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The selective RyR2 inhibitor *ent*-verticilide suppresses atrial fibrillation susceptibility caused by Pitx2 deficiency

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Abstract

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia and a major cause of stroke and morbidity. The strongest genetic risk factors for AF in humans are variants on chromosome 4q25, near the paired-like homeobox transcription factor 2 gene *PITX2*. Although mice deficient in Pitx2 (Pitx2^{+/-}) have increased AF susceptibility, the mechanism remains controversial. Recent evidence has implicated hyperactivation of the cardiac ryanodine receptor (RyR2) in Pitx2 deficiency, which may be associated with AF susceptibility. We investigated pacing-induced AF susceptibility and spontaneous Ca²⁺ release events in Pitx2 haploinsufficient (+/-) mice and isolated atrial myocytes to test the hypothesis that hyperactivity of RyR2 increases susceptibility to AF, which can be prevented by a potent and selective RyR2 channel inhibitor, *ent*-verticilide. Compared with littermate wild-type Pitx2^{+/+}, the frequency of Ca²⁺ sparks and spontaneous Ca²⁺ release events increased in permeabilized and intact atrial myocytes from Pitx2^{+/-} mice. Atrial burst pacing consistently increased the incidence and duration of AF in Pitx2^{+/-} mice. The RyR2 inhibitor *ent*-verticilide significantly reduced the frequency of spontaneous Ca²⁺ release in intact atrial myocytes and attenuated AF susceptibility with reduced AF incidence and duration. Our data demonstrate that RyR2 hyperactivity enhances SR Ca²⁺ leak and AF inducibility in Pitx2^{+/-} mice via abnormal Ca²⁺ handling. Therapeutic targeting of hyperactive RyR2 in AF using *ent*-verticilide may be a viable mechanism-based approach to treat atrial arrhythmias caused by Pitx2 deficiency.

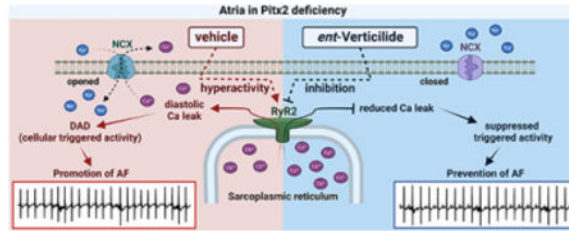
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Disclosures

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Graphical abstract



Keywords

Pitx2; cardiac ryanodine receptor (RyR2); calcium; atrial fibrillation; *ent*-verticilide

1. Introduction

Atrial fibrillation (AF) is the most common cardiac arrhythmia in the general population, with increasing incidence and prevalence worldwide, and associated with increased risk of death, stroke, heart failure, and dementia [1]. Although knowledge of the pathophysiology of AF has expanded greatly during the last decade, effective antiarrhythmic drug therapy to prevent AF remains elusive, likely because the mechanisms leading to AF are multifactorial: electrophysiological, molecular, and structural alterations [2–6].

Although still somewhat controversial, one cellular mechanism proposed to lead to AF initiation and maintenance is abnormal intracellular calcium (Ca²⁺) handling caused by cardiac ryanodine receptor (RyR2) hyperactivity, which is thought to cause rapid focal ectopic activity. RyR2 channels release Ca²⁺ from the sarcoplasmic reticulum (SR) to coordinate cardiac excitation-contraction coupling. However, dysfunctional RyR2-mediated diastolic SR Ca²⁺ release in atrial myocytes is associated with substantially increased AF risk in catecholaminergic polymorphic ventricular tachycardia (CPVT) animal models [7,8]. Additionally, RyR2-mediated triggered activity that contribute to the pathophysiology of AF can occur through enhanced RyR2 Ca²⁺ sensitivity by phosphorylation or oxidation [9–12].

Large-scale genome-wide association studies (GWAS) have identified chromosome 4q25 variants near the paired-like homeodomain transcription factor 2 (*PITX2*) gene as the strongest genetic risk factor for AF in humans [13,14]. Pitx2 is an atrial-selective transcription factor (Pitx2c isoform) that helps establish the left-right asymmetry of the atria during fetal development and is also an upstream transcription regulator of atrial electric function [15]. In mice, heterozygous deletion of Pitx2 (Pitx2^{+/-}) causes increased AF susceptibility when challenged by programmed stimulation [16,17]. Pitx2 haploinsufficiency causes a developmental patterning defect associated with expanded pacemaker gene expression, altered ion channel expression, Ca²⁺ handling, and cell-cell coupling, which have all been associated with AF risk [18–20]. A recent study showed that RyR2 is upregulated and hyperphosphorylated in mutant Pitx2 human and mouse hearts, which suggests that excessive diastolic Ca²⁺ release and the resulting spontaneous electrical activity in atrial cardiomyocytes could be the cause of AF susceptibility in vivo [21]. Mice with heterozygous deletion of the Pitx2 gene are viable without obvious fibrosis or structural

defects [17]. Hence, *Pitx2*^{+/-} mice provide a good model to study the AF biology associated with *Pitx2* loss of function, and test therapeutic efficacy of drugs in a genetically-defined form of AF.

Here, we investigated the AF susceptibility and Ca²⁺ handling in *Pitx2*^{+/-} mice and isolated atrial myocytes to test the hypothesis that hyperactivity of RyR2 causes spontaneous Ca²⁺ release in atrial myocytes and increase susceptibility to AF by promoting DADs and triggered activity. We further hypothesized that Ca²⁺-triggered AF in *Pitx2*^{+/-} mice can be prevented by a mechanism-based approach of suppressing spontaneous Ca²⁺ release with *ent*-verticilide, which is a potent and selective RyR2 Ca²⁺ release channel inhibitor [22].

2. Materials and Methods

2.1. Animal use and isolation of atrial myocytes

The use of animals was approved by the Animal Care and Use Committee of Vanderbilt University Medical Center (animal protocol No. M1900057-00) and performed in accordance with National Institutes of Health guidelines. Male and female paired-like homeodomain 2 transcription factor deficient (*Pitx2*^{+/-}) and their littermate wild type (WT, *Pitx2*^{+/+}) mice at the age between 16 and 30 weeks old were used for all experiments. To measure AF susceptibility, 16–19 week old mice underwent a transesophageal atrial pacing protocol. Mice were euthanized ~2–3 weeks after the in vivo measurements for myocyte isolation and tissue procurement. Atrial myocytes from 20- to 30-week-old *Pitx2*^{+/-} or littermate WT mice were isolated from the heart by collagenase and protease digestion as previously described [7]. Cells were then washed twice by gravity sedimentation for 20 minutes at room temperature in standard Tyrode solution with 0.2 mM CaCl₂. The final suspension contained 0.6 mM Ca²⁺ and the cells were immediately used for intact intracellular Ca²⁺ measurements and Ca²⁺ sparks.

2.2. Ca²⁺ spark measurements in permeabilized atrial myocytes

Ca²⁺ spark measurements from permeabilized atrial cardiomyocytes were carried at as previously reported [23]. Briefly, cells were plated on laminin-coated glass slides and allowed to attach for >10 minutes. Cells were then washed once and permeabilized for one minute in an internal solution containing saponin, to selectively permeabilize the outer membrane. The internal solution contained (in mM) L-aspartic acid potassium salt (120), KCl (15), potassium phosphate (5), HEPES (10), MgCl₂ (0.6), EGTA (0.5), dextran (4%), and CaCl₂ to give a free Ca²⁺ concentration of 54 nM. Free Ca²⁺ was calculated using MaxChelator (<https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/>). After cell permeabilization, cells were incubated in internal solution containing Fluo-4 to detect Ca²⁺ sparks. After 10 minutes, Sparks were recorded using an Olympus inverted confocal microscope equipped with a Yokogawa spinning disk with 50 μm pinhole and 40X silicone objective (1.25 NA). Excitation was achieved via a 100-mW solid state diode laser (488 nm) and the emission was collected using a Hamamatsu CMOS camera. Spark detection analysis was performed using the SparkMaster plugin for ImageJ [24] and statistical analysis was carried out in R using the hierarchical clustering script provided by

Sikkel et al [25]. Data were log₂-transformed prior to analysis (as necessary for parametric tests) and reported as normalized to the WT genotype for that day.

2.3. Intracellular Ca²⁺ measurements in intact atrial myocytes

Atrial myocytes were pre-incubated for 2 hours with DMSO or *ent*-verticilide (0.5 μM). Myocytes were then loaded with Fura-2 acetoxymethyl ester (Fura-2, AM; Invitrogen) as described previously [22]. Briefly, isolated single atrial myocytes were incubated with 2 μM Fura-2, AM for 7 minutes, washed 2 × 10 minutes with normal Tyrode (NT) solution containing 250 μM probenecid. The composition of NT used for Fura-2 loading and washing was (in mM): 134 NaCl, 5.4 KCl, 1.2 CaCl₂, 1 MgCl₂, 10 Glucose, and 10 HEPES (pH adjusted to 7.4 with NaOH). After Fura-2 loading, experiments were conducted in NT solution containing 1 μM isoproterenol and 2 mM CaCl₂ (all solutions retained DMSO or *ent*-verticilide). Atrial myocytes were electrically paced at 3 Hz field stimulation for 20 seconds, followed by no electrical stimulation for 40 seconds for quantification of spontaneous Ca²⁺ release events. Myocytes were then immediately exposed to 10 mM caffeine in NT solution for 5 seconds to estimate total SR Ca²⁺ content. Intracellular Ca²⁺ transients were recorded using a dual-beam excitation fluorescence photometry setup (IonOptix Corp.). All experiments were conducted at room temperature. 3 Hz paced Ca²⁺ transients were analyzed for Ca²⁺ parameters using IonWizard7 data analysis software (IonOptix Corp. Milton, MA).

2.4. Isolation of sarcoplasmic reticulum vesicles

Cardiac SR vesicles were isolated from left-ventricular tissue of fresh porcine hearts as previously described [26]. SR vesicles were flash-frozen and stored at -80 °C.

2.5. Ca-ATPase activity assay

SERCA2a activity was measured in porcine cardiac SR vesicles using an enzyme-linked NADH-coupled ATPase assay. To each well of a 96-well microplate, we applied DMSO or test compound (*ent*-verticilide, thapsigargin), cardiac SR to 10 μg/mL, and assay mix containing 50 mM MOPS (pH 7.0), 100 mM KCl, 1 mM EGTA, 0.2 mM NADH, 1 mM phosphoenolpyruvate, 10 IU/mL of pyruvate kinase, 10 IU/mL of lactate dehydrogenase, and 7 μM of the Ca²⁺ ionophore A23187 (Sigma). CaCl₂ was added to result in free Ca²⁺ of the indicated concentrations. After 20 min of compound contact at room temperature, the assay was started upon addition of MgATP for a final concentration of 5 mM, and absorbance was read at 340 nm in a SpectraMax Plus³⁸⁴ microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

2.6. Transesophageal electrical pacing with surface electrocardiogram (ECG)

Mice were anesthetized with inhaled isoflurane (3% for induction, 1.5–2% for maintenance) while breathing spontaneously and placed in the supine position on a heating pad. Surface electrocardiograms (ECG) were obtained by subcutaneous placement of 27-gauge needles in each limb, and recorded continuously using amplifiers (AD Instruments) and LabChart 8 software. An octopolar 2F electrode catheter (CIB^{ER} MOUSETM; NuMED, Inc) was placed through the mouth in the esophagus, and situated properly for atrial capturing using a

programmable stimulator, followed by measurement of the atrial diastolic capture threshold. Bipolar atrial pacing was performed using a stimulus amplitude twice threshold with a pulse width of 2 ms, as described previously [27]. To test for susceptibility of AF, burst atrial pacing was performed at six different cycle lengths (50, 40, 30, 25, 20, and 15 ms) for 15 seconds, each as described previously [7]. Between all pacing trains, there was a recovery time of 30 seconds. AF episodes were defined as rapid atrial activity and irregular ventricular rate lasting more than 1 second. The duration of AF was measured from the end of the pacing train to the first P wave with regular atrioventricular conduction after termination of AF. The number of induced episodes and total duration of AF were calculated for each animal. If an AF episode lasted for 10 min, the procedure was terminated. DMSO (vehicle group) or *ent*-verticilide (10 mg/kg, intraperitoneal injection) was administered to mice 15 minutes prior to the study. Isoproterenol (1.5 mg/kg, intraperitoneal (i.p.) injection) was administered before the burst atrial pacing. At the end of the procedure, the esophageal catheter and subcutaneous needles were removed, and isoflurane discontinued. The animal was placed in a cage with food and water and observed until recovery.

2.7. Statistical analysis

Statistical analyses were performed using Prism v6.0.1 (GraphPad Software, Inc.). The specific statistical tests used are reported in the figure and table legends. For all data, mean and standard deviation are provided. Results were considered statistically significant if the probability (*p*) value was less than 0.05 as the threshold to reject the null hypothesis. Ca-ATPase data were fit in Prism using the equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + (\text{IC50}/X)^{\text{HillSlope}})$.

3. Results

3.1. Hyperactive RyR2-mediated Ca²⁺ release in permeabilized atrial myocytes from Pitx2^{+/-} mice

Ca²⁺ sparks are elementary sarcoplasmic reticulum (SR) Ca²⁺ release events generated by spontaneous opening of intracellular RyR2 Ca²⁺ release channels [28]. Hence, to test the hypothesis that hyperactivity of RyR2 causes spontaneous Ca²⁺ release in atrial myocytes isolated from the Pitx2 deficient mouse model, we first examined RyR2 activity by measuring Ca²⁺ sparks in permeabilized atrial myocytes with saponin.

Compared to wild-type Pitx2^{+/+} littermates, atrial myocytes from Pitx2^{+/-} mice exhibited a significant increase in Ca²⁺ spark frequency (+/+ veh 1.0, 95% CI 0.96–1.04 vs +/- veh 1.15, 95% CI 1.1–1.2; Fig. 1A) and spark-mediated SR Ca²⁺ leak (+/+ veh 1.0, 95% CI 0.97–1.03 vs +/- veh 1.09, 95% CI 1.05–1.13; Fig. 1D). In addition, the amount of Ca²⁺ released during each Ca²⁺ spark (measured as spark amplitude and spark mass) was also significantly increased in the Pitx2^{+/-} group (amplitude: +/+ veh 1.0, 95% CI 0.98–1.02 vs +/- veh 1.07, 95% CI 1.02–1.11, mass: +/+ veh 1.0, 95% CI 0.97–1.03 vs +/- veh 1.08, 95% CI 1.02–1.13; Fig. 1B & C). We next investigated the effect of the RyR2 selective inhibitor, *ent*-verticilide [22]. Application of 3 μM *ent*-verticilide in Pitx2^{+/-} myocytes significantly reduced the frequency of Ca²⁺ sparks, spark amplitude, and spark mass (Fig. 1A – C), without depletion of SR Ca²⁺ stores (Fig. 1E). Consequently, spark-mediated SR Ca²⁺ leak

was drastically reduced by *ent*-verticilide (Fig. 1D), indicating that Pitx2 deficiency leads to increased RyR2 activity, which is reduced by selective RyR2 inhibition. To determine whether *ent*-verticilide inhibits Ca²⁺ sparks via effects on the cardiac SR Ca²⁺ ATPase (SERCA2a), we next measured the Ca²⁺-dependent ATP hydrolysis in SR microsomes from porcine hearts, in the presence of *ent*-verticilide at concentrations ranging 0.03 – 30 μM. Thapsigargin was used as a positive control for inhibition of SERCA2a. Within the tested [*ent*-verticilide] range, we observed no effect on the Ca²⁺-ATPase activity (Figure 2A). In concurrent control measurements, thapsigargin induced the typical complete inhibition, with low-nanomolar IC₅₀ (Figure 2B).

3.2. RyR2 inhibition with *ent*-verticilide suppresses spontaneous SR Ca²⁺ release in intact atrial myocytes

We next investigated intracellular Ca²⁺ handling in *intact* atrial myocytes. Spontaneous Ca²⁺ release (SCR) events are the cellular mechanism responsible for delayed afterdepolarizations that can trigger premature beats and evoke atrial ectopy and arrhythmogenesis [7]. Intracellular Ca²⁺ was monitored in Fura-2, AM-loaded intact atrial myocytes treated with the beta-adrenergic agonist isoproterenol to simulate catecholaminergic stress. After a rapid pacing train with 3 Hz pacing, we quantified spontaneous diastolic Ca²⁺ release events in isolated myocytes from Pitx2^{+/-} and littermate wild-type (+/+) mice (Fig 3A). Consistent with the increased rate of RyR2-mediated Ca²⁺ sparks (Fig. 1), spontaneous Ca²⁺ release events were significantly increased in Pitx2^{+/-} – compared to Pitx2^{+/+} myocytes, consistent with abnormal Ca²⁺ handling caused by hyperactive RyR2 in Pitx2^{+/-} mice. Inhibition of RyR2 with *ent*-verticilide (0.5 μM) had no effect on Ca²⁺ handling in wild-type Pitx2^{+/+} myocytes, whereas in Pitx2^{+/-} cells, *ent*-verticilide significantly reduced the frequency of SCR events compared to vehicle (veh 6.97 ± 5.62 vs. *ent*-vert 2.36 ± 2.55, p<0.0001; Fig 3B). At the same time, *ent*-verticilide normalized the increased Ca²⁺ transient amplitude (-veh 0.79 ± 0.19 vs. *ent*-vert 0.67 ± 0.14, p=0.0090; Fig 3C) and increased fractional SR Ca²⁺ release (veh 0.87 ± 0.15 vs. *ent*-vert 0.78 ± 0.13, p=0.0293; Fig 3D) observed in Pitx2^{+/-} to values similar to those observed in wild-type atrial myocytes. There were no statistically significant differences in other Ca²⁺ handling parameters such as diastolic Ca²⁺ levels in the cytoplasm, time to peak, decay rate of the Ca²⁺ transient (a measure of sarcoplasmic reticulum Ca²⁺-ATPase [29]), SR Ca²⁺ content or caffeine-induced Ca²⁺ decay kinetics (a measure of Na⁺-Ca²⁺ exchanger function [30]) (Table 1). Ca²⁺ transient alternans – a beat-to-beat change in the Ca²⁺ transient amplitude especially at fast pacing rates – has been associated with increased risk for arrhythmias and sudden cardiac death [31,32]. However, we did not observe Ca²⁺ alternans in any group during 3 Hz pacing, the fastest rate that allowed continuous capture in atrial myocytes. Taken together, Pitx2^{+/-} atrial myocytes exhibit RyR2 hyperactivity resulting in increased spontaneous RyR2-mediated Ca²⁺ release, which can be prevented by RyR2 inhibition with *ent*-verticilide.

3.3. Adrenergic stimulation increases AF susceptibility in Pitx2 deficient mice

Next, we determined *in vivo* AF susceptibility of Pitx2^{+/-} mice using a transesophageal (TE) atrial burst pacing protocol. The TE atrial stimulation protocol is widely used to initiate pacing-induced AF [7,33–35] and is a survival procedure that allows for repeated

measurement in the same mouse. However, TE atrial pacing can cause vagal stimulation (evidenced by atrioventricular (AV) block), which causes AF induction even in normal mice. Hence, pacing episodes with AV block were excluded to increase the specificity of the TE burst pacing protocol [27]. Atrial burst pacing resulted in a significantly higher AF incidence (27.3 % vs 75.0 %, $P=0.039$, Fischer exact test, $n=11$ and 12 mice) and total AF burden (7.6 ± 17.2 sec vs 27.6 ± 33.2 sec, $P=0.027$, Mann-Whitney test) in Pitx2^{+/-} mice compared with wild-type Pitx2^{+/+} littermates. Addition of a catecholamine challenge with isoproterenol (iso) before burst pacing stimulation increased AF burden further in Pitx2^{+/-} mice, and sustained AF was also observed (persistent AF over 300 sec). Fig 4A shows representative ECG traces of pacing-induced AF. Atrial pacing-induced AF was observed in 96 % of Pitx2^{+/-} mice (21 of 22 mice), whereas wild-type littermates had only a 50 % AF incidence (7 of 14 mice; Fig 4B). Moreover, sustained AF – defined as persistent AF lasting more than 300 seconds – was significantly increased in Pitx2^{+/-} mice (+/+ 21.4 % vs. +/- 81.8 %, $P=0.0005$ Fischer Exact Test; Fig 4B). Total AF burden was also increased in Pitx2^{+/-} compared to Pitx2^{+/+} mice (Fig 4C).

On the other hand, ECG parameters such as heart rate, RR, PR, QRS and QT intervals were not statistically different between Pitx2^{+/-} mice and wild-type littermates. TE pacing programmed stimulation was used to determine Wenckebach cycle length (WCL) and atrioventricular effective refractory period (AVERP) at a basic cycle length of 100 msec. There were no significant differences between Pitx2^{+/-} and wild-type littermates (Table 2). Our data corroborate the previously reported AF susceptibility and ECG parameters for these mice [16].

3.4. *ent*-Verticilide prevents pacing-induced AF in Pitx2 deficient mice

To test the hypothesis that RyR2 hyperactivity is the underlying molecular mechanism responsible for pacing induced AF in Pitx2^{+/-} mice, we used the RyR2 inhibitor *ent*-verticilide, which does not block membrane ion channels and has no effect on the cardiac action potential [22]. Both Pitx2^{+/+} and Pitx2^{+/-} mice underwent a TE atrial burst pacing protocol after vehicle injection, followed by the same pacing protocol in the presence of *ent*-verticilide one week later. Intraperitoneal injection of *ent*-verticilide 15 min prior to the TE pacing significantly reduced AF inducibility from 100 % to 65 % in Pitx2^{+/-} mice, whereas there was no significant effect in wild-type Pitx2^{+/+} (veh 54.6% vs. *ent*-vert 45.5%; Fig 5A, B). *ent*-Verticilide significantly reduced the incidence of sustained AF (veh 90 % vs. *ent*-vert 25 %, $P<0.0001$ Fischer Exact Test; Fig 5B) to values similar to wild-type Pitx2^{+/+} mice, but had no effect on sustained AF in wild-type Pitx2^{+/+} mice (veh 27.3% vs. *ent*-vert 27.3%; Fig 5A). *ent*-Verticilide also significantly reduced the total AF duration in Pitx2^{+/-} mice (veh 508.0 ± 183.8 sec vs. *ent*-vert 169.1 ± 233.5 sec; Fig 5D), but had no significant effect in Pitx2^{+/+} mice (veh 148.0 ± 241.1 sec vs. *ent*-vert 154.7 ± 265.4 sec; Fig 5C). These data demonstrate that *ent*-verticilide treatment suppresses both incidence and total duration of AF in Pitx2^{+/-} mice. Taken together, our results indicate that in Pitx2 deficient mice, RyR2 hyperactivity causes AF susceptibility via spontaneous Ca²⁺ release, which can be prevented *in vivo* by the selective RyR2 inhibitor, *ent*-verticilide.

4. Discussion

In this study, we report that Pitx2 deficiency in mice causes RyR2 hyperactivity and spontaneous SR Ca²⁺ release in atrial cardiomyocytes, and AF susceptibility *in vivo*. Given the efficacy of a selective inhibitor of RyR2 both *in vitro* and *in vivo*, our data demonstrate mechanistic causality from enhanced Ca²⁺ wave frequency in single atrial myocytes to AF inducibility in mice. Moreover, our data provide proof-of-concept for therapeutic efficacy of RyR2 inhibition with *ent*-verticilide in Pitx2-linked AF risk.

4.1. Dysfunction of RyR2-mediated Ca²⁺ handling for atrial arrhythmogenesis in Pitx2 deficiency

Human GWAS studies have identified sequence variation within 4q25 as conferring increased susceptibility to AF [13,14,36,37]. The Pitx2 gene is in this region and considered a prime candidate for the development of atrial arrhythmias. Consistent with the hypothesis that loss of function in Pitx2 causes AF risk, heterozygous deletion of Pitx2 in mice causes an increased tendency of atrial flutter and tachycardia, with reduced atrial action potential duration postulated as a mechanism of AF induction [16,17]. Interestingly, we find increased AF risk in Pitx2 mice without a reduction in action potential duration *in vivo* (as evidenced by the normal effective atrial refractory period, Table 2). As such, other mechanisms are probably important. It is known that Pitx2 plays a key role in left-right asymmetry and has regulatory effects on gene networks including microRNAs, and membrane effector genes [38]. Especially, Pitx2 modulates microRNAs and thereby regulates distinct ion channel, gap junction cell-cell communication and beta-adrenergic signaling, resulting in electrical remodeling and calcium abnormalities in humans with 4q25 variants and in mutant mouse models [20,39]. Moreover, Pitx2 has been linked to defective Ca²⁺ homeostasis such as regulating Ca²⁺-handling gene expression via Wnt signaling, increased SERCA2a expression, SR Ca²⁺ load, and RyR2 phosphorylation [18,21], which is pointing to a pivotal role of Pitx2 for AF associated with excessive spontaneous Ca²⁺ release-induced electrical activity. Therefore, we focused in our study on the role of RyR2 for AF inducibility in this mouse model. Our results show that the frequency of Ca²⁺ sparks and RyR2-mediated Ca²⁺ leak is significantly increased in isolated atrial myocytes from Pitx2^{+/-} mice (Fig. 1), which is the first direct evidence of RyR2 hyperactivity in the Pitx2^{+/-} mouse model. In addition, we find that the abnormal RyR2 activity increased diastolic Ca²⁺ release events in intact Pitx2^{+/-} atrial myocytes (Fig. 3). Although we did not directly examine the molecular mechanism for RyR2 hyperactivity here, it is well established that diastolic SR Ca²⁺ leak can be mediated by enhanced RyR2 phosphorylation [21,40,41], increased RyR2 expression [42–44], or SR Ca²⁺ overload [44], which have all been documented in the Pitx2 mouse model [18,20], and which are key contributors to atrial arrhythmogenesis in humans with different forms of AF. Spontaneous SR Ca²⁺ release via RyR2 activates the electrogenic Na⁺-Ca²⁺ exchanger (NCX), resulting in a depolarizing transient inward current that is referred to as delayed afterdepolarization (DAD). DADs can trigger premature action potentials leading to proarrhythmic ectopic activity, and hence AF [45]. Generally, the amplitude of Ca²⁺ transient is dynamically regulated and depends on both the RyR2 open probability and the SR Ca²⁺ content. Our data show that the SR Ca²⁺ content was not changed, whereas the Ca²⁺ transient amplitude was increased (Fig. 3), indicating that the

increased spontaneous Ca²⁺ release events are caused by increased RyR2 open probability rather than SR Ca²⁺ overload [46].

Our transesophageal burst pacing protocol shows a significant increase on AF inducibility in Pitx2 deficient mice. In an AF mouse model that has increased SR Ca²⁺ leak caused by RyR2 mutations, the prevalence of pacing-induced AF was not suppressed by beta-blocker, which was interpreted as evidence that catecholamines did not contribute importantly in triggering AF [8]. In contrast, our data show more frequent and persistent AF on catecholaminergic challenge with isoproterenol in the Pitx2^{+/-} mouse model. This result supports the hypothesis that Pitx2 plays a more complex role beyond RyR2 regulation, which is consistent with Pitx2's well-established regulatory roles in heart development, as well as in a highly complex gene regulatory network including beta-adrenergic signaling [17,18,39]. Nevertheless, our results demonstrate that targeting RyR2 hyperactivity is an effective approach for reducing AF susceptibility in Pitx2 deficiency.

4.2. Effect of RyR2 inhibition with *ent*-verticilide on AF susceptibility

RyR2 dysfunction due to its hyperactivity, mutations, and abnormal regulation by its binding partners, typically manifest as increased SR Ca²⁺ leak and spontaneous Ca²⁺ release events, which ultimately increase AF susceptibility [37,40–42]. This establishes a critical role of RyR2 in atrial arrhythmia risk in humans and mice. Consequently, stabilizing RyR2 and preventing diastolic Ca²⁺ leak could attenuate AF inducibility. Our experimental study here provides proof of concept for this approach in a genetic model of AF susceptibility due to Pitx2 haploinsufficiency.

AF is the most prevalent cardiac arrhythmia requiring antiarrhythmic drug therapy. Rhythm-control therapy has a beneficial effect when the treatment begins early. Current FDA-approved anti-arrhythmic drugs targeting RyR2 have short-term efficacy, but carry substantial risks when used long-term, particularly drug-induced pro-arrhythmia. For instance, an antiarrhythmic drug currently in clinical use, flecainide, effectively suppresses RyR2-mediated spontaneous Ca²⁺ release, but it also blocks a Na channel [47,48]. In addition, the dual RyR2 and Na channel inhibitor R-propafenone prevents AF induction through reduction of spontaneous Ca²⁺ elevation in mouse model compared to S-propafenone (no RyR2 inhibition) [7]. Unfortunately, when tested in humans, both R- and S-propafenone increase the rate of inducible atrial flutter due to Na channel blockade [49]. We previously reported that *ent*-verticilide is effective against ventricular arrhythmias triggered by RyR2-mediated Ca²⁺ release and is more potent as an inhibitor of RyR2 compared with the benchmark compounds dantrolene, tetracaine, and flecainide [22]. Hence, we here tested *ent*-verticilide for efficacy against arrhythmogenic spontaneous Ca²⁺ release and pacing-induced AF. Consistent with our previous study in ventricular cardiomyocytes, we observed the reduction of both spontaneous Ca²⁺ release events and Ca²⁺ transient amplitude in the presence of *ent*-verticilide in intact Pitx2^{+/-} atrial myocytes, without changing the other Ca²⁺ parameters (Figs. 2, 3 and Table 1), demonstrating that *ent*-verticilide selectively inhibits RyR2 hyperactivity without altering SERCA2a function and SR Ca²⁺ content. *ent*-Verticilide inhibition of the spontaneous Ca²⁺ release *in vitro* translated into activity against pacing-induced AF *in vivo* (Fig 5), indicating that *ent*-verticilide effectively attenuates the

susceptibility of atrial, as well as ventricular arrhythmia, by selective inhibition of RyR2-mediated Ca²⁺ release. Furthermore, since RyR2 inhibition with *ent*-verticilide saturates at 25% [22] and does not reduce skeletal muscle function, it has a large therapeutic window *in vivo* [50]. Our experimental data demonstrates that pathophysiological RyR2-mediated Ca²⁺ release triggers pacing-induced AF, and *ent*-verticilide prevents the initiation of AF by selective targeting the RyR2 channel in mouse model. However, research to support the *ent*-verticilide pharmacological relevance in AF patients is still needed.

In summary, the reduction in AF burden and incidence by the RyR2 specific inhibitor *ent*-verticilide supports the hypothesis that diastolic Ca²⁺ leak by resting RyR2 hyperactivity is responsible for AF susceptibility in the setting of Pitx2 deficiency. These results suggest a potential therapeutic role for using a selective RyR2 inhibitor such as *ent*-verticilide to prevent AF.

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Non-standard Abbreviations and Acronyms

RyR2	Cardiac ryanodine receptor
Pitx2	Paired-like homeodomain transcription factor 2
AF	Atrial fibrillation
SR	Sarcoplasmic reticulum
Ca²⁺	Calcium
WT	Wild type
CPVT	Catecholaminergic polymorphic ventricular tachycardia
GWAS	Genome-wide association studies
DAD	Delayed afterdepolarization
NT	Normal Tyrode
SERCA2a	Sarcoplasmic reticulum Ca ²⁺ -ATPase
ECG	Electrocardiogram
SCR	Spontaneous Ca ²⁺ release
TE	Transesophageal
AV	Atrioventricular

WCL	Wenckebach cycle length
AVERP	Atrioventricular effective refractory period
NCX	Sodium-Calcium exchanger

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Highlights

- Pitx2 deficiency causes RyR2 hyperactivity and increased Ca²⁺ spark leak in permeabilized atrial myocytes and atrial fibrillation susceptible in vivo
- Increased spontaneous Ca²⁺ release events in Pitx2 deficient atrial cardiomyocytes were prevented by RyR2 inhibition with *ent*-verticilide
- Adrenergic stimulation aggravated atrial fibrillation susceptibility in Pitx2 deficient mice
- RyR2 inhibition with *ent*-Verticilide effectively prevented pacing-induced atrial fibrillation

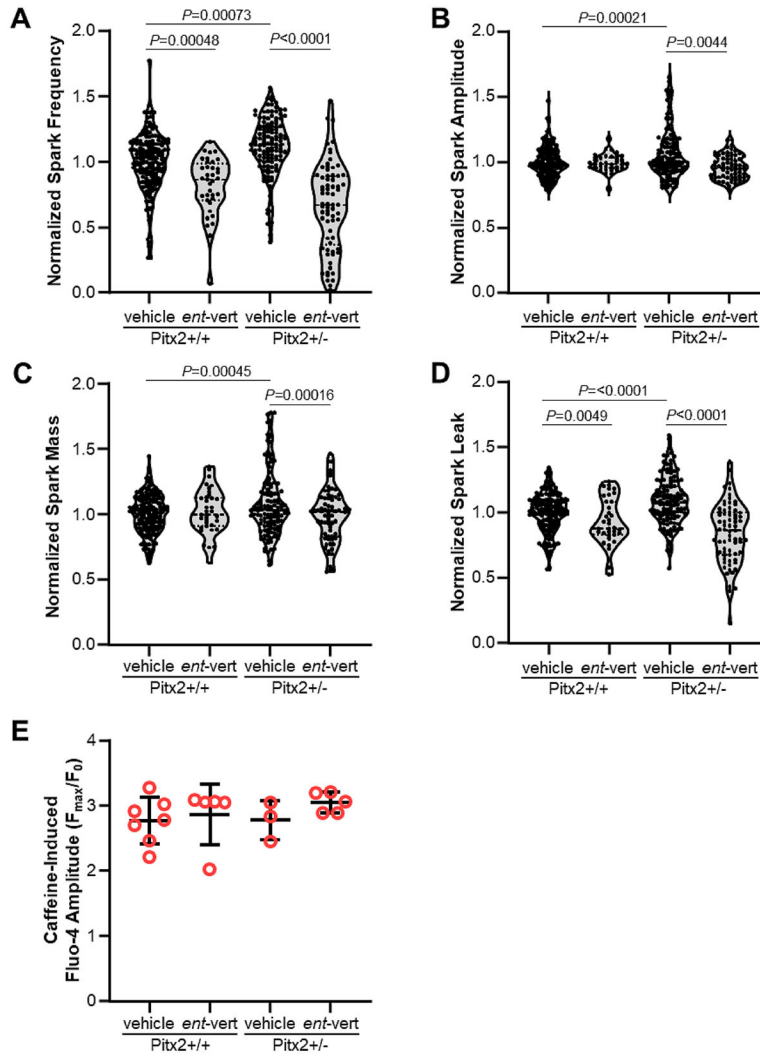


Figure 1. Ca²⁺ spark properties in permeabilized atrial cardiomyocytes. (A) Ca²⁺ spark frequency, (B) amplitude, (C) mass, and (D) leak. Figure panels shown with full range of values and a box bounding the 25th and 75th percentiles with median. N: Pitx2^{+/+} (vehicle) = 168 cells from 6 mice; Pitx2^{+/+} (*ent-vert*) = 36 cells from 2 mice; Pitx2^{+/-} (vehicle) = 127 cells from 6 mice; Pitx2^{+/-} (*ent-vert*) = 70 cells from 4 mice normalized to values from ^{+/+} mice by day of experiment. Bonferroni-adjusted *P* values calculated using hierarchical clustering. (E) SR load measured by caffeine-induced Ca²⁺ transient amplitude. Individual values with mean ± SD.

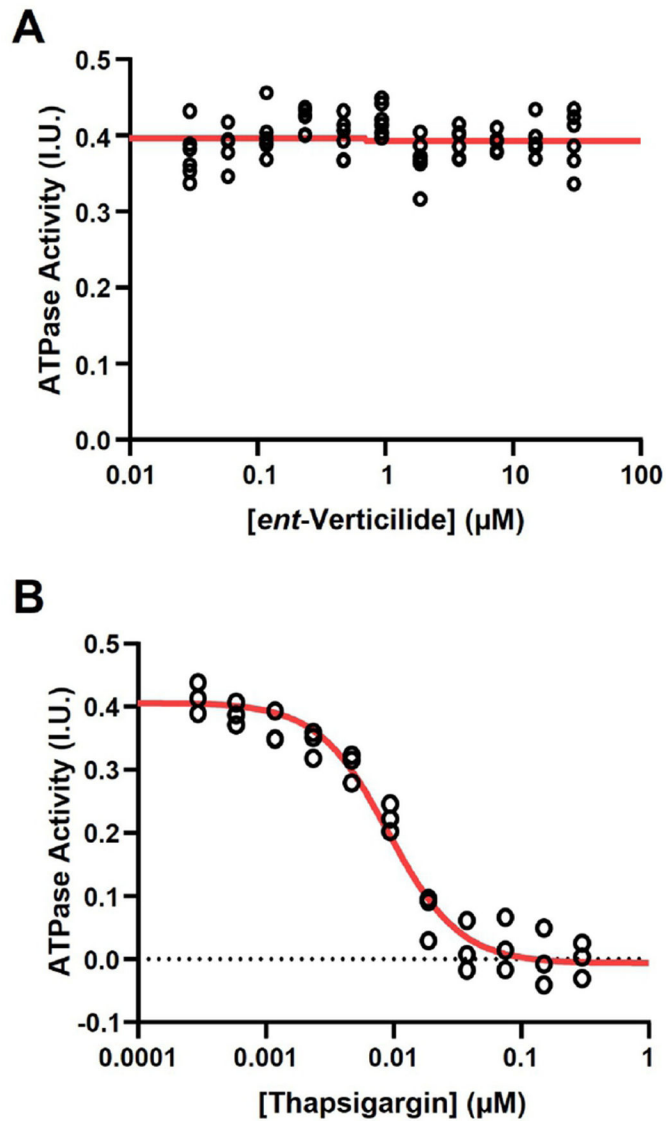


Figure 2. *ent*-Verticilide did not inhibit cardiac sarco(endo)plasmic reticulum Ca²⁺ ATPase (SERCA2a) function.

(A) SERCA2a ATPase activity (calculated as $V_{pCa5.4} - V_{pCa8}$) in the presence of *ent*-verticilide at the indicated concentrations. N = 6 replicates per concentration tested. (B) Positive control: SERCA ATPase activity in the presence of thapsigargin (SERCA2a inhibitor) at the indicated concentrations. N = 3 replicates at each concentration tested. Red lines show the result of fitting the equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + (\text{IC}_{50}/X)^{\text{HillSlope}})$.

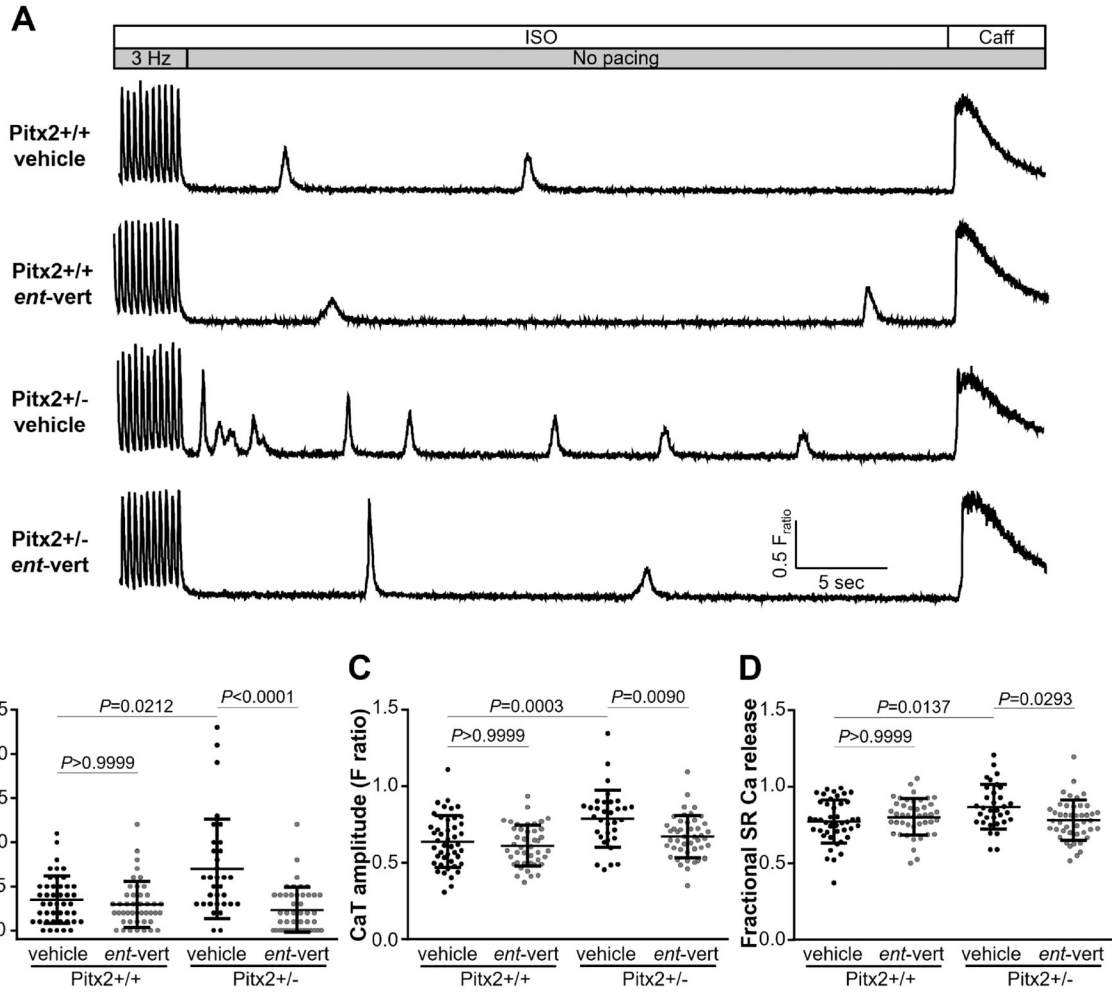


Figure 3. *ent*-Verticilide reduced spontaneous Ca²⁺ release (SCR) in Pitx2^{+/-} atrial myocytes. Isolated intact atrial myocytes were pre-treated with vehicle or *ent*-verticilide for 2.5 hours and loaded with Fura-2 acetoxyethyl to measure spontaneous Ca²⁺ release (SCR) events. (A) Representative Ca²⁺ transient from intact atrial myocytes. Atrial myocytes were field-stimulated at 3 Hz for 20 s followed by 40 s recording of SCR events in 1 μM isoproterenol (ISO) containing Tyrode solution. Application of 10 mM caffeine (Caff) was used to measure SR Ca²⁺ content. Summary data of SCR events (B), Ca²⁺ transient amplitude (C), and fractional SR Ca²⁺ release (D). Data are mean ± SD. n=45, 44, 33 and 45 cells from 4–6 mice in each group. P values calculated using ANOVA multiple comparisons with Dunn’s or Bonferroni’s test.

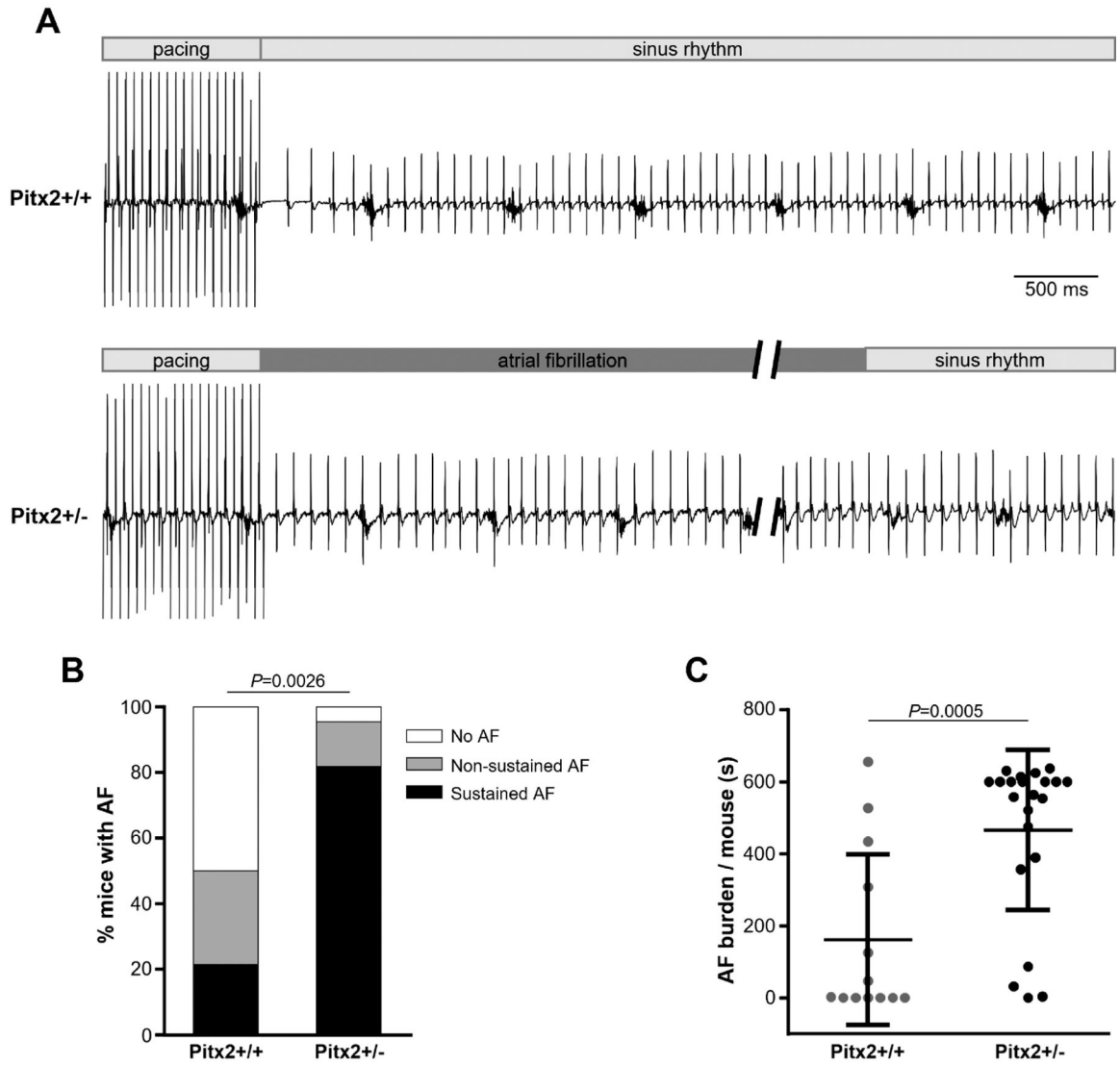


Figure 4. Pitx2^{+/-} mice exhibited increased susceptibility to atrial fibrillation (AF) by transesophageal atrial burst pacing.

(A) Representative ECG traces of littermate WT (upper) and Pitx2^{+/-} (lower) mice after an episode of burst pacing protocol in the presence of isoproterenol (1.5 mg/kg, i.p. injection). Pitx2^{+/-} mice exhibit inducible AF followed by spontaneous conversion to sinus rhythm. (B) Incidence of inducible AF (+/+ 50.0 % vs. +/- 95.5 %; non-sustained AF + sustained AF). Sustained AF was defined as continuous AF over 300 sec induced by any single pacing train (+/+ 21.4 % vs. +/- 81.8 %, $P=0.0005$). P values were obtained using Fisher's exact test. (C) Summary of total AF burden in WT (N=14 mice) and Pitx2 deficiency (N=22 mice). Data are mean \pm SD. P values were obtained using the Mann-Whitney test.

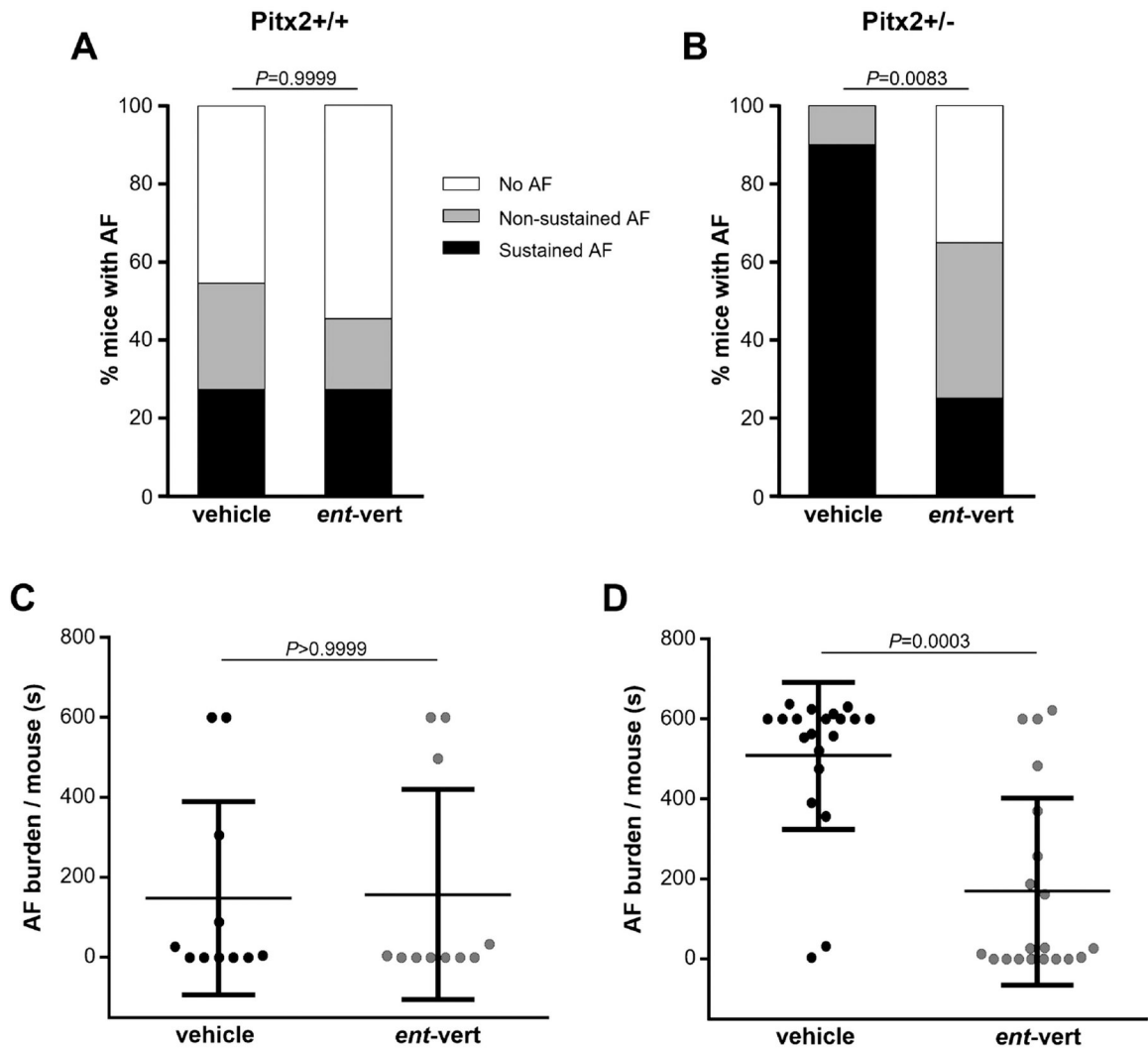


Figure 5. Anti-arrhythmic efficacy of *ent-verticilide* on pacing-induced AF in both *Pitx2^{+/+}* and *Pitx2^{+/-}* mice.

(A, B) Incidence of inducible AF (*Pitx2^{+/+}*: vehicle 54.6 % vs. *ent-vert* 45.5 %, *Pitx2^{+/-}*: vehicle 100.0 % vs. *ent-vert* 65.0 %; non-sustained AF + sustained AF) by burst pacing stimulation in *Pitx2^{+/+}* and *Pitx2^{+/-}* mice. Sustained AF was defined as continuous AF over 300 sec induced by any single pacing train. *ent-Verticilide* significantly reduced sustained AF compared to vehicle group in *Pitx2^{+/-}* mice (vehicle 90.0 % vs. *ent-vert* 25.0 %, $P<0.0001$). (C, D) Summary of total AF burden in *Pitx2^{+/+}* and *Pitx2^{+/-}* mice (N=11 and 20 mice, respectively). Data are mean \pm SD. P values were obtained using Fisher's exact test and Wilcoxon matched-pairs signed rank test.

Table 1.
Intracellular Ca²⁺ measurements in intact atrial myocytes.

Ca²⁺ handling parameters of Fura-2 acetoxymethyl-ester loaded atrial myocytes paced at 3 Hz followed by caffeine (10 mM) application. n=45 cells for +/+ vehicle, 44 cells for +/+ *ent*-verticilide, 33 cells for +/- vehicle, and 45 cells for +/- *ent*-verticilide. All data displayed as mean \pm SD and analyzed using ANOVA multiple comparisons with Bonferroni-adjusted *P* values. CaT – pacing-induced Ca²⁺ transient, Caff.T – caffeine-induced Ca²⁺ transient

	(A) Pitx2+/+ vehicle (n=45)	(B) Pitx2+/+ <i>ent</i> -vert (n=44)	(C) Pitx2+/- vehicle (n=33)	(D) Pitx2+/- <i>ent</i> -vert (n=45)	<i>P</i> values		
					(A) vs (B)	(A) vs (C)	(C) vs (D)
Diastolic Ca²⁺ (F_{Ratio})	0.60 \pm 0.07	0.56 \pm 0.06	0.60 \pm 0.09	0.59 \pm 0.05	0.10	>0.99	>0.99
Time to peak (ms)	30.79 \pm 5.04	29.45 \pm 3.07	29.17 \pm 3.03	29.36 \pm 4.12	0.78	0.54	>0.99
CaT decay rate constant (ms)	114.2 \pm 23.0	123.6 \pm 24.6	114.1 \pm 14.7	114.9 \pm 18.8	0.24	>0.99	>0.99
SR Ca²⁺ content (F_{Ratio})	0.83 \pm 0.17	0.78 \pm 0.16	0.91 \pm 0.15	0.86 \pm 0.09	0.89	0.10	>0.99
Caff.T decay rate constant (s)	2.95 \pm 0.27	2.85 \pm 0.38	2.85 \pm 0.32	2.99 \pm 0.32	>0.99	>0.99	0.53

Table 2.
Baseline cardiac electrophysiological parameters in Pitx^{+/+} and Pitx^{2+/-} mice

ECG, electrocardiogram; RR, time interval between two consecutive R wave peaks; PR, interval from the onset of P wave to the start of QRS complex; QRS, time interval from start of Q wave to end of S wave; QT, interval from start of Q wave to end of T wave; AVERP, atrioventricular nodal effective refractory period; WCL, Wenckebach cycle length. All data displayed as mean \pm SD and analyzed using unpaired *t*-test.

ECG intervals (ms)	Pitx ^{2+/+} (n=14 mice)	Pitx ^{2+/-} (n=22 mice)	<i>P</i> values
Heart rate	564.2 \pm 27.1	563.1 \pm 33.4	0.914
RR	106.6 \pm 5.3	106.9 \pm 6.3	0.869
PR	35.6 \pm 1.8	34.9 \pm 2.1	0.293
QRS	10.4 \pm 0.5	10.5 \pm 0.8	0.755
QT	46.9 \pm 1.9	47.8 \pm 2.4	0.270
AVERP	44.6 \pm 3.6	45.8 \pm 3.7	0.324
WCL	71.7 \pm 2.9	70.5 \pm 3.3	0.290

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