Arrhythmogenic Mechanisms in a Mouse Model of Catecholaminergic Polymorphic Ventricular Tachycardia

Marina Cerrone,* Sami F. Noujaim,* Elena G. Tolkacheva, Arkadzi Talkachou, Ryan O’Connell, Omer Benfenfeld, Justus Anumonwo, Sandeep V. Pandit, Karen Vikström, Carlo Napolitano, Silvia G. Priori, José Jalife

Abstract—Catecholaminergic polymorphic ventricular tachycardia (VT) is a lethal familial disease characterized by bidirectional VT, polymorphic VT, and ventricular fibrillation. Catecholaminergic polymorphic VT is caused by enhanced Ca2+ release through defective ryanodine receptor (RyR2) channels. We used epicardial and endocardial optical mapping, chemical subendocardial ablation with Lugol’s solution, and patch clamping in a knockin (RyR2/RyR2R4496C) mouse model to investigate the arrhythmogenic mechanisms in catecholaminergic polymorphic VT. In isolated hearts, spontaneous ventricular arrhythmias occurred in 54% of 13 RyR2/RyR2R4496C and in 9% of 11 wild-type (P=0.03) littermates perfused with Ca2+ and isoproterenol; 66% of 12 RyR2/RyR2R4496C and 20% of 10 wild-type hearts perfused with caffeine and epinephrine showed arrhythmias (P=0.04). Epicardial mapping showed that monomorphic VT, bidirectional VT, and polymorphic VT manifested as concentric epicardial breakthrough patterns, suggesting a focal origin in the His–Purkinje networks of either or both ventricles. Monomorphic VT was clearly unifocal, whereas bidirectional VT was bifocal. Polymorphic VT was initially multifocal but eventually became reentrant and degenerated into ventricular fibrillation. Endocardial mapping confirmed the Purkinje fiber origin of the focal arrhythmias. Chemical ablation of the right ventricular endocardial cavity with Lugol’s solution induced complete right bundle branch block and converted the bidirectional VT into monomorphic VT in 4 anesthetized RyR2/RyR2R4496C mice. Under current clamp, single Purkinje cells from RyR2/RyR2R4496C mouse hearts generated delayed afterdepolarization–induced triggered activity at lower frequencies and level of adrenergic stimulation than wild-type. Overall, the data demonstrate that the His–Purkinje system is an important source of focal arrhythmias in catecholaminergic polymorphic VT. (Circ Res. 2007;101:1039-1048.)

Key Words: ryanodine receptor ■ CPVT ■ transgenic mice ■ bidirectional ventricular tachycardia ■ sudden cardiac death

Catecholaminergic polymorphic ventricular tachycardia (CPVT) (Online Mendelian Inheritance in Man no. 604772) is an inherited disease leading to arrhythmias and sudden cardiac death.1 The autosomal dominant form has been linked to ryanodine receptor gene (RyR2) mutations, leading to increased spontaneous Ca2+ release from the sarcoplasmic reticulum.2 Typical arrhythmias are bidirectional ventricular tachycardia (BVT) and polymorphic ventricular tachycardia (PVT) that can degenerate into ventricular fibrillation (VF) and thus sudden cardiac death.3 BVT is infrequent, characterized by beat-to-beat 180° alternation of the QRS of the ECG and occurs in CPVT, as well as in digitalis toxicity; thus, it has been inferred that arrhythmogenesis in CPVT is mediated by delayed afterdepolarization (DAD)-induced triggered activity (TA).

Mice heterozygous for the R4496C mutation (RyR2/RyR2R4496C) recapitulate the human phenotype of CPVT by developing BVT, PVT, and/or VF under adrenergic stimulation.4 Recently, Liu et al5 have demonstrated DADs in RyR2/RyR2R4496C mouse ventricular myocytes both in control and in the presence of isoproterenol. However, it remains to be demonstrated whether the arrhythmia originates in the 3D myocardium or in the cable-like Purkinje fiber network. In addition, it is unknown whether the transition from BVT into PVT and VF involves triggered or reentrant mechanisms.

Here we used the RyR2/RyR2R4496C mouse to investigate arrhythmia mechanisms associated with elevated extracellular calcium ([Ca2+]o) and/or catecholaminergic stimuli. Our main objective was to test the idea that arrhythmias in this mouse model, and by inference in CPVT patients, are...
triggered by DADs occurring in Purkinje fibers on the right and left branches of the specialized ventricular conducting system.

**Materials and Methods**

**Animals**

This study conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication no. 85-23, revised 1996). Details of the production of knockin RyR2/RyR2R4496C mice have been published.6

**Optical Mapping Experiments**

Thirty heterozygous RyR2/RyR2R4496C (16 males; 4.2 ± 1 month) and 21 wild-type (WT) (10 males, 4.5 ± 0.9 months) littermates were used. Epicardial and endocardial optical mapping was performed in isolated, Langendorff-perfused hearts. Volume-conducted ECG was recorded; activation and phase maps were generated (see the online data supplement at http://circres.ahajournals.org).6–10

Two different protocols were used to induce ventricular arrhythmias. First, for epicardial mapping, 13 RyR2/RyR2R4496C and 11 WT hearts were perfused with Tyrode’s solution containing 2.7 to 3.6 mmol/L Ca2+ and 100 to 200 nmol/L isoproterenol. Five additional RyR2/RyR2R4496C hearts were perfused with the abovementioned drug-containing solution for endocardial mapping. Second, 12 RyR2/RyR2R4496C and 10 WT hearts were perfused with Tyrode’s solution containing caffeine (1 to 5 mmol/L) and epinephrine (0.1 to 1.6 μmol/L).

**Chemical Subendocardial Ablation**

Mice were anesthetized with Avertin (Sigma) and ventilated through a tracheostomy. Lead I ECG was recorded. IP caffeine (120 mg/kg) and epinephrine (2 mg/kg) were injected as described previously4; several minutes were allowed for arrhythmia initiation.5 Subsequently, a bolus of Lugol’s solution (5 to 7 mL; Humco) or Tyrode’s solution was carefully injected directly into the right ventricular (RV) cavity using a Hamilton syringe inserted through the diaphragm from a minimal abdominal incision. Six RyR2/RyR2R4496C mice (4 females; 5.5 ± 3.8 months) were used in these experiments. For control, we injected 5 to 7 μL of Lugol’s solution in 3 WT (2 females; 4.3 ± 0.5 months) mice and 5 to 7 μL of normal Tyrode’s solution in another set of 3 WT mice (2 females; 4.3 ± 0.5 months) during sinus rhythm (SR). See the online data supplement for details.

**Purkinje Cell Recordings**

Adult RyR2/RyR2R4496C and WT mouse Purkinje cells were obtained by enzymatic dissociation.11 Under whole-cell current-clamp conditions, action potentials were elicited by 5-ms stimuli at ~2X threshold amplitude; resting membrane potential, action potential amplitude, action potential duration (APD90,30,50), and dV/dtmax were determined. DADs and TA were induced by trains of 20 pulses at 1, 5, 10, and 20 Hz in control and in the presence of 30 nmol/L isoproterenol. See the online data supplement for details.

**Statistical Analyses**

Student t test was used to compare normally distributed variables. Cross tabulation with Fisher’s exact test was used for categorical variables. The data are presented as means ± SD. We used the SPSS (version 15.0) or the Origin (version 7.0) statistical packages.

**Results**

**Normal Sequence of Ventricular Activation**

Cerrone et al4 demonstrated that ECG patterns of PVT and BVT may be obtained in RyR2/RyR2R4496C mice under conditions that closely resemble those in CPVT patients. However, understanding arrhythmia mechanisms requires a clear knowledge of the normal sequence of ventricular epicardial activation of the anterior surface of the heart. Figure 1A and 1B shows representative activation maps and volume-conducted ECGs (approximate Lead II) obtained, respectively, from WT and RyR2/RyR2R4496C hearts during SR. C and D in Figure 1 are the mean activation maps of 4 WT (C) and 5 RyR2/RyR2R4496C (D) hearts. These maps show the high reproducibility of the breakthrough patterns, which are nearly identical in the 2 genotypes and similar to those reported for human12 and mouse.6–7,9 Epicardial activation starts with 2 quasismultaneous concentric breakthroughs on the anterior RV and left ventricular (LV) free walls, at sites corresponding to the endocardial insertion of the major Purkinje network branches (see the online data supplement). The wavefronts emanating from such breakthroughs merge at the septum and then propagate upward to activate the rest of the ventricular walls. Thus, in the absence of external stimuli, the isolated RyR2/RyR2R4496C mouse heart shows no abnormalities of ventricular excitation or propagation during SR.

**Ventricular Arrhythmias**

Spontaneous ventricular arrhythmias occurred in 7 of 13 (54%) RyR2/RyR2R4496C and 1 of 11 (9%) WT hearts (P = 0.03) perfused with high Ca2+ and isoproterenol. A total of 41 arrhythmic episodes occurred in RyR2/RyR2R4496C hearts (mean duration, 24±76 seconds). These included 1 episode of BVT, 33 episodes of PVT, and 7 episodes of alternating monomorphic VT (MVT) and PVT. Furthermore, 6 mutant hearts showed 11 episodes of ventricular couplets, with a mean duration of 1.68±0.67 seconds.

Only 1 WT heart had ventricular arrhythmias in this protocol. Of a total of 16 episodes in this heart, 12 were
PVT, 2 were MVT, and 2 alternated between PVT and MVT. The mean duration of the episodes was 26±60 seconds (P=NS versus mutants). The same heart presented 5 episodes of ventricular couplets (mean duration, 2.72±1.78 seconds).

Of hearts perfused with caffeine and epinephrine, 8 of 12 (66%) RyR2/RyR2R4496C and 2 of 10 (20%) WT (P=0.04) showed spontaneous arrhythmias. Twenty arrhythmic episodes occurred in the RyR2/RyR2R4496C hearts, with a mean duration of 62±156 seconds. Of these, 16 were PVT, 2 were focal MVT, and 2 were VF with a mechanism that was clearly reentrant, as demonstrated by the presence of a stable rotor (see below). Four hearts presented 11 episodes of ventricular couplets, with a mean duration of 15.3±36.8 seconds. Of 7 arrhythmic episodes in WT hearts (mean duration, 13±11 seconds), 1 was MVT and 6 were PVT. One WT heart showed 6 episodes of couplets (mean duration, 45.7±52.3 seconds). BVT was not documented in WT hearts.

As a further confirmation of the site of origin of arrhythmic foci in the RyR2/RyR2R4496C hearts, we performed endocardial optical mapping with direct imaging of the Purkinje network in 5 mutant hearts, perfused with 2.7 mmol/L Ca²⁺ and 200 nmol/L isoproterenol. MVT, PVT, and ventricular couplets were recorded and mapped in 3 hearts.

MVT Is Attributable to a Focal Source in the Purkinje Network

In Figure 2, we show examples of MVT occurring in hearts perfused with high Ca²⁺ and 200 nmol/L isoproterenol. The red curves are the upstrokes of the optical single-pixel recording (OSPR) at the earliest activation site during SR and MVT. The black traces are the ECGs, recorded simultaneously. In Figure 2A, the epicardial SR breakthroughs are similar to those in Figure 1. Note that in this experiment, the OSPR corresponding to the asterisk on the RV occurred slightly later than the onset of the QRS. On average (n=4), this signal started 4.3±1 ms before the QRS. In Figure 2B, the ECG is that of an MVT (cycle length=40 ms), where the P waves are absent. The multiphasic QRS complex is suggestive of a left bundle branch block. On the map, an epicardial activation wave appeared repetitively as a highly localized breakthrough on the anterior wall of the RV. Here again, the upstroke of the OSPR of the earliest activation site during SR occurred slightly later than the onset of the QRS. On average (n=4), this signal started 4.3±1 ms before the QRS. In Figure 2C, the ECG is that of an MVT (cycle length=40 ms), where the P waves are absent. The multiphasic QRS complex is suggestive of a left bundle branch block. On the map, an epicardial activation wave appeared repetitively as a highly localized breakthrough on the anterior wall of the RV. This concentric breakthrough pattern was similar to that emerging from the RV during SR. Here again, the upstroke of the OSPR of the earliest activation site in the breakthrough appeared 1.4±1.5 ms (n=4) before the QRS. Figure 2C was obtained from an experiment in which the RV cavity was opened for endocardial mapping during SR. As expected, the initial breakthrough occurred at the His bundle, and the impulse propagated down the right bundle branch (RBB) on the septal
wall to reach the anterior papillary muscle within 4 ms. The endocardial OSPR demonstrated that the upper RBB activated appreciably earlier than the appearance of the QRS. On average (n=4), upper RBB activation occurred 29±1.8 ms before the QRS (P<0.001 versus epicardial SR). Figure 2D was from a different open-RV heart, during MVT (cycle length=50 ms). On the RV endocardial activation map, this tachycardia originates as a focus that fires repetitively from the same location. The upstroke of the OSPR at the asterisk occurred much earlier than the QRS inscription on the lower ECG. On average (n=4) during MVT, endocardial activation occurred 14.5±1.8 ms before the QRS (P<0.001 versus epicardial MVT).

Altogether, the data in Figure 2 demonstrate that the focus responsible for MVT is much closer to the endocardium than the epicardium. The long delay between the endocardial discharge at the OSPR and the onset of the QRS complex likely represents the propagation time from the focus at a distal RV Purkinje fiber, transmurally to the epicardium and to the rest of the ventricular wall.

Bidirectional Tachycardia

One of our objectives was to determine the dynamics of wave propagation that underlie the alternating QRS morphology of BVT in the RyR2/RyR2<sup>R4496C</sup> mouse. Figure 3 shows results from the experiment in which this was done successfully. Figure 3A is the fluorescent image of the heart. Figure 3B depicts data in SR. The ECG shows 10 sinus beats at a cycle length of 179 ms, and the isochrone map illustrates the normal sequence of ventricular epicardial activation during a single beat. The pattern is characterized by 2 focal breakthroughs (asterisks) that originate almost simultaneously on the free walls of the right and left ventricles. The breakthroughs form wavefronts that fully activate the field of view within 2.5 ms (see also Figures 1 and 2B). As discussed previously, these breakthroughs are the sites of epicardial emergence of sinus impulses from the distal ends of the RBB and left bundle branch across the ventricular walls. Figure 3C shows the same RyR2/RyR2<sup>R4496C</sup> mouse heart in the presence of 2.7 mmol/L Ca<sup>2+</sup> and 100 nmol/L isoproterenol. The ECG shows a classic example of BVT with 180° beat-to-beat alternations in the QRS. As shown by the map obtained during beat 1, upward QRS complexes coincided with repetitive, highly localized epicardial breakthroughs on the lateral wall of the RV, with the wavefront propagating slowly toward the LV. In contrast, downward QRS deflections (2) were associated with LV breakthroughs and slow propagation toward the RV. These results strongly support our hypothesis that BVT in RyR2/RyR2<sup>R4496C</sup> mice is triggered by DADs occurring alternately at specific Purkinje fibers on the RBB and left bundle branch.

In addition to BVT, in both protocols, RyR2/RyR2<sup>R4496C</sup> hearts showed ventricular bigeminal couplets, defined as pairs of QRS complexes with alternating morphology followed by a relatively long pause. This arrhythmic pattern is similar to that of BVT, with the exception that, in this case, each couplet is followed by a long pause (see the online data supplement).
Multifocal PVT
In Figure 4, we mapped the endocardial activation of each of 9 consecutive QRS complexes in an episode of nonsustained multifocal PVT (mean cycle length, 35 ms) induced by 2.7 mmol/L Ca$^{2+}$ and 200 nmol/L isoproterenol in an isolated RyR2/RyR2R4496C mouse heart. In Figure 4A, high-resolution image analysis of the initial site of activation for each QRS complex (bottom) demonstrated 8 different foci on the RV septal wall. Beat 5 originated outside the field of view. The ECG shows absence of P waves and varying QRS morphologies from beat to beat reflecting the multifocal origin of the arrhythmia. Figure 4B shows enlarged views of the two areas boxed in A. The image contrast in these areas was enhanced to visualize the Purkinje fibers. Each colored number shows that the initial site of activation for the respective QRS complexes corresponds to the location of a distal, free-running Purkinje fiber. These results establish that PVT in this model is attributable to focal discharges at multiple locations within the specialized conduction system.

PVT to VF Transition
The cause of sudden cardiac death in CPVT patients is degeneration of BVT or PVT into VF. In some RyR2/RyR2R4496C mouse hearts, we documented the transition from PVT to VF. In Figure 5, perfusion with Tyrode’s solution (1.8 mmol/L Ca$^{2+}$; 1 mmol/L caffeine) containing 1 mmol/L epinephrine (1.6 μmol/L) initiated a run of PVT, which manifested as repetitive multifocal
RV epicardial breakthroughs and deteriorated into VF. Figure 5, top left, shows three 10-ms snapshots from the phase map during 1 focal discharge. The middle 3 images show similar maps obtained during the transition from focal to reentrant activity brought about by a wave break formed between the first and second frames. In this episode, the focal activity lasted 1.1 second (frequency, 39 Hz) and the rotor lasted 1.7 second (frequency, 43 Hz). The maps on the bottom demonstrate that VF was maintained by a high-frequency rotor throughout the remainder of the episode (see the recording to the right of the figure).

Subendocardial Ablation Terminates BVT

At least 4 different groups of investigators13–17 have used selective destruction of the Purkinje network by Lugol as a means to determine the importance of the specialized conducting system in the activation of the ventricular endocardium and epicardium and in the transition to VF. Thus, to establish the role of the Purkinje network in the mechanism of BVT, Lugol’s solution was directly injected into the RV cavity of anesthetized WT and RyR2/RyR2R4496C mice. We used anesthetized mice in consideration of the rare occurrence of BVT in the isolated Langendorff-perfused heart. The mice underwent the same protocol of induction of arrhythmias that was used in the previous studies4–5; ie, caffeine (120 mg/kg) and epinephrine (2 mg/kg) IP.

As shown in Figure 6A, when 5 to 7 μL of Lugol’s solution was injected into the RV of an anesthetized WT mouse during SR, the QRS widened as a result of RBB block and the PR interval prolonged slightly, but the SR pattern and the R-R interval were unchanged. QRS prolongation under these conditions was clearly visualized by comparing the complexes shown on the top of Figure 6C at baseline (left) and after Lugol injection (right). In 3 WT mice, the mean QRS duration was 16.6±1.3 ms at baseline, and it increased to 26.8±3.2 ms after Lugol injection (P<0.001). As an additional control, in yet another group of 3 anesthetized WT mice, we injected 5 to 7 μL of normal Tyrode’s solution into the RV cavity. No changes in QRS width were recorded (baseline: 16.5±1.9 versus 16.8±1.9 ms after Tyrode’s solution; P=NS).

We induced BVT in all 4 anesthetized RyR2/RyR2R4496C mice. Figure 6B shows a representative example. The top
ECG was obtained during SR. The middle trace, recorded a few minutes after IP injection of caffeine and epinephrine, shows a classic pattern of BVT with narrow QRS and alternating QRS axis. As clearly illustrated by the bottom trace, in all the cases, after carefully injecting 5 to 7 μL of Lugol’s solution into the RV cavity, BVT was converted to MVT with wide QRS and RBB block (see also bottom traces in Figure 6C). Statistical analysis demonstrated that the QRS widening was highly significant. The mean QRS duration recorded in the RyR2/RyR2R4496C mice in SR was 15.75 ± 1 ms; after Lugol injection, it increased to 33 ± 4.2 ms (P < 0.001). As a further control, RV injection of 5 to 7 μL of Tyrode’s solution in 2 RyR2/RyR2R4496C hearts had no effect on the BVT pattern (data not shown).

These experiments further support our contention that BVT originates at the ventricular Purkinje fibers.

**Single Purkinje Cells From Mutant and WT Mice**

The results thus far, together with previous data in the literature,5,18,19 suggest that focal arrhythmias in the RyR2/RyR2R4496C mouse heart are the result of DAD-induced triggered discharges. In this regard, Liu et al5 demonstrated that single ventricular myocytes from RyR2/RyR2R4496C mice undergo DADs and TA when subjected to high-frequency excitation and isoproterenol. On the other hand, mammalian Purkinje fibers are known to be more sensitive to calcium overload than ventricular myocytes.20 We have therefore performed a group of patch-clamp experiments in isolated Purkinje cells from both RyR2/RyR2R4496C and WT mouse hearts to demonstrate their ability to undergo DADs and TA.

**DADs and TA in Isolated Purkinje Cells**

Nine cells (9 mice) from RyR2/RyR2R4496C and 9 cells (9 mice) from WT hearts were paced with repetitive trains of 20 stimuli at increasing frequencies (1, 5, 10, 20 Hz) in control...
Ca\textsuperscript{2+} the absence of stimuli leading to Ca\textsuperscript{2+} overload. The isolated RyR2/RyR2R4496C mouse heart showed no abnormality in the absence of isoproterenol. WT cells did not. Altogether, the data strongly suggest that the His–Purkinje system is a major source of focal arrhythmias in CPVT.

The RyR2/RyR2R4496C Mouse

The demonstration in various in vitro models that the R4497C mutation results in increased calcium release has led to the speculation that life-threatening arrhythmias may develop in CPVT patients as a consequence of abnormal sarcoplasmic reticulum calcium release.\textsuperscript{2,18,19,21} However, experimental evidence linking this mutation to the ventricular tachycardias that characterize the disease was still lacking. The recent demonstration that the R4496C mutation in RyR2 predisposes the murine heart to BVT, PVT, and VF upon administration of caffeine and adrenergic agonists opened the possibility of investigating the molecular and cellular mechanisms of arrhythmias in CPVT. In this regard, Cerrone et al\textsuperscript{4} demonstrated that PVT and BVT may be elicited in the RyR2/RyR2R4496C mice under conditions that closely resemble those in CPVT patients. In fact, not all RyR2/RyR2R4496C mice developed arrhythmias, which is also consistent with the incomplete penetrance of CPVT in humans. Similarly, Cerrone et al\textsuperscript{4} were unable to find differences in the levels of mutant mRNA when comparing animals that developed arrhythmias with those that remained asymptomatic. The studies presented here naturally extend those observations by providing new insight into the dynamics of epicardial propagation during MVT, BVT, and PVT and demonstrating their focal origin and their close relationship with the epicardial patterns during normal sequence of activation. Furthermore, when selective chemical ablation of 1 of the 2 branches of the Purkinje system was performed, the alternating QRS pattern of BVT converted to a unifocal pattern. These experiments, together with the demonstration that Purkinje cells from the RyR2/RyR2R4496C mouse heart have an abnormally high propensity to undergo DADs and TA, establish that the origin of the arrhythmias lies in the His–Purkinje network.

Mechanism of BVT

BVT is an infrequent arrhythmia that has nevertheless mesmerized electrophysiologists for many years. It is most commonly observed under conditions of digitalis intoxication and in advanced heart disease.\textsuperscript{22} On ECG, BVT is manifested as an alternation in the polarity of the QRS axis in some of the...
leads; the remaining leads may demonstrate changes in morphology. The tachycardia is often regular, occurs in brief salvos, and often resolves spontaneously or may degenerate into PVT or VF. The alternating pattern is usually associated with bundle branch block morphology in the precordial leads, with the alternating QRS complexes differing from each other in amplitude and duration. Since its first description in 1922, several hypotheses have been postulated for the mechanism of BVT, including enhanced automaticity with the existence of 2 separate ventricular foci or even reentry. More recently, the demonstration of RyR2 gain-of-function mutations in patients with familial CPVT has led to the hypothesis that BVT results from TA secondary to the disruption of the normal process of release of Ca2+ from the sarcoplasmic reticulum during EC coupling.

Our experiments extend substantially such a hypothesis by highlighting for the first time the potential role played by the Purkinje fiber network in the mechanism of the focal arrhythmias that characterize both the calcium overloaded and/or adrenergically stimulated RyR2/RyR2R4496C mouse heart and the patient with CPVT. Several pieces of evidence lead us to think that the above hypothesis is correct: (1) the ability of RyR2/RyR2R4496C Purkinje cells to undergo TA at relatively low stimulation frequencies, even in the absence of adrenergic stimuli (see Figure 7); (2) the focal nature of MVT and PVT in the isolated RyR2/RyR2R4496C mouse heart (Figures 2 and 3); (3) the location of the origin of ectopic beats during MVT and PVT in endocardial optical mapping experiments and the relation between the optical signal and the QRS complexes (Figures 2 and 3); (4) the occurrence of alternating RV and LV epicardial breakthroughs accompanying the beat-to-beat changes in QRS axis during bidirectional couples and BVT (see Figure 3 and the online data supplement); and (5) the conversion of BVT into MVT with RBB block configuration on RV endocardial ablation with Lugol’s solution (see Figure 6).

Several different scenarios have been invoked previously to explain the origin of BVT. Accordingly we have performed computer simulations in a 2D as well as in a geometrically realistic 3D mouse heart model to explore the ability of these different scenarios to result in BVT. In general, our experiments reinforce the idea that the arrhythmias are initiated in the specialized conduction system rather than in the muscle. In addition, the simulations enabled us to provide testable predictions for future experiments and also test the validity of alternative hypothesis regarding the mechanisms of maintenance and initiation of BVT. The hypotheses investigated were (1) 2 alternating sources of epicardial ventricular activity located, respectively, 1 in the RV and 1 in the LV; (2) alternating epicardial and endocardial discharges, as previously proposed; (3) a single Purkinje focus with alternating bundle branch block. Detailed descriptions and a discussion about these simulations are presented in the online data supplement.

Although BVT is frequent in conscious and anesthetized mice, the incidence of the arrhythmia in the isolated heart seems quite low. In fact, we could map only 1 example of BVT in an isolated RyR2/RyR2R4496C heart. As such, a crucial role for the autonomic nervous system in promoting ventricular arrhythmias in the in situ RyR2/RyR2R4496C heart that may be absent or attenuated in the denervated heart cannot be disputed. Resolving this issue, however, is well beyond the reach of the present study.

In addition, it needs to be demonstrated in the whole heart that Purkinje cells are not only capable of forming ectopic foci and generating triggered discharges in situ but also of precisely coordinating their activity to give rise to the peculiar ECG patterns of BVT and bidirectional couples. Finally, a role of the autonomic nervous system in modulating such a highly coordinated and predictable activity needs to be verified. Although these questions are clearly outside the scope of the present study, they do offer some tantalizing prospects for research leading to a detailed understanding of the mechanisms of CPVT.

Sources of Funding
This work was supported in part by National Heart, Lung, and Blood Institute grants PO1 HL039707, RO1 HL070074, and RO1 HL060843 (to J.J.); American Heart Association Postdoctoral Fellowships (to M.C. and S.V.P.); and Telethon grants GGP06007 and GGP090466, Fondo per gli Investimenti della Ricerca di Base grants RBNE01XM04_006 and RBLA035A4X_002, and European Union Sixth Framework Programme grant STREP LSHM-CT-2005-018802 (to S.G.P.).

Disclosures
None.

References


Arrhythmogenic Mechanisms in a Mouse Model of Catecholaminergic Polymorphic Ventricular Tachycardia

Marina Cerrone, Sami F. Noujaim, Elena G. Tolkacheva, Arkadzi Talkachou, Ryan O'Connell, Omer Berenfeld, Justus Anumonwo, Sandeep V. Pandit, Karen Vikstrom, Carlo Napolitano, Silvia G. Priori and José Jalife

Circ Res. 2007;101:1039-1048; originally published online September 13, 2007; doi: 10.1161/CIRCRESAHA.107.148064

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/101/10/1039

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2007/09/27/CIRCRESAHA.107.148064.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
ONLINE SUPPLEMENT

EXPERIMENTAL METHODS

**Optical Mapping:** Littermate RyR2/RyR2<sup>R4496C</sup> and wildtype (WT) mice (2-6 months old) mice were heparinized (0.5 U/g IP) and then anesthetized with a ketamine (116mg/kg)/acepromazine (11mg/kg) mixture injected i.p. The heart was rapidly excised through thoracotomy and subsequently connected to a Langendorff-perfusion system to be continuously perfused with warm oxygenated Tyrode’s solution (pH=7.4) with HEPES as buffer, bubbled with 100% O<sub>2</sub>. The temperature of the perfusate was maintained at 36±1° C as described earlier<sup>1</sup>. The heart was placed in the well of a custom-made plastic chamber maintained at 36±1° C and allowed to equilibrate for 10 minutes. The potentiometric dye Di-4-ANEPPS (Molecular Probes) was added to the perfusate as a bolus to achieve a final concentration of 10 µmol/L. We used a optical mapping system<sup>2</sup>;<sup>3</sup> which was composed of a custom-made upright microscope equipped with a high-resolution, 64x64-pixel CCD camera (Dalsa) running at 600 frames per second, and with a spatial resolution of 109 µm<sup>3</sup>. Hearts were unrestrained and no electromechanical uncouplers were used to reduce contraction. The dimensions of our field of view were 7x7mm.

For endocardial optical mapping, after an equilibration period of 10 minutes, 5 isolated, Langendorff-perfused hearts were placed under a dissecting microscope and rotated such that the RV free wall faced the microscope objective. As described earlier<sup>1</sup>, two incisions on the RV free wall were made to expose the RV septal surface. We exercised caution not to lacerate the right coronary artery. To study the patterns of right bundle branch (RBB) activation, the anterior papillary muscle and the septal artery were brought into the field of view (4 x4mm). Blebbistatin (10 µmol/L) was added to reduce motion artefact<sup>4</sup>.

Volume conducted ECGs (pseudo lead I) were recorded at 1 KHz as described elsewhere<sup>5</sup>. As described previously<sup>6</sup>, we calculated the activation time for each pixel time and the maps were subsequently generated.
**Chemical subendocardial ablation.** Littermate RyR2/RyR2<sup>R4496C</sup> and wildtype (WT) were
anesthetized with Avertin (0.025 ml/g, Sigma) and the body temperature was maintained at 37-38°C with
a heating pad (Fine Science tools, Inc.). The trachea was carefully exposed through a cervical incision
and ligated with a 5-0 silk thread. An airway was established by opening a small incision between the
tracheal rings and a 22-gauge Jelco i.v. cannula was carefully inserted into the trachea, secured with the
5-0 silk ligature and connected to a pressure controlled respirator (Kent Scientific). The animal was
ventilated with room air at 110-120 cycles/min. In order to visualize the heart, a small abdominal incision
was performed; the diaphragm was exposed and opened through a medial incision. This manoeuvre
allowed us to access the beating heart, whose right ventricular cavity was injected with 5-7 µL of either
Lugol’s or Tyrode’s solution using an Hamilton syringe. Surface Lead I ECG was recorded. Signals were
amplified and low-pass filtered with a differential amplifier (CyberAmp 380, Axon Instruments), digitized
(MiniDigi 1A, Axon instruments) at 1 KHz and stored for offline analysis.

**Isolation of murine Purkinje cells:** The protocol for isolating Purkinje cells is a modification of that
previously described<sup>7</sup>. Briefly, littermate RyR2/RyR2<sup>R4496C</sup> and wildtype (WT) mice (2-4 months old)
were injected with 1 ml heparin (100 IU/ml i.p.) 20 min before heart excision. Animals were
anesthetized with a mixture of ketamine (116 mg/Kg) and acepromazine (11mg/Kg) i.p. The heart
was quickly removed from the chest and retrogradely perfused through the aorta at a constant flow
(3 ml/min) and 37°C for 4 min with a Ca<sup>2+</sup>-free buffer containing (in mmol/L): 113 NaCl, 4.7 KCl,
1.2 MgSO<sub>4</sub>, 0.6 Na<sub>2</sub>HPO<sub>4</sub>, 0.6 KH<sub>2</sub>PO<sub>4</sub>, 10 KHCO<sub>3</sub>, 12 NaHCO<sub>3</sub>, 10 HEPES, 10 2.3-butanedione
monoxime (BDM, Sigma), 30 taurine, and 5.5 glucose. All solutions were filtered (0.2-µm filter) and
equilibrated with 100% O<sub>2</sub> for at least 20 min before use. Enzymatic digestion was initiated by
adding Collagenase type II (Worthington) (773.4 u/ml), trypsin (0.14 mg/ml), and CaCl<sub>2</sub> (12.5
µmol/L) to the perfusion solution. After 4-5 min of digestion, the ventricles were cut open and Purkinje
fibers/webs were extracted using micropipettes coupled to a suction device. The fibers were teased in a
stopping buffer (perfusion buffer plus 10%fetal bovine serum 12.5 µmol/L and CaCl<sub>2</sub>) to free the Purkinje
cells. Ca\(^{2+}\) concentration was increased gradually to 1 mmol/L. Cells were used for electrophysiological recording within 8 hours after isolation.

Borosilicate glass electrodes were pulled with a Brown-Flaming puller (model P-97), yielding a tip resistance of 3-5 m\(\Omega\) when filled with pipette solution. Data were recorded using an Axoclamp-2A amplifier and the pClamp8 suite of programs (Axon Instruments, Union City, CA). Stimulus pulses were generated by PCI-6013 Basic Multifunctional I/O board (National Instruments, Austin, TX). A custom-written LabView program (National Instruments, Austin, TX) controlled stimulation.

To distinguish isolated Purkinje cells from working myocytes it was important to define their respective morphological features. In panel A of Online Figure 1 we present a light photomicrograph of a branching Purkinje strand dissected from a mouse right ventricle. Panel B shows a higher magnification image of the same strand in which it is possible to visualize individual cells before further teasing the fibers for cell isolation. In Panel C we show two cells with typical Purkinje morphology obtained from the same strand.

In Online Figure 2, confocal fluorescence microscopy was used to further characterize the isolated Purkinje cells and emphasize their different morphology with respect to that of ventricular myocytes. After enzymatic dissociation, both cell types were stained with potentiometric dye Di-8-ANEPPS (20 \(\mu\)mol/L; Molecular Probes) in Tyrode’s solution, for 20 minutes at room temperature. Cells were then visualized under a confocal microscope (LSM-510, Carl Zeiss) at 488-nm excitation. Panel A shows a ventricular myocyte and panel B shows a Purkinje cell. The Purkinje cell is spindle shaped and longer than the ventricular myocyte. Furthermore the Purkinje cell lacks a well-defined T-tubule system, which is clearly visualized as green striations in the image of the ventricular myocyte.

Online Table 1 presents baseline electrophysiological characteristics determined in current clamp experiments from 8 RyR2/RyR2\(^{R4496C}\) and 10 WT Purkinje cells paced at 1 Hz. No significant differences were found in action potential duration, resting membrane potential, action potential
amplitude, or rate of rise of the action potential upstroke. Furthermore, as illustrated in panels A and B of Online Figure 3 pacemaker activity typical of mammalian Purkinje cells was recorded in both WT and mutant cells. The maximum diastolic potential was not significantly different between spontaneously pacing cells of both genotypes (WT: -82.3±2.3 mV vs RyR2/RyR2R4496C: -81±2.2 mV, NS). An important difference, however, was in the ability to undergo delayed after depolarizations (DADs) and triggered activity, which was significantly greater in the RyR2/RyR2R4496C than the WT Purkinje cells. Yet another difference is presented in panels C and D of Online Figure 3. While, as demonstrated in the main manuscript, DADs and TA were often induced by trains of stimuli at 5 and 10 Hz, many RyR2/RyR2R4496C Purkinje cells underwent runs of early afterdepolarizations (EADs, panel C), or generated complex, high frequency oscillations from a relatively low maximum diastolic potential (panel D). Tentatively, we attribute this abnormal behaviour to leakiness of the RyR2 channels leading to calcium overload.

**Bidirectional couplets.** In both protocols, RyR2/RyR2R4496C hearts showed ventricular couplets, defined as pairs of QRS complexes with different morphology followed by a relatively long pause. This ECG pattern is similar to that of BVT and its morphology provides additionally insights to the origin and mechanisms of propagation of BVT.

Online Figure 4 shows examples of couplets elicited in two different configurations by adrenergic stimulation in separate RyR2/RyR2R4496C mouse hearts. In both cases the absence of P waves on the ECG suggests that the rhythm is junctional or idioventricular. In Panel A, the first beat manifests as a breakthrough on the lateral wall of the RV with the emergent wavefront moving toward the LV. During the second beat, the breakthrough appears near the LV apex and the wavefront propagates toward the RV. Of note, the polarity of the ECG complexes of each couplet alternates 180 degrees, reminiscent of the pattern that characterizes BVT8; 9; 9, with the exception that here each couplet is followed by a pause. In Panel B, taken from a different experiment, all ectopic beats originated within the LV. In each couplet the first beat appeared as a breakthrough near the LV apex and the wave propagated upward toward the base and the RV. The second beat
started also as a breakthrough in the LV, but its location on the free wall was different and the wavefront propagated more slowly toward the RV. It is important that, in this case, both QRS complexes on the ECG had the same polarity in the frontal plane. This was consistent with the left-to-right direction of epicardial propagation observed in the activation maps.

**Chemical Endocardial Ablation**

As demonstrated in the main manuscript, the reproducibility of the chemical ablation data in anesthetized mice, together with our optical mapping results in the isolated Langendorff-perfused heart, provide strong evidence that the His-Purkinje network plays an essential role in the genesis and maintenance of BVT in our mouse model. The question remains, however, as to whether the conversion of the ECG pattern from BVT to MVT with wide QRS that is seen after chemical ablation of the subendocardial Purkinje network in the RyR2+/RyR2R4496C mouse heart would be accompanied by disappearance of the alternating breakthrough pattern that is seen in the optical mapping experiment of figure 3 (main manuscript). Unfortunately, after repeated attempts we were unable to induce BVT to address that question in the Langendorff-perfused RyR2/RyR2R4496C mouse heart. Nevertheless, we did demonstrate that bathing the right ventricular cavity of the normal isolated mouse heart with a Lugol’s solution dissolved in Tyrode’s significantly distorted the epicardial sequence of activation during normal sinus rhythm. Online Figure 5 shows results obtained in one such experiment. A Langendorff-perfused and superfused (38°C) mouse heart was positioned such that the anterior wall of the ventricles faced the CCD camera. Panel A shows the control. Both branches of the His-Purkinje system were intact and thus the normal sequence of atrio-ventricular activation during sinus rhythm was accompanied by the quasi-simultaneous appearance of 2 epicardial breakthroughs, one on the right ventricle and the other on the left ventricle, which reflected transmural activation from terminal Purkinje-muscle junctions at the subendocardium of both free walls at every single beat (see also Figure 1 and 2 of main manuscript).

However, as clearly illustrated by the isochrone map in panel B, less than 1 min after bathing the right ventricular cavity with 0.05 ml Lugol’s solution, the breakthrough on the right ventricle disappeared completely. Instead, only the left ventricular breakthrough persisted and epicardial excitation spread slowly toward the right ventricle. As shown by the lower traces, even though the heart rate remained unchanged at ~340 bpm, the
PR interval increased from 37 to 44 ms and the QRS interval increased from 8 ms to 12 ms after Lugol administration. These data confirm that the breakthroughs represent transmural activation that originated in the Purkinje fiber network and strongly support our contention that the data in the anesthetized mouse rule out a ventricular muscle source for CPVT.

SIMULATIONS

Triggered activity in Purkinje versus muscle; a simulation in 2D: The R4496C RyR2 mutation predisposes the murine heart to ventricular tachycardia (VT) and ventricular fibrillation (VF) under conditions that accurately reproduce those eliciting cardiac arrhythmias in patients with catecholaminergic polymorphic ventricular tachycardia (CPVT). To further test the validity of our idea that in the RyR2/RyR2 R4496C mouse heart exposed to Ca$^{2+}$ overload and/or adrenergic input focal arrhythmias such as monomorphic VT (MVT), bidirectional VT (BVT) and polymorphic VT (PVT) occur preferentially in free running Purkinje fibers because of a reduced electrical load by surrounding tissue compared to the three-dimensional ventricular muscle, we carried out numerical simulations in which three small foci of cells containing leaky RyR2 receptors were incorporated in a simplified 2D model consisting of two thin branches (mimicking the specialized cardiac conduction system) connected to a larger area, as illustrated in panel A of Online Figure 6. The model was constructed on a 200x300-element sheet. Numerical experiments of cardiac excitation were based on the mouse action potential model developed by Bondarenko et al$^{10}$. Our laboratory has recently coded for this model and implemented it, as previously published$^{11, 12}$. Since no mathematical model for the mouse Purkinje cell is currently available, we have mimicked the conditions at the Purkinje-muscle junction building a 2D structure of homogeneous isotropic tissue, in which two thin branches were connected to an expanded region, DADs were generated artificially mimicking a large surge of Ca$^{2+}$ release from the SR, via a leaky RyR2 channel. In Panel B of Online Figure 6, the black arrows show the three designated small areas (8×11 computational cells) that were assigned a leak through the RyR2 channel for an initial period of 25 ms. As can be seen by the action potentials on the right, while the central region failed to produce a propagating impulse (red arrow), the DADs at the ends of both thin branches successfully activated the entire
model to give rise to normal mouse action potentials in the central wide area of the array (green arrow). A dynamic representation of these data is presented in the attached video clip (2D.avi). Overall, these simulations predict that even though the R4496C RyR2 mutation predisposes both Purkinje cells and ventricular myocytes to generate both DADs and triggered activity, given the specific conditions of electrical load to which each type of cell is exposed in the heart, DADs are more likely to reach threshold in the one-dimensional Purkinje fiber than in the three-dimensional ventricular muscle.

3D ventricular mouse model: We have created a complete geometrical model of the mouse ventricles, including the His-Purkinje system, using a scaled-down approximation of the canine ventricular structure and muscle fiber organization. The model is based on extensive anatomic measurements that have allowed description of the myocardial units in terms of a 3D field of geometry and of orientation unit vectors, in an interpolated 60-element structure, formulated in a prolate spheroidal coordinate system. We established the canine ventricular myocardial structure after translating the 3D field into a Cartesian coordinate system of 94x94x94 elements with a 1-mm physical distance between nodes. In addition, we constructed the original Purkinje network model based on real microscopic and macroscopic features while at the same time simplifying the electrophysiological properties of the Purkinje system structure. The extensive and asymmetrical network of the murine His-Purkinje system on the endocardial surface of both ventricles was approximated, using data obtained by a line of mice in which the visualization of the His-Purkinje system is possible following a simple staining procedure (for details see). These mice were generously provided by Dr. Glenn Fishman of New York University.

Online Figure 7 presents a panoramic view of the entire Purkinje system (in blue) on the endocardial surface of the right ventricle of an adult mouse heart visualized by LacZ staining. The asterisk indicates the position of a papillary muscle insertion. Images such as this, obtained from whole-mount Lac-Z stained preparations were assembled into a 2D representation of the Purkinje network. The digital description of the Purkinje system was then manually edited for digitization and rescaling errors and correction of incomplete anatomic data. The 2D layer of the Purkinje
model was subsequently connected at discrete sites, representing the Purkinje-muscle junction to another 2D model with ventricular characteristics. In a following step, the 2D Purkinje digital bit-map presentations were superimposed on the 3D structure by laying the bit-map onto the detected endocardial surfaces. The His bundle and the left and right bundle branches were established and connected to the left and right conduction system. Normal and bundle branch block sequences were simulated to assure the correctness of the overall excitation process of the Purkinje-muscle system. Down-scaling the reconstruction of the dog heart\textsuperscript{17} to the size of the mouse heart with a unit-size of 0.1 mm the total number of units was maintained, which allowed the conversion of the kinetics into a detailed ionic type model, where both Purkinje cell and ventricular muscle excitation and recovery have been represented by Hodgkin-Huxley/Markovian type equations with the appropriate time-steps\textsuperscript{10; 19}.

**ECG reconstructions:** In the simulations, unipolar precordial and bipolar limb-lead ECGs were calculated by summing up all the transmembrane intercellular dipoles ($P_i$; i.e. the V gradient) weighted by the distance ($r$) from the electrode and its orientation: \( ECG(t) = -\sum_{\text{grid}} \frac{\bar{P}(t) \cdot \bar{r}}{r^3} \)

The 3D model, including the His-Purkinje network and the corresponding position of each electrode used for the pseudo-ECG is presented in Online Figure 8. Unipolar leads V1-V6 and bipolar leads I-III are represented together with their corresponding ECGs. The bipolar leads were approximated as illustrated by the white triangle in Online Figure 8, by connecting V1 to V6 (Lead I); V1 to VF (Lead II); and V6 to VF (Lead III).

**Simulating Bidirectional Ventricular Tachycardia in 3D.** We have used this new 3D mouse heart model to test if the overall sequence of activation during BVT seen in our optical mapping experimental results may be simulated by alternating excitation of two separate foci in the LBB and in the RBB and to explore whether other configurations of focal activity may also result in both dynamics of wave propagation and ECG appearance simulating BVT.
Online Figure 9 (see also attached videoclips SR_1.avi and SR_1_B.avi) presents reproduction of sinus rhythm in the model. The 3D model was paced repetitively at the His bundle to simulate conditions of sinus rhythm (BCL, 150ms). Panel A shows a sagittal section of the simulated heart to illustrate the site of pacing and the arrangement of the His-Purkinje network on the endocardium. The color map on the right shows the epicardial activation sequence predicted by the model. In panel B the simulated ECG (unipolar lead V1) is illustrated. The total activation time of the anterior epicardium in the model is about 2.25-2.5 msec, which is somewhat faster than the ~3.5 msec observed experimentally (see Figure 1 in the main manuscript). Nevertheless, the numerical model closely reproduces the experimentally demonstrated concentric breakthroughs that characterize the ventricular epicardial activation of both large and small mammals\(^1,20\).

In our experimental results, in the RyR2/RyR2\(^{R4496C}\) mouse heart BVT manifests by RV and LV epicardial breakthroughs that alternate in direction on a beat-to-beat basis. We hypothesize that such pattern will most likely be explained by the alternation in the spontaneous firing of two separate foci in the His-Purkinje network, one in the LV and one in the RV. Online Figure 10 presents the results of this simulation in the 3D model. Panel A shows the sagittal section of the ventricles indicating the two alternating pacing sites. The lead V1 ECG in panel B demonstrates that pacing alternatively in the RBB and in the LBB resulted in a pattern of BVT (cycle length 80 msec) that closely simulated the experimental results (see Figure 3 in the main manuscript). Furthermore the alternating concentric epicardial breakthroughs shown on activation maps in panel C (see also attached videoclips RBB-LBB_epi.avi and RBB-LBB_endo.avi) for beats 1 and 2 demonstrate the close correspondence between the simulation results and the experimental data in the RyR2/RyR2\(^{R4496C}\) mouse heart.

**Alternative hypotheses on the site of origin of Bidirectional Tachycardia.**

1. **RV and LV epicardial foci:** Online Figure 11 and the attached video clip labelled LV/RV-Endo illustrate the data obtained with the obvious first choice of two alternating focal sources of ventricular activity, one located on the epicardium of the RV and the other located on the
epicardium of the LV. As shown by the LII ECG recording presented in the lower panel of Online Figure 11, this configuration also results in a pattern that resembles BVT, with the individual QRS axis rotating 180 degrees from one beat to the next. However, based on the 2D computer simulations presented above, this type of organization would seem unlikely to prevail as a mechanism of BVT in the calcium overloaded or adrenergically stimulated heart. In this regard, it should be noted that the amount of current that was needed to generate the local discharges at each site were more than twice as large as those needed to activate the Purkinje fiber foci of Online Figure 10. Should the epicardial foci correspond to groups of ventricular myocytes with leaky RyR2 channels, the very large electrical load created on them by the surrounding ventricular myocardium would greatly impair their ability to reach threshold on such repetitive and highly regular bases.

2. Alternating epicardial and endocardial discharges: Nam et al\textsuperscript{21} recently proposed that BVT might occur as a consequence of alternation in the origin of ectopic beats between endocardium and epicardium or between epicardium and mid-myocardium. To determine the mechanism of BVT these investigators used a wedge preparation in which they mimicked altered calcium homeostasis using caffeine and epinephrine. We used the 3D mouse model, to determine whether the scenario proposed by Nam et al results in BVT in the whole heart applying stimuli alternating between the endocardium and the epicardium of the LV. As demonstrated in Online Figure 12 (see also video clip EPI/ENDO-Endo), BVT is indeed generated by this configuration. However, the only 2 ECG leads in which it is possible to see a bidirectional pattern are those that face the left ventricle, including precordial leads V5-V6 and standard Lead III. This result clearly does not conform with what has been shown both in patients affected by CPVT\textsuperscript{8, 22}, in which a BVT pattern is visible in all leads of the ECG and in the RyR2/RyR2\textsuperscript{R4496C}mouse \textsuperscript{9}, in which BVT is usually recorded in the right sided leads. Moreover, as demonstrated by the attached video clip labeled EPI/ENDO-Epi, this configuration was incapable of reproducing the pattern of epicardial breakthroughs that was demonstrated in the optical mapping experiment (see Figure 3 in main article), in which there was a clear alternation between RV and LV during BVT.
3. **Single Purkinje focus with alternating right bundle branch block:** The third alternative possibility that we considered was that ectopic bigeminy and BVT may be generated by triggered activity at a single ectopic focus in the right bundle branch with alternating unidirectional exit block toward the RV and one-to-one propagation toward the LV. As illustrated in Online Figure 13, in this simulation conditions were such that an impulse initiated at the RBB (labelled 1) blocked antegradely toward the RV but propagated retrogradely toward the LBB to generate beat 1. The following impulse (labelled 2) propagated in both directions to initiate beat 2. The resulting BVT pattern was very similar to that obtained by alternating focal activation of the RBB and LBB (see Figure 3 in main manuscript and video clip RBB/LBB-Epi). This is clearly visualized in the attached video clip (RBBB-Epi) in which the arrows indicate the direction of propagation and the T-crossed bar signals the occurrence of RBB block. The first epicardial breakthrough appears on the RV and the second breakthrough appears on the LV at the same time that the QRS axis switches from one polarity to the other.

**Reference List**


7. Tolkacheva EG, Vaidyanathan R, Munoz V, Noujaim S, Anumonwo J. Inward rectifier current


Online Table 1: Electrophysiological properties of WT and RyR2/RyR2<sup>R4496C</sup> mouse Purkinje cells at 1 Hz

<table>
<thead>
<tr>
<th>GENOTYPE</th>
<th>APD&lt;sub&gt;50&lt;/sub&gt; (msec)</th>
<th>APD&lt;sub&gt;70&lt;/sub&gt; (msec)</th>
<th>APD&lt;sub&gt;90&lt;/sub&gt; (msec)</th>
<th>Vr (mV)</th>
<th>APA (mV)</th>
<th>dV/dt (V/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype (10 cells)</td>
<td>8.7 ± 2.2</td>
<td>44.7 ± 14.2</td>
<td>116.3 ± 18.1</td>
<td>-83.4 ± 0.6</td>
<td>101.3 ± 2.8</td>
<td>166.9 ± 13.5</td>
</tr>
<tr>
<td>RyR2/RyR2&lt;sup&gt;R4496C&lt;/sup&gt; (8 cells)</td>
<td>10.9 ± 2.1</td>
<td>82.3 ± 23.3</td>
<td>150.7 ± 16.4</td>
<td>-82.5 ± 0.4</td>
<td>107.9 ± 2.6</td>
<td>190.4 ± 11.6</td>
</tr>
</tbody>
</table>

APD<sub>50-90</sub>, action potential duration at 50, 70 and 90% repolarization; Vr, resting membrane potential; APA, action potential amplitude; dV/dt, rate of rise of the action potential upstroke.
Figure Legends

Online Figure 1. A: Photomicrograph (20X) of a Purkinje strand from mouse right ventricle immediately after enzymatic dissociation, immersed in stopping buffer. The boxed section is shown in panel B. B: Close-up at a higher magnification (40X) of the same strand to visualize individual Purkinje cells. C: Two Purkinje cells from the same strand after isolation (40X).

Online Figure 2: Morphology of isolated ventricular myocyte and Purkinje cell. A: Confocal and transillumination image of a ventricular myocyte. The transverse T-tubule system is seen as striation. B: Confocal and transillumination image of a single Purkinje cell isolated from the same mouse heart. Both cells were stained with 20 µmol/L Di-8-ANEPPS.

Online Figure 3: A and B, representative example of spontaneous pacemaker activity in isolated Purkinje cells from WT (A) and RyR2/RyR2^{R4496C} (B) mouse heart. C and D, early afterdepolarizations and sustained triggered activity at relatively low maximum diastolic potentials initiated by trains of stimuli at 5 Hz (C) and 10 Hz (D).

Online Figure 4: Ventricular couplets in RyR2/RyR2^{R4496C} hearts. A: Bi-ventricular couplets. Top and bottom, activation maps of two consecutive beats (arrows) in an example of bidirectional couplets (middle, ECG), resulting from alternating focal activations in RV (top) and LV (bottom). B: Couplets originating in one ventricle. Top and bottom, activation maps of two consecutive beats (arrows) from a different example of ventricular couplets (middle, ECG). Both beats in each couplet originated in LV (arrows). Maps superimposed on the fluorescent heart image. Orange line, interventricular septum.

Online Figure 5. Effect of chemical endocardial ablation on ventricular epicardial sequence of activation. Left, control; right, 1 min after Lugol. Note disappearance of right breakthrough after Lugol.
Online Figure 6. The electrotonic load controls the ability of cells to undergo DAD-induced triggered activity in 1D Purkinje fiber vs 2D ventricular myocardium. A: Structure of the model. Two thin branches mimicking the specialized conduction system are connected to a larger area simulating a ventricular muscle sheet. Three small foci (green) were assigned a transient, 25 ms sarcoplasmic Ca$^{2+}$ leak thorough the RyR2 channel. B, Left: 10-ms isochrone map showing activation sequence upon firing of the Purkinje strands. Right: time course of the action potentials in the three green foci. A DAD initiated focally in the center of the rectangular muscle sheet failed to reach threshold (red arrow). In contrast, the DADs originating at both thin branches, which were subjected to a lesser load from their neighbors, reached threshold and initiated propagated action potentials. Both impulses move in opposite directions at the same speed and collide at the center to initiate an action potential in the muscle’s center (green arrow). See also videoclip: 2D.avi

Online Figure 7. Endocardial right ventricular surface and His-Purkinje system visualized by Lac-Z staining in an adult mouse heart.

Online Figure 8. Endocardial view of the geometrically realistic 3D model of the mouse ventricles and its His-Purkinje system, surrounded by the simulated ECG leads to show their location and the QRS morphology at each lead.

Online Figure 9: Simulating sinus rhythm in mouse model. A: Left: 3D model of heart with His-Purkinje system. Right: Epicardial activation map of sinus beat. Note concentric breakthroughs in RV and LV. B: Pseudo–ECG (lead V1). Compare with Figure 1 in the main manuscript. See also videoclips: SR-1 and SR-1B

Online Figure 10: Bidirectional tachycardia generated by alternating pacing of two separate foci in the LBB and in the RBB. A: 3D model showing sites of focal sequential activation in RBB and LBB; B: pseudo-ECG (lead V1) showing 180° shift in QRS axis. C: epicardial activation maps of beats 1
and 2 showing alternating breakthroughs on LV (beat 1) and RV (beat 2). Compare with Figure 4C in main manuscript. See also videoclips: RBB/LBB_endo.avi and RBB/LBB_epi.avi

Online Figure 11. Bidirectional tachycardia generated by alternating focal epicardial activation in the 3D model as recorded on ECG LII. Stimuli were applied alternatively each beat on epicardial surface of RV and LV. Numbers indicate beat number; blue dots site of stimulation. See also videoclip: LV/RV_Endo.avi.

Online Figure 12. Bidirectional tachycardia generated by alternating focal activation of epicardium and endocardium of LV as recorded on ECG LIII. The pattern was present also on V5 and V6 but not on other leads. Numbers indicate beat number; blue dots site of stimulation. See also videoclips Epi/Endo_endo.avi and Epi/Endo_epi.avi.

Online Figure 13. Bidirectional tachycardia generated by single Purkinje focus with alternating right bundle branch block as recorded on ECG V1. Numbers indicate beat number; blue dots site of stimulation. See text for further details and videoclip RBBB_epi.avi.

**Video-clips:**

SR-1: Normal sequence of endocardial ventricular activation. Sinus rhythm was simulated by pacing repetitively at the His bundle at a cycle length of 150 msec. For pseudo-ECG see Online Figure 9.

SR-1-B: Normal sequence of epicardial ventricular activation. Sinus rhythm was simulated by pacing repetitively at the His bundle at a cycle length of 150 msec. For pseudo-ECG see Online Figure 9.

RBB/LBB-endo: Endocardial activation during bidirectional tachycardia generated by beat-to-beat alternation of focal discharges at RBB and LBB. For pseudo-ECG see Online Figure 10.
RBB/LBB-epi: Epicardial activation during bidirectional tachycardia generated by beat-to-beat alternation of focal discharges at RBB and LBB. For pseudo-ECG see Online Figure 10.

LV/RV-endo: Endocardial activation during bidirectional tachycardia generated by two focal sources located epicardially in the RV and LV. For pseudo-ECG see Online Figure 11.

Epi/Endo-endo: Endocardial pattern of ventricular activation during alternating stimulation of two foci, one on the endocardium and the other on the epicardium of the LV. For pseudo-ECG (lead III, facing the LV) see Online Figure 12.

Epi/Endo-epi: Epicardial pattern of ventricular activation during alternating stimulation of two foci, one on the endocardium and the other on the epicardium of the LV. For pseudo-ECG (lead III, facing the LV) see Online Figure 12.

RBBB-Epi: Endocardial ventricular activation pattern of BVT obtained by pacing a single ectopic focus in the right bundle branch. The impulse propagates with alternating right bundle branch block on a beat-to-beat basis. For pseudo-ECG see Online Figure 13.
Online Figure 3
Online Figure 4
Control

Lugol

Online Figure 5
Online Figure 6
Online Figure 8
Online Figure 9
Online Figure 10
Alternating focal epicardial activation in LV and RV
Alternation of focal activation of LV epicardium and endocardium
Repetitive RBB focal discharges with intermittent RBBB

Online Figure 13