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Pitx2c deficiency confers cellular electrophysiological hallmarks of atrial fibrillation to isolated atrial myocytes

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ABSTRACT

Aims: Atrial fibrillation (AF) has been associated with altered expression of the transcription factor Pitx2c and a high incidence of calcium release-induced afterdepolarizations. However, the relationship between Pitx2c expression and defective calcium homeostasis remains unclear and we here aimed to determine how Pitx2c expression affects calcium release from the sarcoplasmic reticulum (SR) and its impact on electrical activity in isolated atrial myocytes.

Methods: To address this issue, we applied confocal calcium imaging and patch-clamp techniques to atrial myocytes isolated from a mouse model with conditional atrial-specific deletion of Pitx2c.

Results: Our findings demonstrate that heterozygous deletion of Pitx2c doubles the calcium spark frequency, increases the frequency of sparks/site 1.5-fold, the calcium spark decay constant from 36 to 42 ms and the wave frequency from none to 3.2 min^{-1}. Additionally, the cell capacitance increased by 30% and both the SR calcium load and the transient inward current (I_{TI}) frequency were doubled. Furthermore, the fraction of cells with spontaneous action potentials increased from none to 44%. These effects of Pitx2c deficiency were comparable in right and left atrial myocytes, and homozygous deletion of Pitx2c did not induce any further effects on sparks, SR calcium load, I_{TI} frequency or spontaneous action potentials.

Conclusion: Our findings demonstrate that heterozygous Pitx2c deletion induces defects in calcium homeostasis and electrical activity that mimic derangements observed in right atrial myocytes from patients with AF and suggest that Pitx2c deficiency confers cellular electrophysiological hallmarks of AF to isolated atrial myocytes.

1. Introduction

A number of electrophysiological, molecular, and structural alterations take place in the fibrillating atria that favor the maintenance and self-perpetuation of the arrhythmia [1,2]. The electrophysiological alterations include defective calcium homeostasis [3–6] and sarcoplasmic reticulum (SR) [3,4,6,7]. Thus, patients with AF have a high incidence of spontaneous SR calcium release [3]. This, in turn, has been associated with deficient phosphodiesterase [8] or phosphatase activity [9], causing excessive protein kinase A [4,10] or calmodulin kinase type II-dependent phosphorylation of the cardiac ryanodine receptor (RyR2) [7,11,12]. Moreover, spontaneous calcium release can induce afterdepolarizations in patients with AF [7], and occur at rest or in electrically stimulated human atrial myocytes [13,14]. In recent years, genome-wide association studies have identified a large number of single nucleotide polymorphisms (SNP)s that are associated with an increase in the risk of AF [15–17], and that could contribute to the derangements in calcium homeostasis observed in AF. Among these variants, those located in the 4q25 region remain among the most significant [18], and because of their location near the locus of the bicoid-related homeodomain transcription factor Pitx2, it was suggested that alterations in Pitx2c expression or function could be a molecular link

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between risk SNPs at 4q25 and AF [15]. However, a number of studies that examined Pitx2 expression in patients with 4q25 risk variants [19,20] or with AF [20–22], failed to find a clear correlation between risk SNPs or AF and Pitx2c expression. Indeed, some studies report a reduction in Pitx2c levels in patients with AF [22] while others report an increase in Pitx2c expression [21]. Moreover, the latter study shows that the L-type calcium current (I_{Ca}) density is inversely correlated with Pitx2c expression while atrial-specific deletion of Pitx2c in a mouse model decreases Pitx2c mRNA expression in the left atria and reduces I_{Ca} density in left atrial myocytes [23]. On the other hand, we recently showed that the 4q25 risk SNP rs13143308T increases the incidence of spontaneous calcium release events and afterdepolarizations in human atrial myocytes from patients without AF to levels observed in patients with AF without affecting the I_{Ca} density [24]. Moreover, one study did show that another 4q25 risk SNP rs259510 reduces Pitx2c expression [25]. However, the rs259510 and rs13143308 SNPs are not genetically linked. Therefore, to establish how Pitx2c deficiency affects key mechanisms regulating intracellular calcium levels and electrical activity, we performed confocal calcium imaging and patch-clamp analysis in atrial myocytes from a mouse model with conditional atrial specific hetero- or homozygous deletion of Pitx2c. This model has previously been shown to present pathological features related to AF such as atrial dilation, depolarization of the resting membrane potential, missing p-waves in the ECG and characteristics of atrioventricular block [22]. Moreover, because Pitx2c expression is 100-fold higher in the left than in the right atrium and because functional electrophysiological studies in human atrial myocytes are primarily performed in right atrial samples, we also assessed if there were functional differences among left and right atrial myocytes from WT and Pitx2c deficient mice.

2. Methods

2.1. Transgenic mouse model and myocyte isolation

The Pitx2 floxed and NppaCre transgenic mouse lines have been described previously [22,23]. Wild-type Cre- controls (NppaCre-Pitx2 +/+) and atrial-specific heterozygous (NppaCre+Pitx2 +/-) or homozygous (NppaCre+Pitx2-/-) mice were used in this study. NppaCre+Pitx2 + /- display a single copy of Pitx2 while on NppaCre+Pitx2-/- mice both Pitx2 alleles have been deleted on the NppaCre expression domain. Right and left atrial myocytes were isolated from 3 to 6 months-old mice. Briefly, mice were anesthetized (I.P. medetomidine 1 mg/kg and ketamine 75 mg/kg) and heparinized (I.P. 25 IU/ml). Twenty minutes later, after cervical dislocation, the heart was quickly excised and arrested in ice-cold Ca2 + -free Tyrode solution. Hearts were then Langendorff perfused with a Ca^{2+}-free Tyrode solution containing 0.5 mg/ml collagenase type II (Bohringer), 0.1 mg/ml proteinase (Sigma-Aldrich) and 0.5 mg/ml fatty acid-free BSA (Sigma-Aldrich) during approximately 8 min. Afterwards, the left and right atrial chambers were dissected and cut into small pieces, washed twice in Ca^{2+}-free Tyrode solution by gentle agitation, and transferred back into a fresh enzymatic solution for approximately 8 min. This process was repeated several times until an adequate number of elongated atrial myocytes with clear striations had been released. Subsequently, cells were pooled for the right and the left atrium, centrifuged for 5 min at 500 rpm, resuspended in Tyrode solution, and the [Ca^{2+}] was gradually increased to 1 mM. The experimentation with mouse atrial myocytes conforms to the Guide for the Care and Use of Laboratory Animals, and was approved by the Bioethical Committee at Hospital de Santa Creu i Sant Pau.

2.2. Patch-clamp technique

I_{Ca} was elicited by a 200 ms depolarization to 0 mV followed by repolarization to − 80 mV, and spontaneous I_{TI} currents were measured at holding potentials of − 80 or − 50 mV using whole cell voltage-clamp in the perforated patch configuration with a HEKA EPC-10 amplifier (HEKA Elektronik Dr. Schultze, Lambrecht/Pfalz, Germany). Na-currents were eliminated by a 50 ms depolarization from a holding potential of − 80 mV to − 50 mV prior to activation of I_{Ca}. Series resistance compensation was not performed. The extracellular solution contained (in mM): NaCl 127, TEA 5, HEPES 10, $NaHCO_3$ 4, NaH_2PO_4 0.33, glucose 10, pyruvic acid 5, $CaCl_2$ 2, $MgCl_2$ 1.8, (pH = 7.4). The pipette solution contained (in mM): aspartic acid 109, CsCl 47, Mg_2ATP 3, $MgCl_2$ 1, Na2phosphocreatine 5, Li_2GTP 0.42, HEPES 10 (pH = 7.2 with CsOH) and 250 μg/ml amphotericin B. Experiments were done at room temperature and began when the series resistance did not decrease further, and cells were discarded if it was larger than 6 times the pipette resistance. The I_{Ca} amplitude was determined as the difference between the peak inward current and the current at the end of the depolarization.

The SR calcium load was determined from the charge carried by the current elicited by rapid exposure to 10 mM caffeine for 5–10 s. The charge was converted to amoles (10^{-18} mol) of calcium, assuming $3Na^+$ is exchanged for $1Ca^{2+}$, and normalized to the cell capacitance. Erroneous measurement(s) were eliminated, giving rise to variations in the total number of experiments for different currents. Most of these cases occurred because exposure of myocytes to caffeine produced a strong contraction that increased the leakage current or caused irreversible cell contracture.

Membrane potentials were measured in the current-clamp configuration using K^+-containing intra and extracellular media. The external medium contained (in mM): NaCl 136, KCl 4, NaH_2PO_4 0.33, $NaHCO_3$ 4, $CaCl_2$ 2, $MgCl_2$ 1.6, HEPES 10, Glucose 5, pyruvic acid 5, (pH = 7.4). The internal medium contained (in mM): aspartic acid 109, KCl 47, Mg_2ATP 3, $MgCl_2$ 1, Na_2phosphocreatine 5, Li_2GTP 0.42, HEPES 10 (pH = 7.2 with KOH) and 250 μg/ml amphotericin B. The holding current was varied in order to assess the amplitude and frequency of spontaneous membrane depolarizations at different resting membrane potentials. The average holding current necessary to clamp the membrane potential at − 80 mV was − 1.08 ± 0.06 pA/pF. Results were pooled in 10 mV intervals from − 85 to − 76 mV; − 75 to − 66 mV and from − 65 to − 56 mV.

2.3. Confocal calcium imaging

To visualize intracellular calcium levels, cells were loaded with 2.5 μM fluo-4 AM. Images (512 ×140 pixels) were recorded at 90 Hz in 3 recordings lasting 30 s with 30 s rest between each recording, using a resonance-scanning confocal microscope with a 63x glycerol-immersion objective (Leica SP5 AOBS, Wetzlar, Germany). Fluo-4 was excited at 488 nm and emission was measured between 500 and 650 nm with a Leica Hybrid Detector. Laser power was set to 20% of 100 mW and then attenuated to 4%. Experiments were done at room temperature.

To detect calcium sparks, a wavelet-based detection method was applied to the normalized time-dependent fluorescence signal zi(t) at every pixel in order to detect candidates for Ca^{2+} release events as previously described [26]. Subsequently, candidate sparks were filtered based on a threshold for their relative amplitude (0.25), a time to peak between 0 and 100 ms, a duration at half of the maximum amplitude between 10 and 200 ms, an exponential decay constant of the spark between 15 and 200 ms and a minimum of 0.5 for the goodness-of-fit R^2 of the exponential fit to the decay. To eliminate the sparks that occurred within a Ca^{2+} wave, events with a high threshold (0.5 above baseline or greater) were eliminated. Spark candidates detected within a radius of less than 3 μm and less than 30 ms apart were considered a single event. The program allows visual inspection of the calcium signal in consecutive images in order to manually validate or reject doubtful events. The program also allowed merging of consecutive recordings from the same cell. After supervised validation and merging, sparks that coincided spatially (within a radius of 2 μm) were pooled into a common spark site and calcium traces were generated for each spark site. Sparks were characterized by their amplitude, duration, i.e. the Full Duration at Half Maximum (FDHM) and width, i.e. the Full Width at Half Maximum

(FWHM). The spark frequency was calculated as the number of sparks/cell/s or normalized to the cell area within the confocal plane and expressed as sparks/1000 μm^2/s. The number of spark sites was expressed as sites/1000 μm^2. The spark frequency per site was given as sparks/site/s.

Statistics Unless otherwise stated, values were averaged for each mouse and given as mean±s.e.m. as indicated. Statistical significance was evaluated using Fisher's exact test for categorical data. Student's t-test was used for paired or unpaired comparisons, and ANOVA was used for comparison of multiple effects as indicated. Statistically significant effects are indicated with p-values or * : $p < 0.05$, * *: $p < 0.01$; * ** : $p < 0.001$.

3. Results

3.1. Pitx2c deficiency increases local and global calcium release events

To determine the impact of Pitx2c deficiency on the activity of the ryanodine receptor (RyR2) that releases calcium from the sarcoplasmic reticulum into the cytosol when opening, we used confocal calcium imaging in right atrial myocytes from WT and mice with hetero- and homozygous Pitx2c deletion. As shown in Fig. 1A-D, Pitx2c deletion increased the density of calcium sparks, which was primarily due to a significant increase in the number of sparks per site. Moreover, heterozygous deletion (hereafter referred to as Pitx2c deficiency) induced the same effect as homozygous deletion of Pitx2c. Consequently, Pitx2c deficiency also reduced the distance from a spark to its nearest neighbor (Fig. 1E). On the other hand, Pitx2c deficiency did neither affect the cross-sectional area of the cell (Fig. 1F) nor the spark amplitude or width (Fig. 1G-H), but it did slow down the decay of the calcium sparks

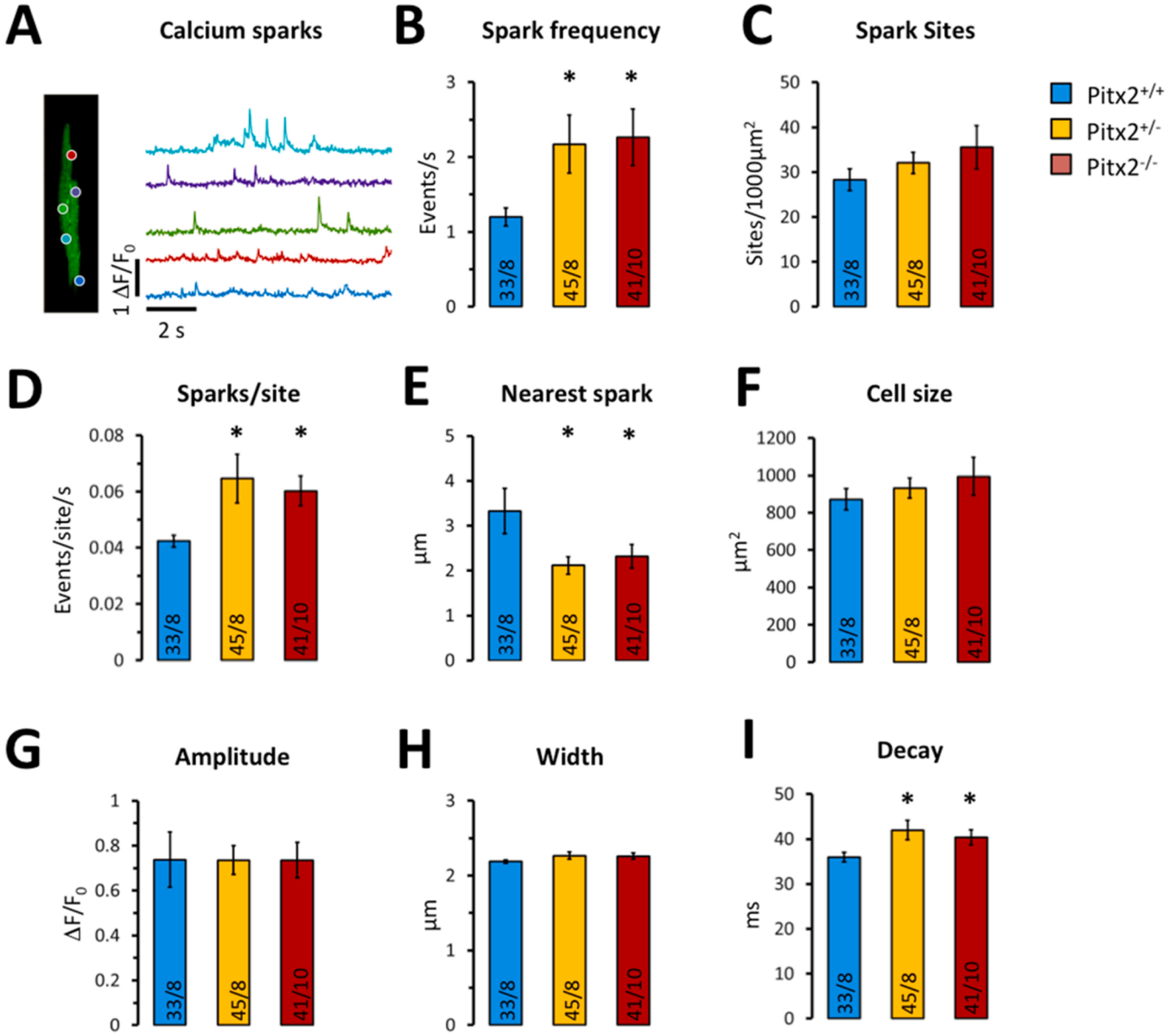


Fig. 1. Effects of Pitx2c deletion on calcium spark frequency and properties. **A** Spark recordings in a right atrial myocyte. Sparks were recorded in right atrial myocytes from 8 $Pitx2^{+/+}$, 8 $pitx2^{+/-}$, and 10 $pitx2^{-/-}$ mice. **B-D** Impact of Pitx2c deletion on calcium spark density (B), spark site density (C) and calcium sparks per site (D). **E-I** Impact of Pitx2c deletion on calcium spark amplitude (E), rate of rise (F), duration at half maximum (G), decay (H) and width (I). The number of cells/mice is given for each bar. Statistical significance was determined using ANOVA analysis with Holm-Sidak post-test. * $p < 0.05$.

(Fig. 1I).

Together, these changes in the incidence, decay, and distance between sparks in Pitx2c deficient myocytes would be expected to favor the fusion of sparks into calcium waves. In line with this, Fig. 2A-B shows that only myocytes from mice with Pitx2c deficiency displayed calcium waves or spontaneous calcium transients. Moreover, Fig. 2A demonstrates that calcium waves are capable of eliciting spontaneous calcium transients. Furthermore, Fig. 2C-D shows that Pitx2c deficiency also increased the frequency of spontaneous transient inward currents significantly. To determine if the higher incidence of spontaneous calcium release events concurred with increased SR calcium content, we measured the caffeine releasable SR calcium load in myocytes from WT and Pitx2c deficient mice at rest. Fig. 3A-B shows that the time integral of the caffeine-induced current was significantly higher in Pitx2$^{+/-}$ and Pitx2c$^{-/-}$ than in WT mice. To determine how Pitx2c deficiency affected the SR calcium loading capacity, we measured the caffeine-induced current after 1, 2, 5, 10 and 20 stimulation pulses. Fig. 3C shows the relationship between pulse number and SR calcium loading. Fitting data with a Hill equation revealed that the same number of pulses were required to achieve half-maximal loading in WT and KO myocytes (12.3 ± 4.4 and 12.4 ± 5.6 respectively). However, estimated maximal SR calcium loading was higher for KO than for WT ($p < 0.05$, $n = 10$). Measurements of the I_{Ca} density revealed that it could neither account for the higher SR calcium load nor the higher incidence of calcium release-induced electrical activity in KO mice, since Pitx2c deletion significantly reduced the I_{Ca} density (Fig. 4).

3.2. Pitx2c deficiency induces spontaneous action potentials

To determine if the higher incidence of spontaneous calcium waves and transients was translated into afterdepolarizations or spontaneous action potentials (APs), we determined their incidence at rest and during electrical stimulation. As shown in Fig. 5A, Pitx2c$^{+/+}$ mice had only small amplitude afterdepolarizations at − 80 mV while Pitx2c$^{+/-}$ mice displayed spontaneous action potentials. Fig. 5B shows that the fraction of myocytes with spontaneous APs increased to 0.23 in Pitx2c$^{+/-}$ and 0.64 in Pitx2c$^{-/-}$ mice ($p < 0.001$). Moreover, Fig. 5C shows that the incidence of spontaneous APs increased at depolarized membrane potentials ($p < 0.001$) and the incidence was significantly higher in Pitx2c$^{+/-}$ ($p < 0.01$) and in Pitx2c$^{-/-}$ mice ($p < 0.001$). Fig. 5D shows that myocytes from mice with Pitx2c deficiency also displayed spontaneous APs during electrical stimulation and Fig. 5E shows that the fraction of myocytes with spontaneous APs increased to 0.44 and 0.46 in Pitx2c$^{+/-}$ and Pitx2c$^{-/-}$ mice respectively ($p = 0.009$).

3.3. The impact of Pitx2c deficiency is similar in right and left atrial myocytes

Because Pitx2c expression in the left atrium is 100-fold higher than in the right atrium [27] and because there could be differences in calcium homeostasis at baseline in the two atrial chambers; we assessed calcium regulatory mechanisms and electrical activity in both right and left atrial myocytes from WT and Pitx2c deficient mice. Comparison of calcium spark properties in WT mice revealed no differences between right and left atrial myocytes (Table 1). Similarly, electrophysiological measurements of cell capacitance, L-type calcium current density and SR calcium load were comparable in right and left atrial myocytes. This is illustrated in Fig. 6, which also shows that even though deletion of Pitx2c affects key features of calcium homeostasis and electrical activity, the effects are similar in right and left atrial myocytes.

4. discussion

4.1. Main findings

This study is the first to analyze the impact of conditional atrial-specific deletion of Pitx2c on spontaneous calcium release and electrical activity in mouse right and left atrial myocytes. Our findings

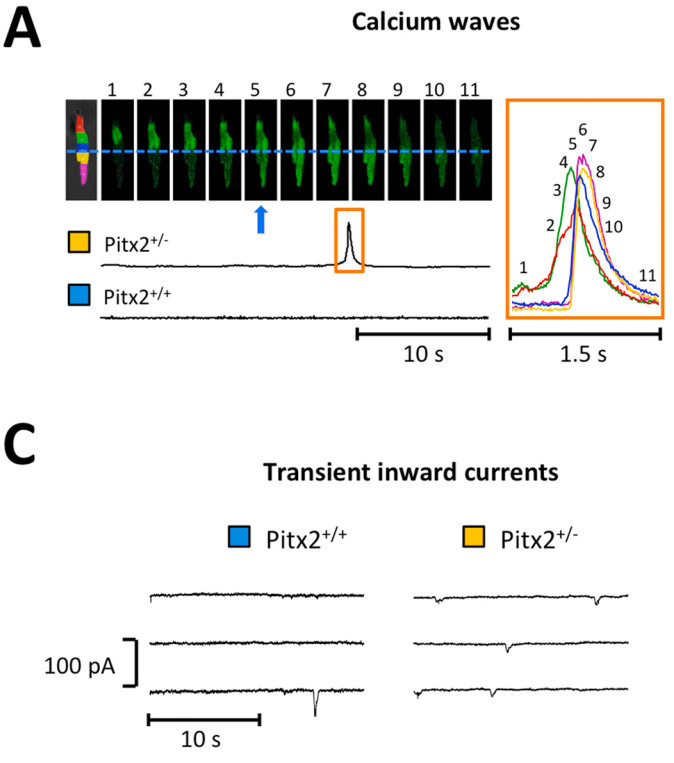
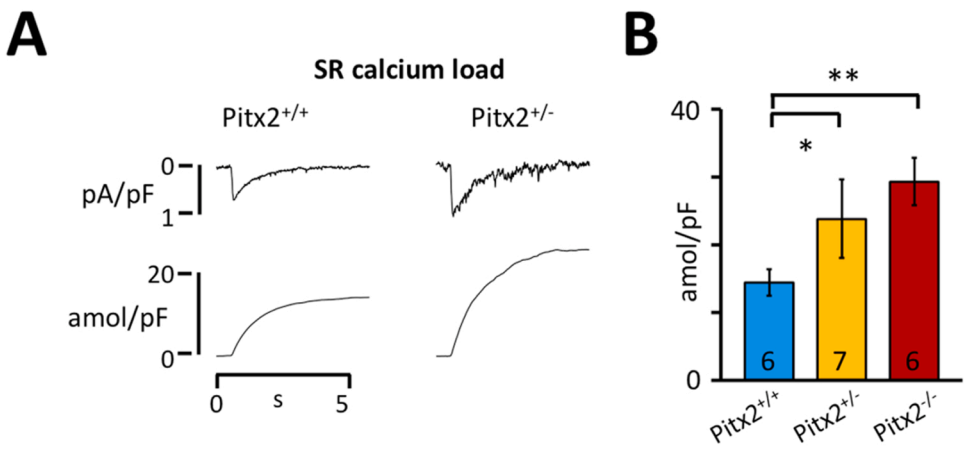


Fig. 2. Effect of Pitx2c deficiency on the incidence of calcium waves and I_{TI}s at rest. **A** Consecutive calcium images recorded in a right atrial myocyte from a Pitx2c$^{+/-}$ mouse showing the propagation of a calcium wave that triggers the induction of a spontaneous calcium transient (at the blue arrow / dashed line) **B** Frequency of spontaneous calcium release events (waves or transients). No events were observed in 21 myocytes from 6 Pitx2$^{+/+}$ mice. **C** Representative transient inward current (I_{TI}) recordings at − 80 mV in right atrial myocytes from WT and Pitx2c$^{+/-}$ mice. **D** I_{TI} frequency in WT, Pitx2c$^{+/-}$ and Pitx2c$^{-/-}$ mice. The number of cells/mice is given for each bar. Statistical significance was determined using unpaired t-test or ANOVA analysis with Holm-Sidak post-test. * $p < 0.05$; * * $p < 0.01$.



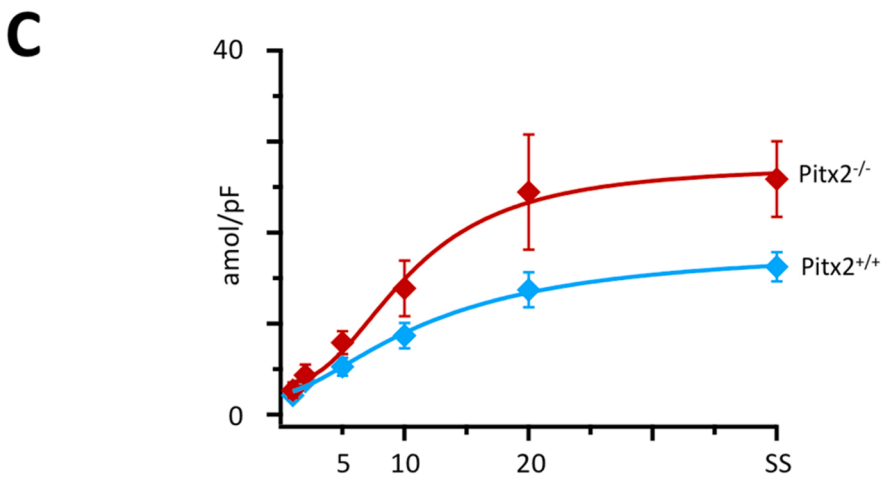


Fig. 3. Impact of Pitx2c deletion on SR calcium loading. **A** Representative caffeine-induced transient inward currents ($I_{NCX,Caf}$) and their time integral. **B** Caffeine releasable SR calcium load in right atrial myocytes. The time integral of $I_{NCX,Caf}$ was converted to atto- (10^{-18}) moles and normalized to the cell capacitance. The number of mice is given for each bar. **C** Dependency of SR calcium loading on the number of stimulation pulses after clearance of the SR calcium content. $I_{NCX,Caf}$ at steady-state (SS) is shown for reference. Fitting of the raw data with a Hill equation yielded a similar baseline for $Pitx2c^{+/+}$ and $Pitx2c^{-/-}$ myocytes (2.3 ± 1.4 vs. 2.4 ± 1.3), slope (1.7 ± 0.9 vs. 1.8 ± 1.2) and number of pulses to half-maximal loading (12.3 ± 4.4 vs. 12.4 ± 5.6). The maximal load was significantly higher for $Pitx2c^{-/-}$ myocytes (29.4 ± 8.4 vs. 18.4 ± 3.9 amol/pF; n = 11 mice, $p < 0.01$). Statistical significance is given as * $p < 0.05$; * * $p < 0.01$.

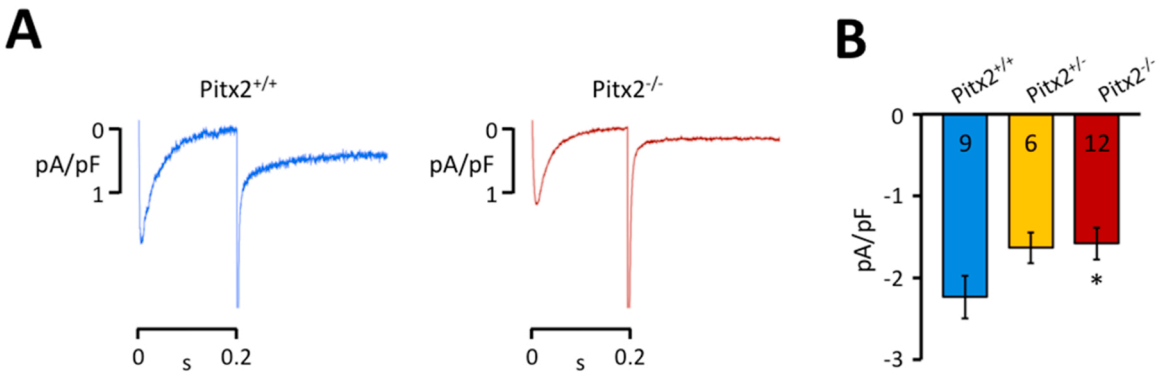


Fig. 4. Impact of Pitx2c deletion on L-type calcium current. **A** Representative I_{Ca} recordings in right atrial myocytes from a $Pitx2c^{+/+}$ and a $Pitx2c^{-/-}$ mouse. **B** Mean right atrial I_{Ca} density. Currents were normalized to the cell capacitance and given in pA/pF. The number of mice is given for each bar. Statistical significance is given as * $p < 0.05$.

demonstrate that heterozygous atrial-specific Pitx2c deletion increases the incidence of calcium sparks, waves and SR calcium loading, which is translated into a higher frequency of I_{TI} and spontaneous membrane potentials in both resting and paced myocytes. These findings, summarized in Fig. 7, mimic alterations in calcium homeostasis and electrical activity observed in human right atrial myocytes from patients with AF or that carry the 4q25 risk variant rs13143308 [24] and support the notion that Pitx2c deficiency can mediate functional defects of 4q25 risk variants that are also recognized hallmarks of AF [3,7]. Furthermore, our findings show that even though Pitx2c expression is 100-fold lower in the right atrium than in the left atrium [27], the functional electrophysiological properties at baseline and the impact of Pitx2c deficiency is similar in right and left atrial myocytes.

4.2. Impact of Pitx2c deficiency on calcium homeostasis

Because the *PITX2* locus is located near single nucleotide polymorphisms (SNPs) on chromosome 4q25 associated with risk of AF, changes in Pitx2c activity has been proposed to underlie molecular alterations in carriers of 4q25 risk variants [15,25]. The present findings confirm this notion, showing that conditional heterozygous atrial-specific deletion of Pitx2c induces changes in the intracellular calcium homeostasis that closely mimic alterations observed in human right atrial myocytes that carriers at least one rs13143308 risk allele.

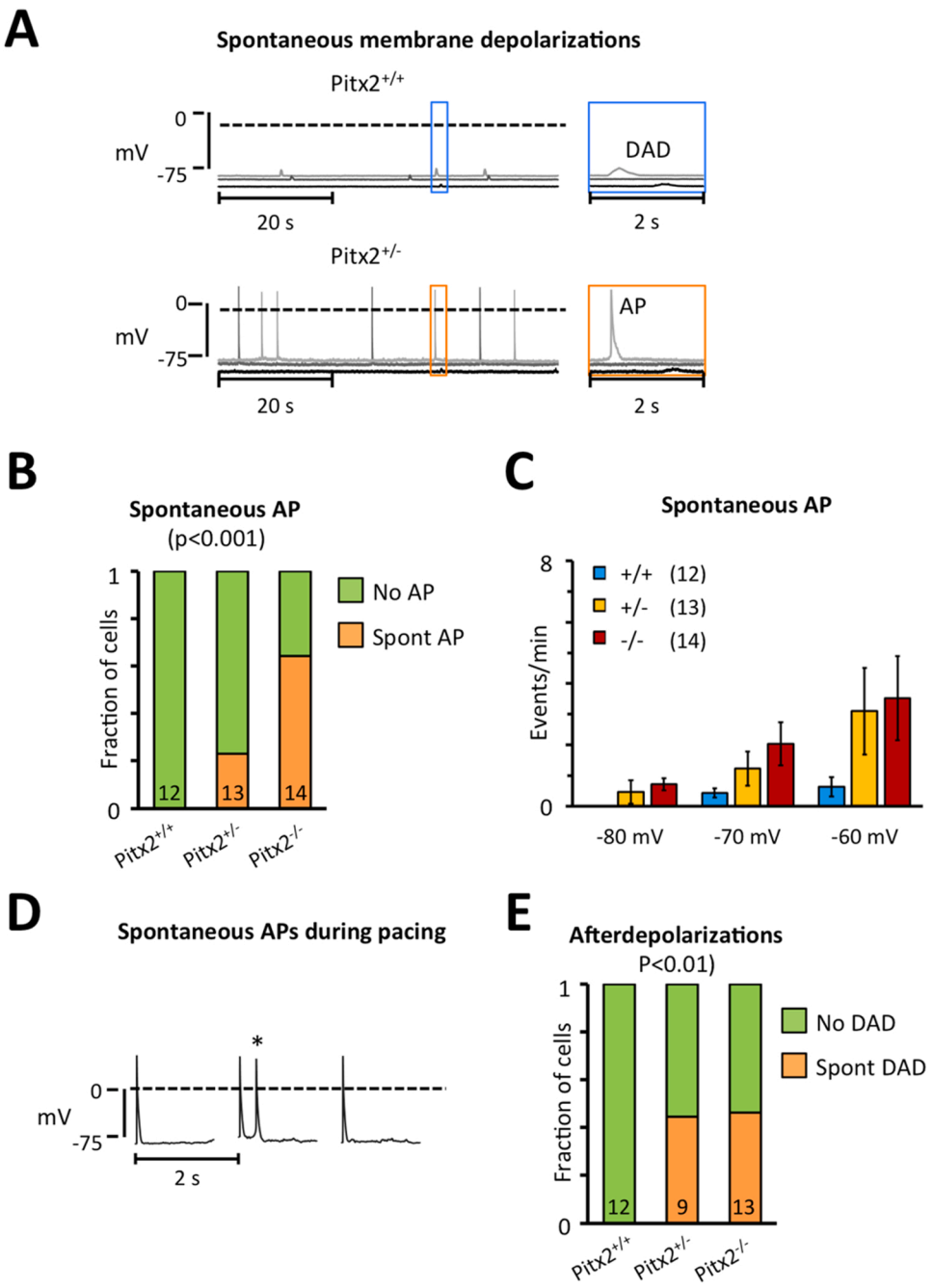


Fig. 5. Impact of Pitx2c deletion on spontaneous membrane depolarizations. **A** Representative membrane potential recordings. **B** Fraction of right atrial cells with spontaneous action potentials (APs). The number of cells is given for each bar. **C** Incidence of spontaneous APs at different resting potentials (given below bars). **D** Recording of spontaneous membrane potentials (*) in a myocyte paced at 0.5 Hz. **E** Fraction of cells with spontaneous APs when paced at 0.5 Hz. The p-value above bars in B and E indicates that the fraction of cells with spontaneous APs was significantly different among the three groups (Chi-Square Test).

Table 1
Comparison of calcium spark properties in right atrial (R.A.) and left atrial (L.A.) myocytes.

	ampl	Bl	mass	FDHM	FWHM	tau	sites	area	freq	dens	Spk/site
RA	**0.738**	**0.309**	**17.3**	**41.7**	**2.18**	**35.7**	**33.5**	**872**	**1.20**	**1.45**	**0.0414**
sem	0.241	0.082	5.3	3.6	0.05	1.8	6.4	112	0.23	0.38	0.0032
LA	**0.734**	**0.310**	**18.2**	**40.6**	**2.20**	**35.2**	**34.5**	**909**	**1.11**	**1.13**	**0.0405**
sem	0.177	0.074	3.9	1.5	0.06	1.5	10.9	181	0.20	0.26	0.0017

Mean values for 33 right atrial (RA) myocytes from 8 mice and 35 left atrial (LA) myocytes from 8 mice are given in bold with the standard error (sem) below. Abbreviations: ampl: amplitude (ΔF/F0); Bl: baseline (F0); mass: time integral of the calcium transient; FDHM: full duration at half maximum (ms); FWHM: full width at half maximum (μm); tau: decay time constant (ms); sites: spark sites/1000μm2; area: cell area (μm2); freq: frequency (sparks/s/cell); dens: density (sparks/s/1000μm2); spk/site: sparks/s/site.

This includes a higher incidence of sparks, SR calcium load, I_{TI}s and spontaneous APs at rest [24], findings that are also observed in patients with AF [3,4,7,28], suggesting that Pitx2c deficiency confers cellular electrophysiological hallmarks of AF. Our findings also show that heterozygous deletion of Pitx2c is sufficient to induce near-maximal changes in calcium spark frequency and properties, suggesting that

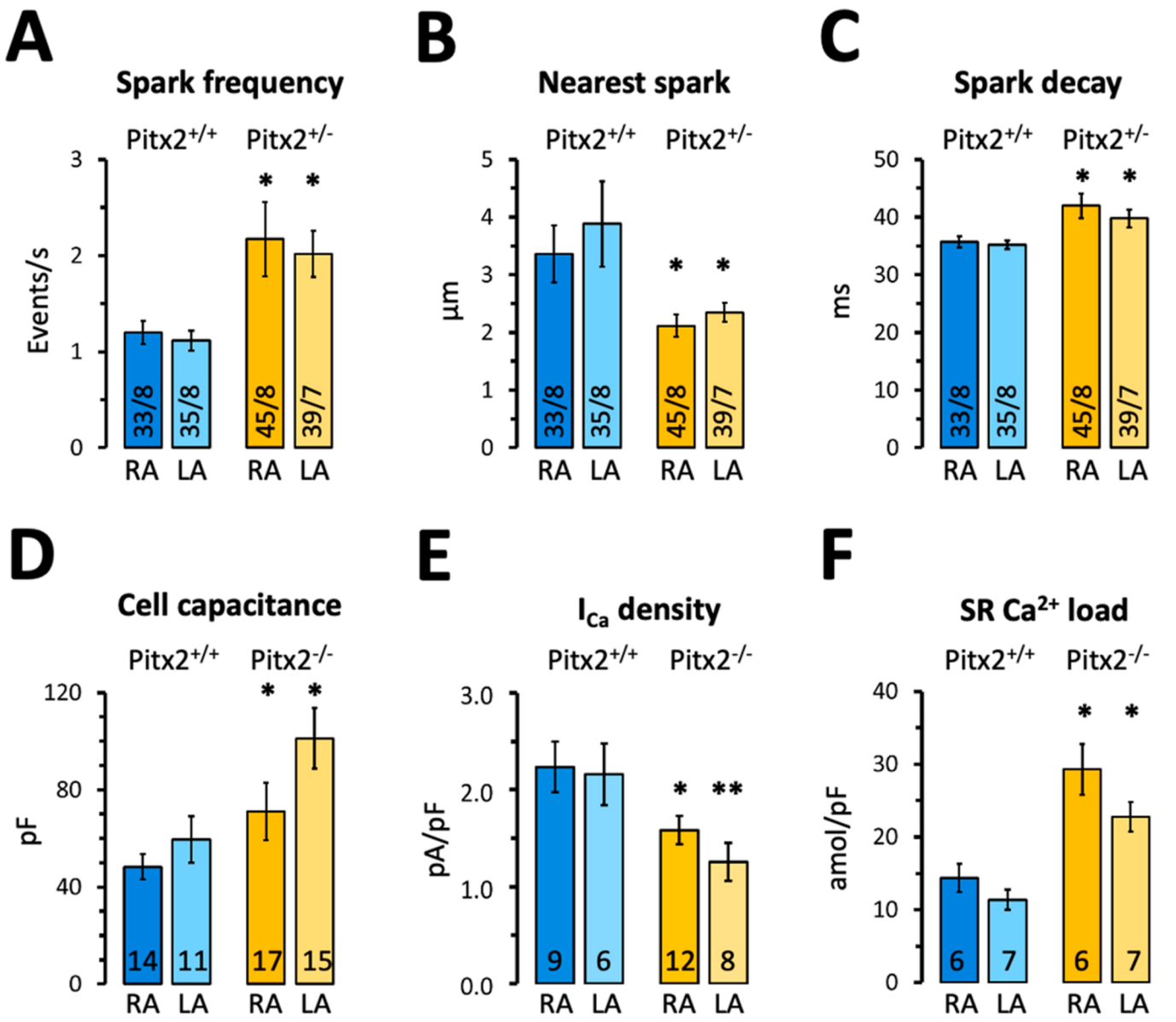


Fig. 6. Functional impact of Pitx2c deficiency in right and left atrial myocytes. **A** Spark frequency. **B** Distance to nearest spark. **C** Spark decay constant. The number of cells/mice is given for each column. **D** Cell capacitance. **E** I_{Ca} density **F** Caffeine releasable SR calcium load. The number of mice is given for each bar. RA: right atrium. LA: Left atrium. Statistical significance for differences between $Pitx2^{+/+}$ mice and mice with Pitx2 deletion is indicated with * $p < 0.05$, * * $p < 0.01$.

the effect of the *PITX2* locus on calcium homeostasis could be dominant. In line with this notion, we have previously shown that heterozygous deletion of Pitx2c in our mouse model reduces pitx2c mRNA expression levels by more than 75% and a recent study reported early sarcomeric and metabolic defects in zebrafish with heterozygous deletion of Pitx2c [29]. Furthermore, a dominant effect would be required for Pitx2c to play a significant role in 4q25 risk SNPs that have been associated with alterations in function [24,25,30] or responses to treatment [31–34]. Thus, the majority of patients enrolled in these studies would be heterozygous carriers of the risk allele(s) studied. Also in line with this notion, point mutations in the Pitx2c homeodomain causing loss of Pitx2c function induce alterations in the expression of key calcium regulatory proteins and SR calcium loading [35]. On the other hand, a previous study did show a dose-dependent modulation of mRNA levels for several calcium regulatory proteins in Pitx2c deficient mice [23]. However, because Pitx2c simultaneously changes the expression of multiple calcium regulatory proteins, it is possible that the net effect is near maximal in heterozygotes.

4.3. *Impact of Pitx2c deficiency on spontaneous electrical activity*

The stimulation of spontaneous calcium release in Pitx2c deficient myocytes could by itself account for the observed membrane depolarizations by activating electrogenic Na^{+}-Ca^{2+} exchange [3,7]. However, it might also be potentiated by Pitx2c-dependent modulation of mechanisms that determine the spatial distribution of calcium regulatory proteins, which has recently been shown to be pivotal in regulating the incidence and amplitude of calcium-release induced afterdepolarizations [26]. In this regard, we previously reported that Pitx2c deletion increases mRNA levels of adenosine A_{2A} and β2-adrenergic receptors [23], which are located near the sarcolemma, have been shown to increase spontaneous calcium release from individual RyR2 clusters [36], and has been associated with defective calcium homeostasis in myocytes from patients with AF [4,28,37].

Spontaneous electrical activity could also be potentiated by Pitx2c-mediated modulation of ion channels regulating, among others, the resting membrane potential or excitability. In this regard, previous

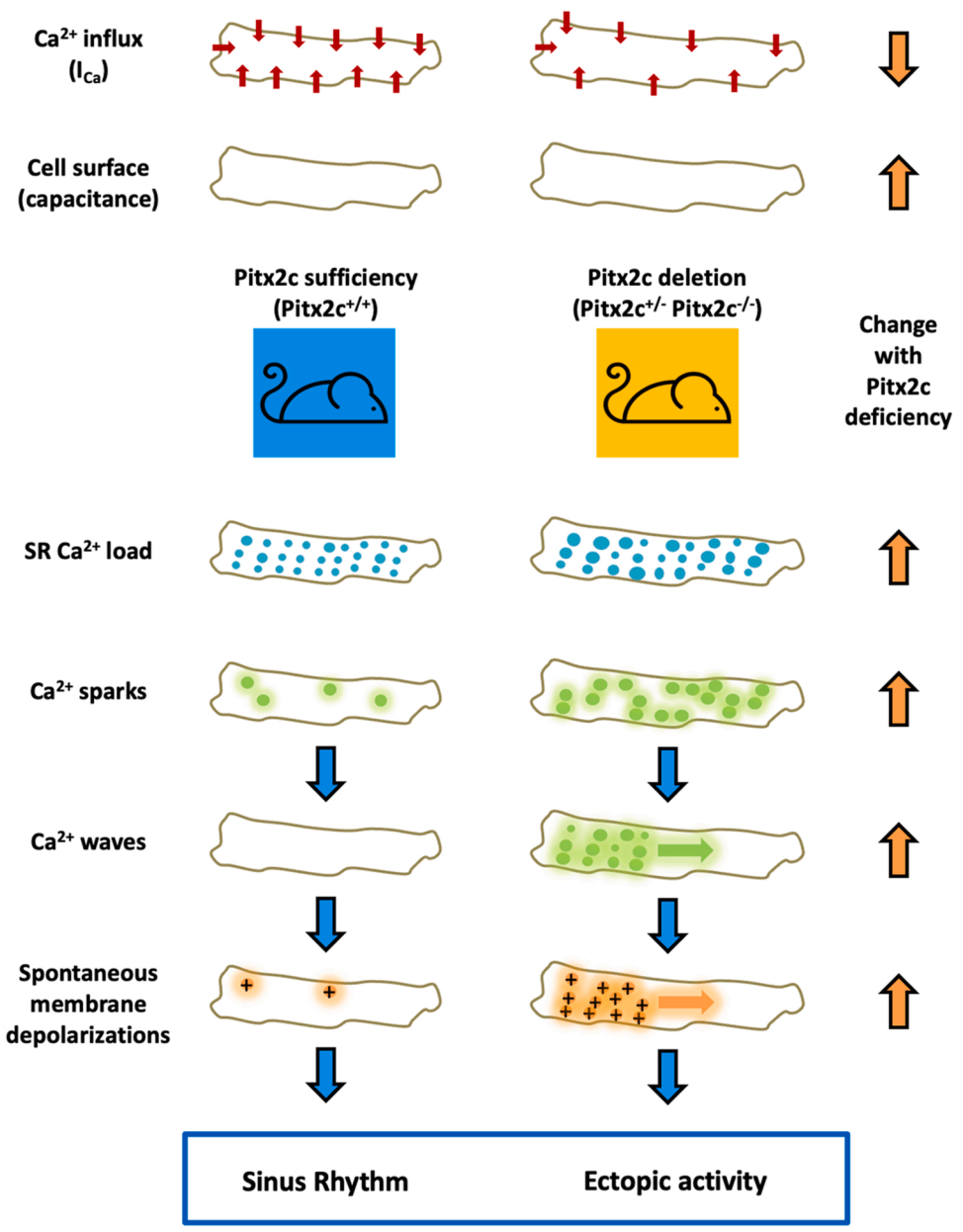


Fig. 7. Functional impact of Pitx2c deletion on calcium homeostasis in mouse atrial myocytes. Comparison of cell surface area, L-type calcium current and calcium regulatory mechanisms underlying the induction of spontaneous membrane depolarizations in atrial myocytes from $Pitx2^{+/+}$ mice and Pitx2c deficient mice ($Pitx2^{+/-}$ or $Pitx2c^{-/-}$). The impact of Pitx2c deficiency is shown on the right. Changes were similar in right and left atrial myocytes. Moreover, the changes in SR calcium homeostasis observed in $Pitx2^{+/-}$ mice closely mimic the alterations observed in carriers of the risk SNP rs13143308 [24] that is thought to modify Pitx2 activity, or in patients with paroxysmal AF [6].

studies using the present mouse model of Pitx2c deficiency have associated it with depolarization of the resting membrane potential linked to reduced expression of the inwardly rectifying I_{K1} channel [22]. This, in turn, would reduce the amplitude of a depolarization necessary to trigger a spontaneous action potential. Pitx2c deletion also reduced mRNA levels of the calcium-activated potassium channel SK3[23], which would diminish the ability of this channel to repolarize calcium release-induced membrane depolarizations. These Pitx2c-mediated alterations could therefore contribute to increase the incidence of spontaneous action potentials observed in Pitx2c deficient myocytes at rest and during electrical pacing. In addition, a higher incidence of I_{TI}s in Pitx2c deficient mice may also lower the frequency threshold for the induction of alternating beat-to-beat responses in human atrial myocytes. This phenomenon has previously been shown to precede atrial fibrillation [38] and reported to occur in myocytes carrying point mutations in the Pitx2c homeodomain that induce loss of Pitx2c function [35].

4.4. *Impact of Pitx2c deficiency in right and left atrial myocytes*

Because Pitx2c expression is 100 fold higher in the left atrium than in the right atrium [27], the functional impact of atrial-specific Pitx2c deletion could be different in right and left atrial myocytes. However, it has previously been shown that atrial-specific deletion of Pitx2c leads to enlargement of both right and left atrial chambers at different embryonic stages and comparable changes in the expression of different ionic channels in adult mice [22]. In line with this, we here find that the Pitx2c deletion increases the cell capacitance (a measure of the cell surface area) in both right and left atrial myocytes. On the other hand, chamber-specific differences in mRNA expression have previously been reported for some calcium regulatory proteins and channels in myocytes with Pitx2c deletion [23]. In contrast to this, we do not find any significant differences in the impact of Pitx2c deletion on these parameters between left and right atrial myocyte (see tables 3–4). Together these findings suggest that even though the Pitx2c mRNA expression level is 100-fold lower in the right atrium, it is the relative change in the Pitx2c levels that determine the functional impact. Our findings also reveal a

similar activity of calcium regulatory mechanisms and electrical activity in left and right mouse atrial myocytes, suggesting that concerns about chamber-specific differences in calcium homeostasis is a minor issue in mouse atria.

4.5. Clinical implications and conclusions

In conclusion, our findings demonstrate that Pitx2c deficiency mimics alterations in calcium homeostasis and electrical activity observed in human right atrial myocytes carrying the 4q25 risk variant rs13143308T and support the notion that Pitx2c deficiency could underlie these functional defects. Moreover, increased cell capacitance, reduced L-type calcium current as well as a higher incidence of calcium sparks, I_{TI}s and spontaneous membrane depolarizations, observed in atrial myocytes from Pitx2c deficient mice, are all well-established pathological alterations in human atrial myocytes from patients with AF, suggesting that Pitx2c deficiency confers cellular electrophysiological hallmarks of AF to isolated atrial myocytes.

Author contributions

Designing study: DF, LH-M. Acquiring data: CT, SAS, AH, EL-V, LH-M. Analyzing and interpreting data: CT, SAS, AH, EL-V, RB, AA, DF, LH-M. Writing the manuscript: LH-M.

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CRediT authorship contribution statement

Carmen Tarifa: Performed Research; Analyzed Data. **Selma A. Serra:** Performed Research; Analyzed Data. **Adela Herraiz-Martínez:** Performed Research; Analyzed Data. **Estefanía Lozano-Velasco:** Performed Research; Analyzed Data. **Raúl Benítez:** Analyzed Data; Acquired Funding. **Amelia Aránega:** Analyzed Data; Acquired Funding. **Diego Franco:** Designed Research; Analyzed Data; Acquired Funding, Edited & Revised Draft. **Leif Hove-Madsen:** Designed Research; Performed Research; Analyzed Data; Wrote Draft; Acquired Funding; Edited & Revised Draft.

Conflict of interest statement

None declared.

Data Availability

Data will be made available on request.

References

[1] R. Wakili, N. Voigt, S. Kaab, D. Dobrev, S. Nattel, Recent advances in the molecular pathophysiology of atrial fibrillation, J. Clin. Invest 121 (2011) 2955–2968, https://doi.org/46315[pii]10.1172/JCI46315.

[2] M.C. Wijffels, C.J. Kirchhof, R. Dorland, M.A. Allessie, Atrial fibrillation begets atrial fibrillation. A study in awake chronically instrumented goats, Circulation 92 (1995) 1954–1968. ⟨http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7671380⟩.

[3] L. Hove-Madsen, A. Llach, A. Bayes-Genis, S. Roura, E. Rodriguez Font, A. Aris, J. Cinca, Atrial fibrillation is associated with increased spontaneous calcium release from the sarcoplasmic reticulum in human atrial myocytes, Circulation 110 (2004) 1358–1363. ⟨http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15313939⟩.

[4] A. Llach, C.E. Molina, C. Prat-Vidal, J. Fernandes, V. Casado, F. Ciruela, C. Lluis, R. Franco, J. Cinca, L. Hove-Madsen, Abnormal calcium handling in atrial fibrillation is linked to up-regulation of adenosine A2A receptors, Eur. Heart J. 32 (2011), https://doi.org/10.1093/eurheartj/ehq464.

[5] D.R. Van Wagoner, A.L. Pond, M. Lamorgese, S.S. Rossie, P.M. McCarthy, J. M. Nerbonne, Atrial L-type Ca2+ currents and human atrial fibrillation, Circ. Res 85 (1999) 428–436. ⟨http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10473672⟩.

[6] N. Voigt, J. Heijman, Q. Wang, D.Y. Chiang, N. Li, M. Karck, X.H. Wehrens, S. Nattel, D. Dobrev, Cellular and molecular mechanisms of atrial arrhythmogenesis in patients with paroxysmal atrial fibrillation, Circulation 129 (2014) 145–156, https://doi.org/CIRCULATIONAHA.113.006641 [pii]10.1161/CIRCULATIONAHA.113.006641.

[7] N. Voigt, N. Li, Q. Wang, W. Wang, A.W. Trafford, I. Abu-Taha, Q. Sun, T. Wieland, U. Ravens, S. Nattel, X.H. Wehrens, D. Dobrev, Enhanced sarcoplasmic reticulum Ca2+ leak and increased Na+-Ca2+ exchanger function underlie delayed afterdepolarizations in patients with chronic atrial fibrillation, Circulation 125 (2012) 2059–2070, https://doi.org/CIRCULATIONAHA.111.067306 [pii]10.1161/CIRCULATIONAHA.111.067306.

[8] C.E. Molina, J. Leroy, W. Richter, M. Xie, C. Scheitrum, I.O. Lee, C. Maack, C. Rucker-Martin, P. Donzeau-Gouge, I. Verde, A. Llach, L. Hove-Madsen, M. Conti, G. Vandecasteele, R. Fischmeister, Cyclic adenosine monophosphate phosphodiesterase type 4 protects against atrial arrhythmias, https://doi.org/S0735-1097(12)01145-X [pii], J. Am. Coll. Cardiol. 59 (2012) 2182–2190, https://doi.org/10.1016/j.jacc.2012.01.060.

[9] A. El-Armouche, P. Boknik, T. Eschenhagen, L. Carrier, M. Knaut, U. Ravens, D. Dobrev, Molecular determinants of altered Ca2+ handling in human chronic atrial fibrillation, Circulation 114 (2006) 670–680, https://doi.org/CIRCULATIONAHA.106.636845 [pii]10.1161/CIRCULATIONAHA.106.636845.

[10] J.A. Vest, X.H. Wehrens, S.R. Reiken, S.E. Lehnart, D. Dobrev, P. Chandra, P. Danilo, U. Ravens, M.R. Rosen, A.R. Marks, Defective cardiac ryanodine receptor regulation during atrial fibrillation, Circulation 111 (2005) 2025–2032. ⟨http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15851612⟩.

[11] M.G. Chelu, S. Sarma, S. Sood, S. Wang, R.J. van Oort, D.G. Skapura, N. Li, M. Santonastasi, F.U. Muller, W. Schmitz, U. Schotten, M.E. Anderson, M. Valderrabano, D. Dobrev, X.H. Wehrens, Calmodulin kinase II-mediated sarcoplasmic reticulum Ca2+ leak promotes atrial fibrillation in mice, J. Clin. Invest 119 (2009) 1940–1951. ⟨http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19603549⟩.

[12] S. Neef, N. Dybkova, S. Sossalla, K.R. Ort, N. Fluschnik, K. Neumann, R. Seipelt, F. A. Schondube, G. Hasenfuss, L.S. Maier, CaMKII-dependent diastolic SR Ca2+ leak and elevated diastolic Ca2+ levels in right atrial myocardium of patients with atrial fibrillation, https://doi.org/CIRCRESAHA.109.203836 [pii], Circ. Res 106 (2010) 1134–1144, https://doi.org/10.1161/CIRCRESAHA.109.203836.

[13] C.E. Molina, A. Llach, A. Herraiz-Martínez, C. Tarifa, M. Barriga, R.F. Wiegerinck, J. Fernandes, N. Cabello, A. Vallmitjana, R. Benitéz, J. Montiel, J. Cinca, L. Hove-Madsen, Prevention of adenosine A2A receptor activation diminishes beat-to-beat alternation in human atrial myocytes, Basic Res. Cardiol. 111 (2016) 5, https://doi.org/10.1007/s00395-015-0525-2.

[14] A. Llach, C.E. Molina, J. Fernandes, J. Padró, J. Cinca, L. Hove-Madsen, Sarcoplasmic reticulum and L-type Ca^{2+} channel activity regulate the beat-to-beat stability of calcium handling in human atrial myocytes, J. Physiol. 589 (2011) 3247–3262, https://doi.org/10.1113/jphysiol.2010.197715.

[15] D.F. Gudbjartsson, D.O. Arnar, A. Helgadottir, S. Gretarsdottir, H. Holm, A. Sigurdsson, A. Jonasdottir, A. Baker, G. Thorleifsson, K. Kristjansson, A. Palsson, T. Blondal, P. Sulem, V.M. Backman, G.A. Hardarson, E. Palsdottir, A. Helgason, R. Sigurjonsdottir, J.T. Sverrisson, K. Kostulas, M.C. Ng, L. Baum, W.Y. So, K. S. Wong, J.C. Chan, K.L. Furie, S.M. Greenberg, M. Sale, P. Kelly, C.A. MacRae, E. E. Smith, J. Rosand, J. Hillert, R.C. Ma, P.T. Ellinor, G. Thorgeirsson, J.R. Gulcher, A. Kong, U. Thorsteinsdottir, K. Stefansson, Variants conferring risk of atrial fibrillation on chromosome 4q25, Nature 448 (2007) 353–357, https://doi.org/nature06007 [pii]10.1038/nature06007.

[16] X. Liu, F. Wang, A.C. Knight, J. Zhao, J. Xiao, Common variants for atrial fibrillation: results from genome-wide association studies, Hum. Genet. 131 (2012) 33–39, https://doi.org/10.1007/s00439-011-1052-3.

[17] M.F. Sinner, P.T. Ellinor, T. Meitinger, E.J. Benjamin, S. Kaab, Genome-wide association studies of atrial fibrillation: past, present, and future, Cardiovasc. Res. 89 (2011) 701–709, https://doi.org/10.1093/cvr/cvr001.

[18] P.T. Ellinor, K.L. Lunetta, C.M. Albert, N.L. Glazer, M.D. Ritchie, A.V. Smith, D. E. Arking, M. Müller-Nurasyid, B.P. Krijthe, S.A. Lubitz, J.C. Bis, M.K. Chung, M. Dörr, K. Ozaki, J.D. Roberts, J.G. Smith, A. Pfeufer, M.F. Sinner, K. Lohman, J. Ding, N.L. Smith, J.D. Smith, M. Rienstra, K.M. Rice, D.R. Van Wagoner, J. W. Magnani, R. Wakili, S. Clauss, J.I. Rotter, G. Steinbeck, L.J. Launer, R. W. Davies, M. Borkovich, T.B. Harris, H. Lin, U. Völker, H. Völzke, D.J. Milan, A. Hofman, E. Boerwinkle, L.Y. Chen, E.Z. Soliman, B.F. Voight, G. Li, A. Chakravarti, M. Kubo, U.B. Tedrow, L.M. Rose, P.M. Ridker, D. Conen, T. Tsunoda, T. Furukawa, N. Sotoodehnia, S. Xu, N. Kamatani, D. Levy, Y. Nakamura, B. Parvez, S. Mahida, K.L. Furie, J. Rosand, R. Muhammad, B. M. Psaty, T. Meitinger, S. Perz, H.-E. Wichmann, J.C.M. Witteman, W.H.L. Kao, S. Kathiresan, D.M. Roden, A.G. Uitterlinden, F. Rivadeneira, B. McKnight, M. Sjögren, A.B. Newman, Y. Liu, M.H. Gollob, O. Melander, T. Tanaka, B.H. C. Stricker, S.B. Felix, A. Alonso, D. Darbar, J. Barnard, D.I. Chasman, S. R. Heckbert, E.J. Benjamin, V. Gudnason, S. Kääb, Meta-analysis identifies six new susceptibility loci for atrial fibrillation, Nat. Genet. 44 (2012) 670–675, https://doi.org/10.1038/ng.2261.

[19] S.R. Gore-Panter, J. Hsu, P. Hanna, A.M. Gillinov, G. Pettersson, D.W. Newton, C. S. Moravec, D.R. Van Wagoner, M.K. Chung, J. Barnard, J.D. Smith, Atrial fibrillation associated chromosome 4q25 variants are not associated with PITX2c expression in human adult left atrial appendages, PLoS One 9 (2014), e86245, https://doi.org/10.1371/journal.pone.0086245.

[20] F. Syeda, A.P. Holmes, T.Y. Yu, S. Tull, S.M. Kuhlmann, D. Pavlovic, D. Betney, G. Riley, J.P. Kucera, F. Jousset, J.R. de Groot, S. Rohr, N.A. Brown, L. Fabritz, P. Kirchhof, PITX2 modulates atrial membrane potential and the antiarrhythmic effects of sodium-channel blockers, J. Am. Coll. Cardiol. 68 (2016) 1881–1894, https://doi.org/10.1016/j.jacc.2016.07.766.

[21] M. Perez-Hernandez, M. Matamoros, A. Barana, I. Amoros, R. Gomez, M. Nunez, S. Sacristan, A. Pinto, F. Fernandez-Aviles, J. Tamargo, E. Delpon, R. Caballero, Pitx2c increases in atrial myocytes from chronic atrial fibrillation patients enhancing IKs and decreasing ICa,L, Cardiovasc Res. 109 (2016) 431–441, https://doi.org/cvv280 [pii]10.1093/cvr/cvv280.

[22] A. Chinchilla, H. Daimi, E. Lozano-Velasco, J.N. Dominguez, R. Caballero, E. Delpon, J. Tamargo, J. Cinca, L. Hove-Madsen, A.E. Aranega, D. Franco, PITX2 insufficiency leads to atrial electrical and structural remodeling linked to arrhythmogenesis, Circ. Cardiovasc Genet 4 (2011) 269–279, https://doi.org/CIRCGENETICS.110.958116 [pii]10.1161/CIRCGENETICS.110.958116.

[23] E. Lozano-Velasco, F. Hernandez-Torres, H. Daimi, S.A. Serra, A. Herraiz, L. Hove-Madsen, A. Aranega, D. Franco, Pitx2 impairs calcium handling in a dose-dependent manner by modulating Wnt signalling, Cardiovasc Res. 109 (2016) 55–66, https://doi.org/cvv207 [pii]10.1093/cvr/cvv207.

[24] A. Herraiz-Martínez, A. Llach, C. Tarifa, J. Gandía, V. Jiménez-Sabado, E. Lozano-Velasco, S.A. Serra, A. Vallmitjana, E. Vázquez Ruiz de Castroviejo, R. Benítez, A. Aranega, C. Muñoz-Guijosa, D. Franco, J. Cinca, L. Hove-Madsen, The 4q25 variant rs13143308T links risk of atrial fibrillation to defective calcium homoeostasis, Cardiovasc. Res. 115 (2019) 578–589, https://doi.org/10.1093/cvr/cvy215.

[25] J. Ye, N.R. Tucker, L.-C. Weng, S. Clauss, S.A. Lubitz, P.T. Ellinor, A functional variant associated with atrial fibrillation regulates PITX2c expression through TFAP2a, Am. J. Hum. Genet. 99 (2016) 1281–1291, https://doi.org/10.1016/j.ajhg.2016.10.001.

[26] C. Tarifa, A. Vallmitjana, V. Jiménez-Sábado, M. Marchena, A. Llach, A. Herraiz-Martínez, H. Godoy-Marín, C. Nolla-Colomer, A. Ginel, X. Viñolas, J. Montiel, F. Ciruela, B. Echebarria, R. Benítez, J. Cinca, L. Hove-Madsen, The spatial distribution of calcium sparks determines their ability to induce afterdepolarizations in human atrial myocytes, JACC Basic Transl. Sci. (2022), https://doi.org/10.1016/j.jacbts.2022.07.013.

[27] P. Kirchhof, P.C. Kahr, S. Kaese, I. Piccini, I. Vokshi, H.H. Scheld, H. Rotering, L. Fortmueller, S. Laakmann, S. Verheule, U. Schotten, L. Fabritz, N.A. Brown, 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. 4 (2011) 123–133, https://doi.org/CIRCGENETICS.110.958058 [pii]10.1161/CIRCGENETICS.110.958058.

[28] A. Herraiz-Martínez, C. Tarifa, V. Jiménez-Sábado, A. Llach, H. Godoy-Marín, H. Colino, C. Nolla-Colomer, S. Casabella, P. Izquierdo-Castro, I. Benítez, R. Benítez, E. Roselló-Díez, E. Rodríguez-Font, X. Viñolas, F. Ciruela, J. Cinca, L. Hove-Madsen, Influence of sex on intracellular calcium homeostasis in patients with atrial fibrillation, Cardiovasc. Res. (2021), https://doi.org/10.1093/cvr/cvab127.

[29] M.M. Collins, G. Ahlberg, C.V. Hansen, S. Guenther, R. Marín-Juez, A.M. Sokol, H. El-Sammak, J. Piesker, Y. Hellsten, M.S. Olesen, D.Y.R. Stainier, P. R. Lundegaard, Early sarcomere and metabolic defects in a zebrafish *pitx2c* cardiac arrhythmia model, Proc. Natl. Acad. Sci. 116 (2019) 24115–24121, https://doi.org/10.1073/pnas.1913905116.

[30] G. Ahlberg, L. Andreasen, J. Ghouse, L. Bertelsen, H. Bundgaard, S. Haunsø, J. H. Svendsen, M.S. Olesen, Genome-wide association study identifies 18 novel loci associated with left atrial volume and function, Eur. Heart J. 42 (2021) 4523–4534, https://doi.org/10.1093/eurheartj/ehab466.

[31] D. Husser, V. Adams, C. Piorkowski, G. Hindricks, A. Bollmann, Chromosome 4q25 variants and atrial fibrillation recurrence after catheter ablation, J. Am. Coll. Cardiol. 55 (2010) 747–753, https://doi.org/S0735-1097(09)04083-2 [pii] 10.1016/j.jacc.2009.11.041.

[32] W.-S. Choe, J.H. Kang, E.-K. Choi, S.Y. Shin, S.A. Lubitz, P.T. Ellinor, S. Oh, H. E. Lim, A genetic risk score for atrial fibrillation predicts the response to catheter ablation, Korean Circ. J. 49 (2019) 338, https://doi.org/10.4070/kcj.2018.0161.

[33] G.R. Wong, C.J. Nalliah, G. Lee, A. Voskoboinik, S. Prabhu, R. Parameswaran, H. Sugumar, R.D. Anderson, L.-H. Ling, A. McLellan, R. Johnson, P. Sanders, P. M. Kistler, D. Fatkin, J.M. Kalman, Genetic susceptibility to atrial fibrillation is associated with atrial electrical remodeling and adverse post-ablation outcome, JACC Clin. Electrophysiol. 6 (2020) 1509–1521, https://doi.org/10.1016/j.jacep.2020.05.031.

[34] M.B. Shoemaker, A. Bollmann, S.A. Lubitz, L. Ueberham, H. Saini, J. Montgomery, T. Edwards, Z. Yoneda, M.F. Sinner, A. Arya, P. Sommer, J. Delaney, S.K. Goyal, P. Saavedra, A. Kanagasundram, S.P. Whalen, D.M. Roden, G. Hindricks, C.R. Ellis, P.T. Ellinor, D. Darbar, D. Husser, Common genetic variants and response to atrial fibrillation ablation, Circ. Arrhythm. Electrophysiol. 8 (2015) 296–302, https://doi.org/CIRCEP.114.001909 [pii]10.1161/CIRCEP.114.001909.

[35] A. Herraiz-Martínez, C. Tarifa, E. Lozano-Velasco, V. Jiménez-Sábado, S. Casabella, F. Hernández-Torres, H. Daimi, E. Vázquez Ruiz de Castroviejo, E. Delpón, R. Caballero, A. Aránega, D. Franco, L. Hove-Madsen, Novel PITX2 homeodomain-contained mutations from ATRIAL fibrillation patients deteriorate calcium homeostasis, Hearts 2 (2021) 251–271, https://doi.org/10.3390/hearts2020020.

[36] C. Nolla-Colomer, S. Casabella-Ramon, V. Jimenez-Sabado, A. Vallmitjana, C. Tarifa, A. Herraiz-Martínez, A. Llach, M. Tauron, J. Montiel, J. Cinca, S.R. W. Chen, R. Benitez, L. Hove-Madsen, β2–adrenergic stimulation potentiates spontaneous calcium release by increasing signal mass and co-activation of ryanodine receptor clusters, Acta Physiol. (2021), https://doi.org/10.1111/apha.13736.

[37] S. Casabella-Ramón, V. Jiménez-Sábado, C. Tarifa, S. Casellas, T.T. Lu, P. Izquierdo-Castro, I. Gich, M. Jiménez, A. Ginel, J.M. Guerra, S.R.W. Chen, R. Benítez, L. Hove-Madsen, Impact of R-carvedilol on β2-adrenergic receptor-mediated spontaneous calcium release in human atrial myocytes, Biomedicines 10 (2022) 1759, https://doi.org/10.3390/biomedicines10071759.

[38] S.M. Narayan, F. Bode, P.L. Karasik, M.R. Franz, Alternans of atrial action potentials during atrial flutter as a precursor to atrial fibrillation, Circulation 106 (2002) 1968–1973, https://doi.org/10.1161/01.CIR.0000037062.35762.B4.