of humans with chronic AF (CAF) [17]. They observed an increased PITX2c expression in CAF patients. Contrary to Tao et al. they published that an increase in PITX2c expression increases transcription of KCNQ1 and KCNE1 genes and increases I_{Ks} [17]. I_{Ks} is reported to be an important current for repolarisation in the heart [66]. However, the results of Perez-Hernandez et al. are contrary to a number of other published data [8, 27, 34]. Li et al. comment on Perez-Hernandez et al. that there might be several reasons for the discrepancies. First, Perez-Hernandez et al. investigate right atrial (RA) appendages. PITX2c expression is described to be higher in the LA [8, 27]. Investigation of PITX2c, KCNQ1 and KCNE1 expression and I_{Ks} density of AF patients in the LA would be very interesting. Additionally, they investigated CAF patients. PITX2c expression might be up-regulated for compensation, or due to drug therapy [67]. In the end, Perez-Hernandez et al. confirm that PITX2c expression somehow plays a role in AF. Whether it is due to up-regulation in RA or down-regulation in LA remains to be established.

Pitx CKO LA cardiomyocytes are reported to have a reduced mRNA expression of Kcnj10 and Kcnj11 [34]. Mutation in Kcnj10 is known to cause EAST syndrome [68]. Kcnj10 and Kcnj11 are ATP-sensitive inward rectifier potassium channels, which play a role in insulin secretion [69]. Changes in mRNA in these two genes are unlikely to have an impact on APD unless the Pitx2 deficiency also causes modifications in metabolism and impairs ATP generation. This is an interesting area for future consideration.

However, there were neither changes in I_{to}, nor in $I_{Ksteadystate}$, (figure 11) in Pitx2c$^{+/-}$ LA cardiomyocytes. Especially in the physiological range of an atrial action potential, which is below 0mV, there is no genotype-dependent difference in WT or Pitx2c$^{+/-}$

cardiomyocytes in the transient outward rectifier (I_{to}), or in the delayed rectifiers ($I_{Kstead-ystate}$). Interestingly, a depolarised resting membrane potential of -60mV instead of -70mV reduces K^+ outflow of I_{Kpeak}, $I_{Ksteadystate}$ and I_{to}, which would again promote a prolongation of APD.

5.7 Depolarised RMP in Pitx2c$^{+/-}$ LA cardiomyocytes could be caused by a change in background (TASK) currents but not inward rectifier K$^+$ currents.

Pitx2c$^{+/-}$ mice display a depolarised RMP, leading to a greater antiarrhythmic effect of flecainide (see figure 6) [32]. I_{K1} is known to play a crucial part in maintaining the RMP in cardiomyocytes [70]. It is reported that in NppaCre$^+$Pitx2$^{-/-}$ LA cardiomyocytes Kcnj2, Kcnj4 and Kcnj12 mRNA expression is reduced (figure 2) [8]. Furthermore Kir2.1, which is encoded by Kcnj2 is reduced, too [8]. Kcnj2, encoding Kir2.1, Kcnj4, encoding Kir2.3, and Kcnj12, encoding for Kir2.2, are reported to contribute to I_{K1} [71]. Therefore, it was hypothesised, that the reduction in Kcnj2, Kcnj4 and Kcnj12 would reduce I_{K1} and in doing so, depolarise the RMP.

However, the data of this study indicates that I_{K1} is unaltered in Pitx2c$^{+/-}$ LA cardiomyocytes (figure 14). It would be interesting whether NppaCre$^+$Pitx2$^{-/-}$ LA cardiomyocytes, which have a reduced Kcnj2, Kcnj4 and Kcnj12 expression, show a reduced I_{K1}.

Next, I_{KACh}, an acetylcholine regulated inward rectifier K$^+$ current, was investigated (figure 17). Induction of I_{KACh} in pathology is reported to promote AF by shortening APD and hyperpolarising the atrial RMP [71, 72]. In CAF patients acetylcholine esterase activity was observed to be significantly reduced, leading to an indirect upregulation of I_{KACh} [37]. If I_{KACh} is constitutively active under physiological conditions (figure 21), then a reduction in I_{KACh} could explain a depolarised RMP in Pitx2c$^{+/}$. However, the findings in this study suggest that there is no genotype dependent-difference in I_{KACh} (figure 17).

I_{KACh} is enhanced in both genotypes at test potentials more negative than -80mV (around the E_K). At potentials above -60mV I_{KACh} was reduced, allowing fewer K$^+$ ions to leave the cardiomyocyte. Again, this would tend to prolong APD in Pitx2c$^{+/-}$ cardiomyocytes that have a more positive RMP.

Pitx2c$^{+/-}$ and NppaCre$^+$Pitx2$^{-/-}$ LA cardiomyocytes both have a depolarised RMP. It was also hypothesised that a change in background K$^+$ currents e.g. TASK channel currents could account for the depolarisation of RMP [32]. These channels are encoded by a number of genes, including Kcnk3 (TASK-1), kcnk5 (TASK-2) and kcnk9 (TASK-3). It is now known that Kcnk5 and TASK-2 expression is depleted in Pitx2c$^{+/-}$ LA tissue. Recently, it

was also shown, that the background TASK current is reduced in Pitx2c$^{+/-}$ LA cardiomyocytes [32]. In view of this, it is therefore proposed that the more positive RMP observed in heterozygous Pitx2c$^{+/-}$ LA cardiomyocytes is due to a decrease in TASK current rather than modifications in I_{K1} or I_{KACh}.

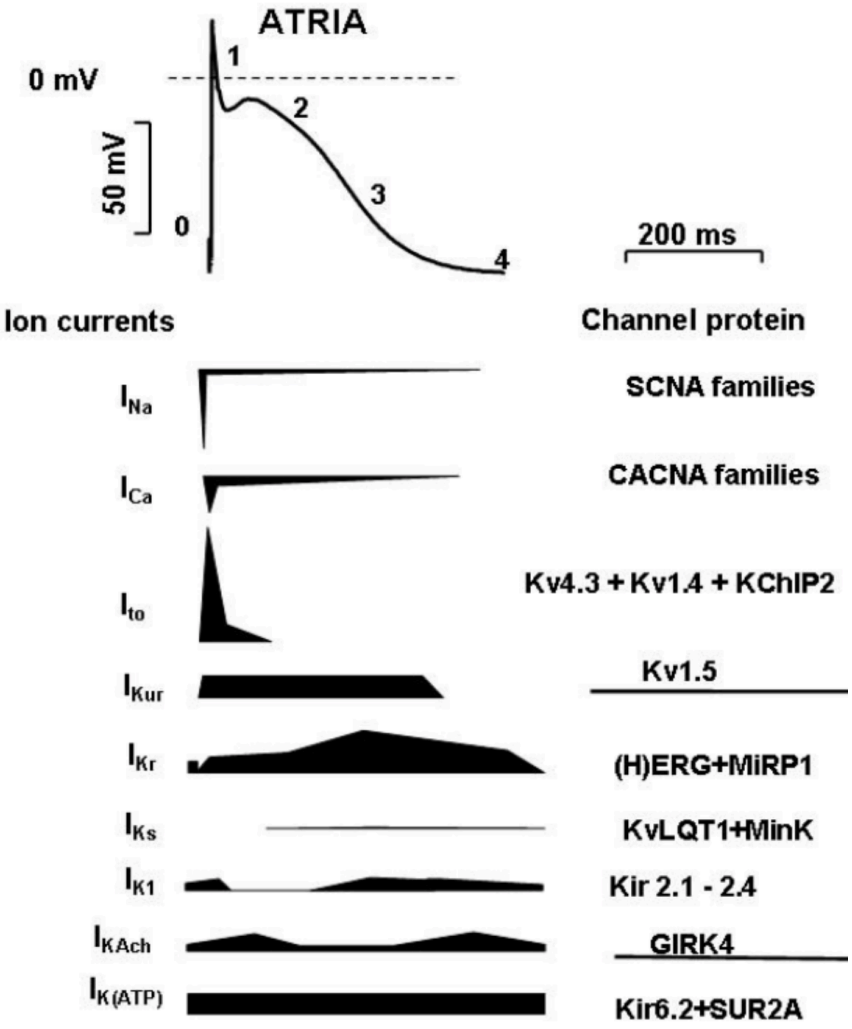


Figure 20: Currents contributing to an atrial action potential. Adapted from Jost, Norbert. (2010). Cardiac action potential and the underlying ion currents. 11-27.

5.8 Conclusion

The following mechanism is proposed for the enhanced efficacy of flecainide in Pitx2c$^{+/-}$ deficient LA: A reduction in Pitx2c causes a reduced expression of ion channels providing the background K^+ outward current, e.g. a decreased expression of Kcnk5. K^+ accumulates intracellularly and depolarises the RMP, which in turn reduces the amount of available Na^+ channels in rested state, leading to a smaller APA. This more positive RMP also enhances the sodium channel blocking action of flecainide, leading to greater prolongation of the ERP and enhanced protection against inducible atrial arrhythmia [32].

This finding could be a first step towards a personalised medicine in AF. If validated in humans, then PITX2c-deficient patients could be prescribed flecainide in preference to other AADs.

5.9 Study Limitations

Mice were used for the experiments. There is a difference between human and murine electrophysiology of the heart. Why the Pitx2c$^{+/-}$ mouse model was used, is described in the introduction. Still, it is not possible to adopt the results of other mouse models without a second thought, as Pitx2c is a transcription factor and regulates the expression of multiple genes, which might influence each other. It is recommended to investigate human LA cardiomyocytes with a lack of PITX2c. At the moment the only option of harvesting human LA cardiomyocytes is via surgery. As surgery on healthy individuals is unethical, there is currently very little control data available.

Additionally, patch-clamp experiments were conducted at room temperature. This was to reduce the magnitude of I_{Na} which allowed for more reliable voltage control. Conducting future experiments in physiological 37°C is recommended in order to measure action potentials in the current clamp configuration. Under these conditions, it is predicted that a more positive RMP, would increase the degree of V_{max} (dV dt^{-1} peak) inhibition caused by flecainide. This would be an important next step, either to be performed in AF LA cardiomyocytes with varying PITX2 expression or in atrial cardiac myocytes derived from human stem cells.

6. Bibliography:

1. Gudbjartsson DF, Arnar DO, Helgadottir A, Gretarsdottir S, Holm H, Sigurdsson A, Jonasdottir A, Baker A, Thorleifsson G, Kristjansson K, Palsson A, Blondal T, Sulem P, Backman VM, Hardarson GA, Palsdottir E, Helgason A, Sigurjonsdottir R, Sverrisson JT, Kostulas K, Ng MC, Baum L, So WY, Wong KS, Chan JC, Furie KL, Greenberg SM, Sale M, Kelly P, MacRae CA, Smith EE, Rosand J, Hillert J, Ma RC, Ellinor PT, Thorgeirsson G, Gulcher JR, Kong A, Thorsteinsdottir U, and Stefansson K, *Variants conferring risk of atrial fibrillation on chromosome 4q25.* Nature, 2007. **448**(7151): p. 353-7.
2. Fuster V, Ryden LE, Cannom DS, Crijns HJ, Curtis AB, Ellenbogen KA, Halperin JL, Le Heuzey JY, Kay GN, Lowe JE, Olsson SB, Prystowsky EN, Tamargo JL, Wann S, Smith SC, Jr., Jacobs AK, Adams CD, Anderson JL, Antman EM, Halperin JL, Hunt SA, Nishimura R, Ornato JP, Page RL, Riegel B, Priori SG, Blanc JJ, Budaj A, Camm AJ, Dean V, Deckers JW, Despres C, Dickstein K, Lekakis J, McGregor K, Metra M, Morais J, Osterspey A, Tamargo JL, Zamorano JL, American College of Cardiology/American Heart Association Task Force on Practice G, European Society of Cardiology Committee for Practice G, European Heart Rhythm A, and Heart Rhythm S, *ACC/AHA/ESC 2006 Guidelines for the Management of Patients with Atrial Fibrillation: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines and the European Society of Cardiology Committee for Practice Guidelines (Writing Committee to Revise the 2001 Guidelines for the Management of Patients With Atrial Fibrillation): developed in collaboration with the European Heart Rhythm Association and the Heart Rhythm Society.* Circulation, 2006. **114**(7): p. e257-354.
3. Go AS, Hylek EM, Phillips KA, Chang Y, Henault LE, Selby JV, and Singer DE, *Prevalence of diagnosed atrial fibrillation in adults: national implications for rhythm management and stroke prevention: the AnTicoagulation and Risk Factors in Atrial Fibrillation (ATRIA) Study.* JAMA, 2001. **285**(18): p. 2370-5.
4. Stewart S, Hart CL, Hole DJ, and McMurray JJ, *A population-based study of the long-term risks associated with atrial fibrillation: 20-year follow-up of the Renfrew/Paisley study.* Am J Med, 2002. **113**(5): p. 359-64.
5. Hart RG and Halperin JL, *Atrial fibrillation and stroke : concepts and controversies.* Stroke, 2001. **32**(3): p. 803-8.

6. Nattel S, *New ideas about atrial fibrillation 50 years on.* Nature, 2002. **415**(6868): p. 219-26.
7. Fox CS, Parise H, D'Agostino RB, Sr., Lloyd-Jones DM, Vasan RS, Wang TJ, Levy D, Wolf PA, and Benjamin EJ, *Parental atrial fibrillation as a risk factor for atrial fibrillation in offspring.* JAMA, 2004. **291**(23): p. 2851-5.
8. Chinchilla A, Daimi H, Lozano-Velasco E, Dominguez JN, Caballero R, Delpon E, Tamargo J, Cinca J, Hove-Madsen L, Aranega AE, and Franco D, *PITX2 insufficiency leads to atrial electrical and structural remodeling linked to arrhythmogenesis.* Circ Cardiovasc Genet, 2011. **4**(3): p. 269-79.
9. Paludan-Muller C, Svendsen JH, and Olesen MS, *The role of common genetic variants in atrial fibrillation.* J Electrocardiol, 2016.
10. Roselli C, Chaffin MD, Weng LC, Aeschbacher S, Ahlberg G, Albert CM, Almgren P, Alonso A, Anderson CD, Aragam KG, Arking DE, Barnard J, Bartz TM, Benjamin EJ, Bihlmeyer NA, Bis JC, Bloom HL, Boerwinkle E, Bottinger EB, Brody JA, Calkins H, Campbell A, Cappola TP, Carlquist J, Chasman DI, Chen LY, Chen YI, Choi EK, Choi SH, Christophersen IE, Chung MK, Cole JW, Conen D, Cook J, Crijns HJ, Cutler MJ, Damrauer SM, Daniels BR, Darbar D, Delgado G, Denny JC, Dichgans M, Dorr M, Dudink EA, Dudley SC, Esa N, Esko T, Eskola M, Fatkin D, Felix SB, Ford I, Franco OH, Geelhoed B, Grewal RP, Gudnason V, Guo X, Gupta N, Gustafsson S, Gutmann R, Hamsten A, Harris TB, Hayward C, Heckbert SR, Hernesniemi J, Hocking LJ, Hofman A, Horimoto A, Huang J, Huang PL, Huffman J, Ingelsson E, Ipek EG, Ito K, Jimenez-Conde J, Johnson R, Jukema JW, Kaab S, Kahonen M, Kamatani Y, Kane JP, Kastrati A, Kathiresan S, Katschnig-Winter P, Kavousi M, Kessler T, Kietselaer BL, Kirchhof P, Kleber ME, Knight S, Krieger JE, Kubo M, Launer LJ, Laurikka J, Lehtimaki T, Leineweber K, Lemaitre RN, Li M, Lim HE, Lin HJ, Lin H, Lind L, Lindgren CM, Lokki ML, London B, Loos RJF, Low SK, Lu Y, Lyytikainen LP, Macfarlane PW, Magnusson PK, Mahajan A, Malik R, Mansur AJ, Marcus GM, Margolin L, Margulies KB, Marz W, McManus DD, Melander O, Mohanty S, Montgomery JA, Morley MP, Morris AP, Muller-Nurasyid M, Natale A, Nazarian S, Neumann B, Newton-Cheh C, Niemeijer MN, Nikus K, Nilsson P, Noordam R, Oellers H, Olesen MS, Orho-Melander M, Padmanabhan S, Pak HN, Pare G, Pedersen NL, Pera J, Pereira A, Porteous D, Psaty BM, Pulit SL, Pullinger CR, Rader DJ, Refsgaard L, Ribases M, Ridker PM, Rienstra M, Risch L, Roden DM, Rosand J, Rosenberg MA, Rost N, Rotter JI, Saba S, Sandhu

RK, Schnabel RB, Schramm K, Schunkert H, Schurman C, Scott SA, Seppala I, Shaffer C, Shah S, Shalaby AA, Shim J, Shoemaker MB, Siland JE, Sinisalo J, Sinner MF, Slowik A, Smith AV, Smith BH, Smith JG, Smith JD, Smith NL, Soliman EZ, Sotoodehnia N, Stricker BH, Sun A, Sun H, Svendsen JH, Tanaka T, Tanriverdi K, Taylor KD, Teder-Laving M, Teumer A, Theriault S, Trompet S, Tucker NR, Tveit A, Uitterlinden AG, Van Der Harst P, Van Gelder IC, Van Wagoner DR, Verweij N, Vlachopoulou E, Volker U, Wang B, Weeke PE, Weijs B, Weiss R, Weiss S, Wells QS, Wiggins KL, Wong JA, Woo D, Worrall BB, Yang PS, Yao J, Yoneda ZT, Zeller T, Zeng L, Lubitz SA, Lunetta KL and Ellinor PT, *Multi-ethnic genome-wide association study for atrial fibrillation.* Nat Genet, 2018.

11. Mohanty S, Santangeli P, Bai R, Di Biase L, Mohanty P, Pump A, and Natale A, *Variant rs2200733 on chromosome 4q25 confers increased risk of atrial fibrillation: evidence from a meta-analysis.* J Cardiovasc Electrophysiol, 2013. **24**(2): p. 155-61.
12. Gudbjartsson DF, Arnar DO, Helgadottir A, Gretarsdottir S, Holm H, Sigurdsson A, Jonasdottir A, Baker A, Thorleifsson G, Kristjansson K, Palsson A, Blondal T, Sulem P, Backman VM, Hardarson GA, Palsdottir E, Helgason A, Sigurjonsdottir R, Sverrisson JT, Kostulas K, Ng MCY, Baum L, So WY, Wong KS, Chan JCN, Furie KL, Greenberg SM, Sale M, Kelly P, MacRae CA, Smith EE, Rosand J, Hillert J, Ma RCW, Ellinor PT, Thorgeirsson G, Gulcher JR, Kong A, Thorsteinsdottir U, and Stefansson K, *Variants conferring risk of atrial fibrillation on chromosome 4q25.* Nature, 2007. **448**(7151): p. 353-U9.
13. Parvez B, Shoemaker MB, Muhammad R, Richardson R, Jiang L, Blair MA, Roden DM, and Darbar D, *Common genetic polymorphism at 4q25 locus predicts atrial fibrillation recurrence after successful cardioversion.* Heart Rhythm, 2013. **10**(6): p. 849-55.
14. Shoemaker MB, Bollmann A, Lubitz SA, Ueberham L, Saini H, Montgomery J, Edwards T, Yoneda Z, Sinner MF, Arya A, Sommer P, Delaney J, Goyal SK, Saavedra P, Kanagasundram A, Whalen SP, Roden DM, Hindricks G, Ellis CR, Ellinor PT, Darbar D, and Husser D, *Common genetic variants and response to atrial fibrillation ablation.* Circ Arrhythm Electrophysiol, 2015. **8**(2): p. 296-302.
15. Ye J, Tucker NR, Weng LC, Clauss S, Lubitz SA, and Ellinor PT, *A Functional Variant Associated with Atrial Fibrillation Regulates PITX2c Expression through TFAP2a.* Am J Hum Genet, 2016. **99**(6): p. 1281-1291.

16. Gore-Panter SR, Hsu J, Hanna P, Gillinov AM, Pettersson G, Newton DW, Moravec CS, Van Wagoner DR, Chung MK, Barnard J, and Smith JD, *Atrial Fibrillation associated chromosome 4q25 variants are not associated with PITX2c expression in human adult left atrial appendages.* PLoS One, 2014. **9**(1): p. e86245.
17. Perez-Hernandez M, Matamoros M, Barana A, Amoros I, Gomez R, Nunez M, Sacristan S, Pinto A, Fernandez-Aviles F, Tamargo J, Delpon E, and Caballero R, *Pitx2c increases in atrial myocytes from chronic atrial fibrillation patients enhancing IKs and decreasing ICa,L.* Cardiovasc Res, 2016. **109**(3): p. 431-41.
18. Parvez B, Vaglio J, Rowan S, Muhammad R, Kucera G, Stubblefield T, Carter S, Roden D, and Darbar D, *Symptomatic response to antiarrhythmic drug therapy is modulated by a common single nucleotide polymorphism in atrial fibrillation.* J Am Coll Cardiol, 2012. **60**(6): p. 539-45.
19. Parvez B, Chopra N, Rowan S, Vaglio JC, Muhammad R, Roden DM, and Darbar D, *A common beta1-adrenergic receptor polymorphism predicts favorable response to rate-control therapy in atrial fibrillation.* J Am Coll Cardiol, 2012. **59**(1): p. 49-56.
20. Lu MF, Pressman C, Dyer R, Johnson RL, and Martin JF, *Function of Rieger syndrome gene in left-right asymmetry and craniofacial development.* Nature, 1999. **401**(6750): p. 276-8.
21. Campione M, Steinbeisser H, Schweickert A, Deissler K, van Bebber F, Lowe LA, Nowotschin S, Viebahn C, Haffter P, Kuehn MR, and Blum M, *The homeobox gene Pitx2: mediator of asymmetric left-right signaling in vertebrate heart and gut looping.* Development, 1999. **126**(6): p. 1225-34.
22. Nakamura T and Hamada H, *Left-right patterning: conserved and divergent mechanisms.* Development, 2012. **139**(18): p. 3257-62.
23. Gage PJ, Suh H, and Camper SA, *Dosage requirement of Pitx2 for development of multiple organs.* Development, 1999. **126**(20): p. 4643-51.
24. Schweickert A, Campione M, Steinbeisser H, and Blum M, *Pitx2 isoforms: involvement of Pitx2c but not Pitx2a or Pitx2b in vertebrate left-right asymmetry.* Mech Dev, 2000. **90**(1): p. 41-51.
25. Kitamura K, Miura H, Miyagawa-Tomita S, Yanazawa M, Katoh-Fukui Y, Suzuki R, Ohuchi H, Suehiro A, Motegi Y, Nakahara Y, Kondo S, and Yokoyama M, *Mouse Pitx2 deficiency leads to anomalies of the ventral body wall, heart, extra-*

and periocular mesoderm and right pulmonary isomerism. Development, 1999. **126**(24): p. 5749-58.

26. Mommersteeg MT, Hoogaars WM, Prall OW, de Gier-de Vries C, Wiese C, Clout DE, Papaioannou VE, Brown NA, Harvey RP, Moorman AF, and Christoffels VM, *Molecular pathway for the localized formation of the sinoatrial node.* Circ Res, 2007. **100**(3): p. 354-62.
27. Kirchhof P, Kahr PC, Kaese S, Piccini I, Vokshi I, Scheld HH, Rotering H, Fortmueller L, Laakmann S, Verheule S, Schotten U, Fabritz L, and Brown NA, *PITX2c is expressed in the adult left atrium, and reducing Pitx2c expression promotes atrial fibrillation inducibility and complex changes in gene expression.* Circ Cardiovasc Genet, 2011. **4**(2): p. 123-33.
28. Kahr PC, Piccini I, Fabritz L, Greber B, Scholer H, Scheld HH, Hoffmeier A, Brown NA, and Kirchhof P, *Systematic analysis of gene expression differences between left and right atria in different mouse strains and in human atrial tissue.* PLoS One, 2011. **6**(10): p. e26389.
29. Li G, Liu X, Xing C, Zhang H, Shimeld SM, and Wang Y, *Cerberus-Nodal-Lefty-Pitx signaling cascade controls left-right asymmetry in amphioxus.* Proc Natl Acad Sci U S A, 2017. **114**(14): p. 3684-3689.
30. Tao G, Kahr PC, Morikawa Y, Zhang M, Rahmani M, Heallen TR, Li L, Sun Z, Olson EN, Amendt BA, and Martin JF, *Pitx2 promotes heart repair by activating the antioxidant response after cardiac injury.* Nature, 2016. **534**(7605): p. 119-23.
31. Nadadur RD, Broman MT, Boukens B, Mazurek SR, Yang X, van den Boogaard M, Bekeny J, Gadek M, Ward T, Zhang M, Qiao Y, Martin JF, Seidman CE, Seidman J, Christoffels V, Efimov IR, McNally EM, Weber CR, and Moskowitz IP, *Pitx2 modulates a Tbx5-dependent gene regulatory network to maintain atrial rhythm.* Sci Transl Med, 2016. **8**(354): p. 354ra115.
32. Syeda F, Holmes AP, Yu TY, Tull S, Kuhlmann SM, Pavlovic D, Betney D, Riley G, Kucera JP, Jousset F, de Groot JR, Rohr S, Brown NA, Fabritz L, and Kirchhof P, *PITX2 Modulates Atrial Membrane Potential and the Antiarrhythmic Effects of Sodium-Channel Blockers.* J Am Coll Cardiol, 2016. **68**(17): p. 1881-1894.
33. Lozano-Velasco E, Hernandez-Torres F, Daimi H, Serra SA, Herraiz A, Hove-Madsen L, Aranega A, and Franco D, *Pitx2 impairs calcium handling in a dose-dependent manner by modulating Wnt signalling.* Cardiovasc Res, 2016. **109**(1): p. 55-66.

34. Tao Y, Zhang M, Li L, Bai Y, Zhou Y, Moon AM, Kaminski HJ, and Martin JF, *Pitx2, an atrial fibrillation predisposition gene, directly regulates ion transport and intercalated disc genes.* Circ Cardiovasc Genet, 2014. **7**(1): p. 23-32.
35. Syeda F, Kirchhof P, and Fabritz L, *PITX2-dependent gene regulation in atrial fibrillation and rhythm control.* J Physiol, 2017. **595**(12): p. 4019-4026.
36. Wakili R, Voigt N, Kaab S, Dobrev D, and Nattel S, *Recent advances in the molecular pathophysiology of atrial fibrillation.* J Clin Invest, 2011. **121**(8): p. 2955-68.
37. Dobrev D and Ravens U, *Remodeling of cardiomyocyte ion channels in human atrial fibrillation.* Basic Res Cardiol, 2003. **98**(3): p. 137-48.
38. Langenbacher A and Chen JN, *Calcium signaling: a common thread in vertebrate left-right axis development.* Dev Dyn, 2008. **237**(12): p. 3491-6.
39. McCloskey C, Jones S, Amisten S, Snowden RT, Kaczmarek LK, Erlinge D, Goodall AH, Forsythe ID, and Mahaut-Smith MP, *Kv1.3 is the exclusive voltage-gated K+ channel of platelets and megakaryocytes: roles in membrane potential, Ca2+ signalling and platelet count.* J Physiol, 2010. **588**(Pt 9): p. 1399-406.
40. Ordog B, Brutyo E, Puskas LG, Papp JG, Varro A, Szabad J, and Boldogkoi Z, *Gene expression profiling of human cardiac potassium and sodium channels.* Int J Cardiol, 2006. **111**(3): p. 386-93.
41. Manabe I, Tsuboi M, Ahmmed GU, Sasaki N, Ohtahara A, Yamamoto Y, Hiroe K, Yoshida A, Hisatome I, and Shigemasa C, *Expression of Shaker-type voltage-gated potassium channel genes in the guinea-pig.* Res Commun Mol Pathol Pharmacol, 1998. **99**(1): p. 33-40.
42. Marionneau C, Couette B, Liu J, Li H, Mangoni ME, Nargeot J, Lei M, Escande D, and Demolombe S, *Specific pattern of ionic channel gene expression associated with pacemaker activity in the mouse heart.* J Physiol, 2005. **562**(Pt 1): p. 223-34.
43. Liu C, Liu W, Palie J, Lu MF, Brown NA, and Martin JF, *Pitx2c patterns anterior myocardium and aortic arch vessels and is required for local cell movement into atrioventricular cushions.* Development, 2002. **129**(21): p. 5081-91.
44. DiFrancesco D, Ferroni A, and Visentin S, *Barium-induced blockade of the inward rectifier in calf Purkinje fibres.* Pflugers Arch, 1984. **402**(4): p. 446-53.
45. Holmes AP, Yu TY, Tull S, Syeda F, Kuhlmann SM, O'Brien SM, Patel P, Brain KL, Pavlovic D, Brown NA, Fabritz L, and Kirchhof P, *A Regional Reduction in Ito and IKACh in the Murine Posterior Left Atrial Myocardium Is Associated with Action*

Potential Prolongation and Increased Ectopic Activity. PLoS One, 2016. **11**(5): p. e0154077.

46. Yu TY, Syeda F, Holmes AP, Osborne B, Dehghani H, Brain KL, Kirchhof P, and Fabritz L, *An automated system using spatial oversampling for optical mapping in murine atria. Development and validation with monophasic and transmembrane action potentials.* Prog Biophys Mol Biol, 2014. **115**(2-3): p. 340-8.
47. Hodgkin AL and Huxley AF, *A quantitative description of membrane current and its application to conduction and excitation in nerve.* J Physiol, 1952. **117**(4): p. 500-44.
48. Anno T and Hondeghem LM, *Interactions of flecainide with guinea pig cardiac sodium channels. Importance of activation unblocking to the voltage dependence of recovery.* Circ Res, 1990. **66**(3): p. 789-803.
49. Liu H, Atkins J, and Kass RS, *Common molecular determinants of flecainide and lidocaine block of heart Na+ channels: evidence from experiments with neutral and quaternary flecainide analogues.* J Gen Physiol, 2003. **121**(3): p. 199-214.
50. Burashnikov A, Belardinelli L, and Antzelevitch C, *Atrial-selective sodium channel block strategy to suppress atrial fibrillation: ranolazine versus propafenone.* J Pharmacol Exp Ther, 2012. **340**(1): p. 161-8.
51. Syed TM and Halperin JL, *Left atrial appendage closure for stroke prevention in atrial fibrillation: state of the art and current challenges.* Nat Clin Pract Cardiovasc Med, 2007. **4**(8): p. 428-35.
52. Bartos DC, Grandi E, and Ripplinger CM, *Ion Channels in the Heart.* Compr Physiol, 2015. **5**(3): p. 1423-64.
53. Van Wagoner DR, Pond AL, Lamorgese M, Rossie SS, McCarthy PM, and Nerbonne JM, *Atrial L-type Ca2+ currents and human atrial fibrillation.* Circ Res, 1999. **85**(5): p. 428-36.
54. Christ T, Boknik P, Wohrl S, Wettwer E, Graf EM, Bosch RF, Knaut M, Schmitz W, Ravens U, and Dobrev D, *L-type Ca2+ current downregulation in chronic human atrial fibrillation is associated with increased activity of protein phosphatases.* Circulation, 2004. **110**(17): p. 2651-7.
55. Brundel BJ, van Gelder IC, Henning RH, Tuinenburg AE, Deelman LE, Tieleman RG, Grandjean JG, van Gilst WH, and Crijns HJ, *Gene expression of proteins influencing the calcium homeostasis in patients with persistent and paroxysmal atrial fibrillation.* Cardiovasc Res, 1999. **42**(2): p. 443-54.

56. Bannister ML, Thomas NL, Sikkel MB, Mukherjee S, Maxwell C, MacLeod KT, George CH, and Williams AJ, *The mechanism of flecainide action in CPVT does not involve a direct effect on RyR2.* Circ Res, 2015. **116**(8): p. 1324-35.
57. Sikkel MB, Collins TP, Rowlands C, Shah M, O'Gara P, Williams AJ, Harding SE, Lyon AR, and MacLeod KT, *Flecainide reduces Ca(2+) spark and wave frequency via inhibition of the sarcolemmal sodium current.* Cardiovasc Res, 2013. **98**(2): p. 286-96.
58. Sikkel MB, Collins TP, Rowlands C, Shah M, O'Gara P, Williams AJ, Harding SE, Lyon AR, and MacLeod KT, *Triple mode of action of flecainide in catecholaminergic polymorphic ventricular tachycardia: reply.* Cardiovasc Res, 2013. **98**(2): p. 327-8.
59. Steele DS, Hwang HS, and Knollmann BC, *Triple mode of action of flecainide in catecholaminergic polymorphic ventricular tachycardia.* Cardiovasc Res, 2013. **98**(2): p. 326-7.
60. Watanabe H, Chopra N, Laver D, Hwang HS, Davies SS, Roach DE, Duff HJ, Roden DM, Wilde AA, and Knollmann BC, *Flecainide prevents catecholaminergic polymorphic ventricular tachycardia in mice and humans.* Nat Med, 2009. **15**(4): p. 380-3.
61. Liu N, Denegri M, Ruan Y, Avelino-Cruz JE, Perissi A, Negri S, Napolitano C, Coetzee WA, Boyden PA, and Priori SG, *Short communication: flecainide exerts an antiarrhythmic effect in a mouse model of catecholaminergic polymorphic ventricular tachycardia by increasing the threshold for triggered activity.* Circ Res, 2011. **109**(3): p. 291-5.
62. Chandy KG, Wulff H, Beeton C, Pennington M, Gutman GA, and Cahalan MD, *K+ channels as targets for specific immunomodulation.* Trends Pharmacol Sci, 2004. **25**(5): p. 280-9.
63. Riazanski V, Becker A, Chen J, Sochivko D, Lie A, Wiestler OD, Elger CE, and Beck H, *Functional and molecular analysis of transient voltage-dependent K+ currents in rat hippocampal granule cells.* J Physiol, 2001. **537**(Pt 2): p. 391-406.
64. Peroz D, Rodriguez N, Choveau F, Baro I, Merot J, and Loussouarn G, *Kv7.1 (KCNQ1) properties and channelopathies.* J Physiol, 2008. **586**(7): p. 1785-9.
65. O'Neal WT, Efird JT, Kamel H, Nazarian S, Alonso A, Heckbert SR, Longstreth WT, Jr., and Soliman EZ, *The association of the QT interval with atrial fibrillation*

and stroke: the Multi-Ethnic Study of Atherosclerosis. Clin Res Cardiol, 2015. **104**(9): p. 743-50.

66. Osteen JD, Sampson KJ, and Kass RS, *The cardiac IKs channel, complex indeed.* Proc Natl Acad Sci U S A, 2010. **107**(44): p. 18751-2.
67. Li N, Dobrev D, and Wehrens XH, *PITX2: a master regulator of cardiac channelopathy in atrial fibrillation?* Cardiovasc Res, 2016. **109**(3): p. 345-7.
68. Abdelhadi O, Iancu D, Stanescu H, Kleta R, and Bockenhauer D, *EAST syndrome: Clinical, pathophysiological, and genetic aspects of mutations in KCNJ10.* Rare Dis, 2016. **4**(1): p. e1195043.
69. Proverbio MC, Mangano E, Gessi A, Bordoni R, Spinelli R, Asselta R, Valin PS, Di Candia S, Zamproni I, Diceglie C, Mora S, Caruso-Nicoletti M, Salvatoni A, De Bellis G, and Battaglia C, *Whole genome SNP genotyping and exome sequencing reveal novel genetic variants and putative causative genes in congenital hyperinsulinism.* PLoS One, 2013. **8**(7): p. e68740.
70. Ibarra J, Morley GE, and Delmar M, *Dynamics of the inward rectifier K+ current during the action potential of guinea pig ventricular myocytes.* Biophys J, 1991. **60**(6): p. 1534-9.
71. Ehrlich JR, *Inward rectifier potassium currents as a target for atrial fibrillation therapy.* J Cardiovasc Pharmacol, 2008. **52**(2): p. 129-35.
72. Liu L and Nattel S, *Differing sympathetic and vagal effects on atrial fibrillation in dogs: role of refractoriness heterogeneity.* Am J Physiol, 1997. **273**(2 Pt 2): p. H805-16.

Eidesstattliche Versicherung

„Ich, Stefan Michael Kuhlmann, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Defining the molecular cause of an altered response to anti-arrhythmic drugs in Pitx2-dependent atrial fibrillation“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
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Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der obenstehenden gemeinsamen Erklärung mit dem Betreuer angegeben sind (siehe Preamble). Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s. o.) und werden von mir verantwortet.
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Datum Unterschrift

Anteilserklärung

Stefan Michael Kuhlmann hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: Syeda F, Holmes AP, Yu TY, Tull S, Kuhlman SM, Pavlovic D, Betney D, Riley G, Kucera JP, Jousset F, de Groot JR, Rohr S, Brown NA, Fabritz L, and Kirchhof P, PITX2 Modulates Atrial Membrane Potential and the Antiarrhythmic Effects of Sodium-Channel Blockers. J Am Coll Cardiol, 2016. 68(17): p. 1881-1894.
Contribution of Stefan Michael Kuhlmann:
Stefan Michael Kuhlmann designed, performed and analysed patch-clamp and high-resolution optical mapping experiments. He measured I_{Na} and I_{K1} current in left atrial cardiomyocytes in wildtype and Pitx2c deficient mice and in human endothelial kidney cells by patch-clamp technique and Ca^{2+} transients using the high-resolution optical mapping setup and contributed to writing the paper. His data are published in figure 5, figure 6 A and B.

Publikation 2: Holmes AP, Yu TY, Tull S, Syeda F, Kuhlmann SM, O'Brien SM, Patel P, Brain KL, Pavlovic D, Brown NA, Fabritz L, and Kirchhof P, A Regional Reduction in Ito and IKACh in the Murine Posterior Left Atrial Myocardium Is Associated with Action Potential Prolongation and Increased Ectopic Activity. PLoS One, 2016. 11(5): p. e0154077.
Contribution of Stefan Michael Kuhlmann:
Stefan Michael Kuhlmann contributed to the performance and analysis of patch-clamp and high-resolution optical mapping experiments. He contributed to measurement and analysis of K^+ currents I_{Kpeak}, I_{to}, $I_{Ksteadystate}$, I_{K1} and I_{KACh} in wild-type mice, analysed action potential duration using the high-resolution optical mapping setup and contributed to writing the paper. He analysed data for figure 4, and contributed to figure 6 and 7.

Unterschrift, Datum und Stempel des betreuenden Hochschullehrers	Unterschrift des Doktoranden

Lebenslauf Stefan Michael Kuhlmann

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Komplette Publikationsliste

1. Syeda F, Holmes AP, Yu TY, Tull S, Kuhlman SM, Pavlovic D, Betney D, Riley G, Kucera JP, Jousset F, de Groot JR, Rohr S, Brown NA, Fabritz L, and Kirchhof P, PITX2 Modulates Atrial Membrane Potential and the Antiarrhythmic Effects of Sodium-Channel Blockers. J Am Coll Cardiol, 2016. 68(17): p. 1881-1894.

2. Holmes AP, Yu TY, Tull S, Syeda F, Kuhlmann SM, O'Brien SM, Patel P, Brain KL, Pav-lovic D, Brown NA, Fabritz L, and Kirchhof P, A Regional Reduction in Ito and IKACh in the Murine Posterior Left Atrial Myocardium Is Associated with Action Potential Prolongation and Increased Ectopic Activity. PLoS One, 2016. 11(5): p. e0154077.

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