Sudden Infant Death Syndrome in Mice With an Inherited Mutation in RyR2

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Background—Mutations in the cardiac ryanodine receptor gene (RyR2) have been recently identified in victims of sudden infant death syndrome. The aim of this study was to determine whether a gain-of-function mutation in RyR2 increases the propensity to cardiac arrhythmias and sudden death in young mice.

Methods and Results—Incidence of sudden death was monitored prospectively in heterozygous knock-in mice with mutation R176Q in RyR2 (R176Q/+/). Young R176Q/+ mice exhibited a higher incidence of sudden death compared with wild-type littermates. Optical mapping of membrane potentials and intracellular calcium in 1- to 7-day-old R176Q/+ and wild-type mice revealed an increased incidence of ventricular ectopy and spontaneous calcium releases in neonatal R176Q/+ mice. Surface ECGs in 3- to 10-day-old mice showed that R176Q/+ mice developed more ventricular arrhythmias after provocation with epinephrine and caffeine. Intracardiac pacing studies in 12- to 18-day-old mice revealed the presence of an arrhythmogenic substrate in R176Q/+ compared with wild-type mice. Reverse transcription–polymerase chain reaction and Western blotting showed that expression levels of other calcium handling proteins were unaltered, suggesting that calcium leak through mutant RyR2 underlies arrhythmogenesis and sudden death in young R176Q/+ mice.

Conclusions—Our findings demonstrate that a gain-of-function mutation in RyR2 confers an increased risk of cardiac arrhythmias and sudden death in young mice and that young R176Q/+ mice may be used as a model for elucidating the complex interplay between genetic and environmental risk factors associated with sudden infant death syndrome. (Circ Arrhythm Electrophysiol. 2009;2:677-685.)

Key Words: sudden infant death syndrome ■ calcium ■ focal activity ■ ryanodine receptors ■ ventricular arrhythmias

Sudden infant death syndrome (SIDS) is a multifactorial disorder in which newborn infants die during the first year of life, which is unexpected by history and in which a full postmortem examination fails to demonstrate a cause of death.1,2 Despite the success of the “Back to Sleep” campaign, the incidence of SIDS remains unacceptably high and efforts have been redirected from identification of associated factors to determination of the causative mechanisms.3 Maron et al4 and Schwartz and Segantini5 were the first to suggest the possibility of arrhythmogenic factors contributing to SIDS. This theory was confirmed 2 decades later, when mutations in genes linked to inherited arrhythmia syndromes were identified in victims of this syndrome.6 Based on these postmortem molecular analyses, it is now estimated that 10% to 15% of SIDS cases can be attributed to inherited mutations in ion channels and associated subunits linked to fatal cardiac arrhythmias.7,8

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Recently, mutations in the intracellular calcium (Ca2+) release channel/ryanodine receptor gene RyR2 were identified in victims of SIDS.9 Single-channel recordings revealed that SIDS-associated mutations in RyR2 result in aberrant channel openings during diastole, when Ca2+ is normally sequestered in the sarcoplasmic reticulum.9,10 This gain-of-function phenotype observed for SIDS-associated RyR2 mutant channels was similar to that observed for RyR2 channels with mutations identified in older children and adults with an inherited arrhythmia syndrome, catecholaminergic polymorphic ventricular tachycardia (CPVT).10,11 CPVT is an inherited disorder characterized by exercise- and stress-induced ventricular...
tachycardias associated with syncope and sudden cardiac death. It is estimated that >50% of all CPVT cases are caused by genetic mutations in RyR2.\textsuperscript{11} It has also been suggested that some RyR2 mutations may cause arrhythmogenic right ventricular dysplasia type 2 (ARVD-2), a condition characterized by fibro-fatty degeneration of the right ventricle and ventricular arrhythmias.\textsuperscript{12} However, it is currently controversial whether RyR2 mutations actually cause right ventricular structural remodeling and ARVD-2.\textsuperscript{13,14}

In this study, we used a knock-in mouse model of the R176Q mutation in RyR2 to examine the hypothesis that a gain-of-function mutation in RyR2 results in SIDS caused by cardiac arrhythmias. This mutation was previously identified in a 15-year-old boy with sudden arrhythmogenic cardiac death.\textsuperscript{13} Our results revealed an increased incidence of sudden unexpected death in R176Q/+ knock-in mice during the first weeks of life. Using optical mapping experiments, we found an increased incidence of spontaneous calcium release events and ectopic electrical activity in young R176Q/+ mice hearts. Electrophysiological recordings demonstrated cardiac arrhythmias in R176Q/+ mice associated with sudden cardiac death. Moreover, intracardiac pacing studies revealed the presence of an arrhythmogenic substrate due to the R176Q/+ mutation in RyR2. This study describes the first mouse model for SIDS caused by cardiac arrhythmias and provides evidence for a causal link between mutations in RyR2 and ventricular tachycardia in young mice.

Methods

Mouse Strains, Animal Care, and Genotyping

All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine conforming to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). R176Q/+ mice were created as described and mated with wild-type (WT) littermates on a C57B16 background.\textsuperscript{15} Mating cages were surveyed, and all dead pups between postnatal days 1 to 24 were collected daily and genotyped for sex\textsuperscript{16} and the R176Q mutation as described in the online supplement.\textsuperscript{15} The postnatal developmental stages of mice are not as clearly defined as in humans. Because weaning occurs in mice at 12 to 18 days of age, the youngest mice we could reliably perform these studies. A 1.1F octapolar catheter (Scisense Inc, London, Ontario, Canada) was advanced through the internal jugular vein into the right ventricle. After baseline recordings were made, pacing thresholds were determined using a programmed electric stimulator (STG3008, Multi Channel Systems, Reutlingen, Germany) and stimulation was delivered at 0.2-ms pulse width, at twice the capture threshold. A drive cycle length of 90 ms followed by a decrementing single extrastimulus was used to determine inducibility of ventricular tachycardia.\textsuperscript{15} Episodic of premature ventricular contractions were defined as 1 to 3 QRS complexes not preceded by atrial activity. Nonsustained ventricular tachycardia was defined as 4 to 9 QRS complexes not preceded by atrial activity, whereas ventricular tachycardia was defined as >9 QRS complexes not preceded by atrial activity.\textsuperscript{20} The pacing protocols were repeated after administration of 0.5 mg/kg i.p. isoproterenol.\textsuperscript{10}

Histological Procedures

Mouse hearts were fixed with 10% buffered formalin, sectioned longitudinally (5 μm), and stained with either hematoxylin and eosin for cell morphology or Masson trichrome for interstitial fibrosis.

Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from 10-day-old neonatal hearts using TRIzol (Invitrogen). Frozen hearts were mechanically homogenized in 1.0 mL TRIzol reagent. Total RNA was dissolved in 30 μL RNasefree H2O, and RNA concentration was determined by measuring absorbance at 260 nm using a DU 530 UV/Vis spectrophotometer (Beckman Coulter, Fullerton, Calif). For each sample, 1.0 μg of RNA was used as a template for reverse transcription using oligo(dt)\textsubscript{12-18} primer and SuperScript II reverse transcriptase (Invitrogen) in a 50-μL total volume to generate first-strand cDNA. The cDNA (2.5 μL for each sample) was then amplified by polymerase chain reaction (PCR) in a 50-μL total volume, using Taq DNA polymerase (Invitrogen) and primers specific for RyR2, the α-subunit of the cardiac L-type voltage-dependent Ca\textsuperscript{2+} channel (Cav1.2), Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX1), sarcolemmal/endothelial reticulum Ca\textsuperscript{2+} ATPase (SERCA2a), and phospholamban. Ribosomal protein L7 mRNA levels were determined as control for equal sample input and amplification.\textsuperscript{21} As a control for genomic DNA contamination,
the same reverse-transcription (RT)-PCR amplification was performed in the absence of reverse transcriptase.

Western Blotting
Western blotting was performed as previously described. Mouse ventricular lysates were subjected to electrophoresis on 6% (for Cav1.2, RyR2, SERCA2a, NCX1, and GAPDH) and 20% (for PLN) acrylamide gels, and transferred onto polyvinyl difluoride membranes. Proteins were detected using antibodies against Cav1.2 (1:200; Sigma), RyR2 (1:5,000; Affinity BioReagents), SERCA2a (1: 500; Santa Cruz Biotechnology), PLN (1:5,000, Affinity BioReagents), NCX1 (1:500; Swant), and GAPDH (1:5000; Millipore). Membranes were incubated with secondary antibodies conjugated to Alexa-Fluor 680 (Invitrogen Molecular Probes) or IR800Dye (Rockland Immunochemicals), and bands were quantified using densitometry (Odyssey System).

Statistical Analysis
Results are expressed as mean ± SEM. Student t test, χ², or Fisher exact test was applied when appropriate. A Kaplan–Meier survival analysis was done to estimate the incidence of sudden death. P < 0.05 was considered statistically significant.

Results
Higher Incidence of SIDS in R176Q/+ Knock-in Mice
Sudden infant death syndrome is defined clinically as the death of an infant under the age of 1 year. To determine the effects of the inherited R176Q mutation in RyR2 on postnatal survival, we prospectively assessed the incidence of sudden death in live births generated in mating cages containing 1 WT and 1 R176Q/+ knock-in mouse. Mating schemes included similar numbers of male (n = 12) and female (n = 9) R176Q/+ breeders, mated with WT littermates in each case, respectively. During the 24-month study, 48 (of 487) live-born neonates died during postnatal days 1 to 24. The total number of R176Q/+ mice (n = 248) born was similar to that of WT mice (n = 239) (P = 0.798), suggesting that the R176Q mutation does not cause embryonic lethality. After PCR genotyping of deceased pups (supplemental Figure 1), we found a greater incidence of sudden death during postnatal days 1 to 24 in R176Q/+ neonates (31/248; 12.5%) compared with WT littermates (17/239; 7.1%) (P = 0.047) (Figure 1). Interestingly, most deaths in both R176Q/+ and WT pups occurred during the first 4 neonatal days, whereas fewer mice died during the subsequent three weeks. Between months 1 and 6, mortality incidence in a different cohort of mice was 1 of 72 in WT mice and none of 102 for sedentary R176Q/+ mice. Thus, there was no increased mortality rate in R176Q/+ mice beyond the infant period.

Absence of Structural Heart Disease and ARVD in R176Q/+ Mice
A detailed postmortem examination was performed on dead 1-day-old R176Q/+ (n = 8) and WT (n = 4) mice. In addition, we electively euthanized 2 whole litters of 1-day-old mice born to WT and R176Q/+ parents (9 R176Q/+ and 7 WT neonatal mice) for postmortem and histological analysis. The autopsy findings confirmed that all dissected mice (both sudden death victims and electively euthanized mice) were born alive, as they all had milk in their digestive system. Microdissection revealed the absence of macroscopic abnormalities, such as organ malformation, cleft palate, incomplete sternum fusion, diaphragmatic hernia, spina bifida, lymph node hyperplasia, or tumors. In particular, no abnormalities were noted in the hearts and great vessels in any of the autopsied mice. There were no differences between the heart weight-to-body weight ratios between R176Q/+ mice (0.0089 ± 0.0020, n = 8) and WT mice (0.0091 ± 0.0028, n = 4; P = 0.935) that died suddenly on postnatal day 1. Moreover, those heart weight-to-body weight ratios were similar to those of WT and R176Q/+ mice electively euthanized on day 1 after birth. Thus, a macroscopic pathological examination revealed no obvious morphological abnormalities in the pups that died suddenly or mice that were electively autopsied at the same age.

To determine whether the R176Q mutation in RyR2 causes remodeling of the neonatal heart, we performed a detailed histological examination of electively euthanized 1-day-old WT and R176Q/+ mice (n = 4 per genotype). Sections stained with hematoxylin and eosin revealed the absence of ventricular enlargement and myofiber disorganization (data not shown). Because some patients with the R176Q mutation in RyR2 exhibited right ventricular abnormalities believed to be associated with ARVD,12 a Masson trichrome staining was performed that revealed the absence of fibrosis in the right ventricle of R176Q/+ mice (Figure 2A and 2B). To exclude structural heart disease as a cause of sudden unexpected death in R176Q/+ mice, we also performed a histological analysis of the hearts dissected from deceased neonatal mice (8 R176Q/+ and 4 WT mice). Although the quality of these sections was lower because the dead mice had been frozen before processing for histology, Masson trichrome staining appeared to exclude right ventricular dysplasia and/or interstitial fibrosis as a cause of death (Figure 2C and 2D).

Unaltered Expression Levels of Calcium Handling Proteins in R176Q/+ Mice
We also examined the possibility that the R176Q/+ mutation in RyR2 resulted in compensatory changes in other major Ca²⁺-handling proteins that could be proarrhythmogenic. RT-PCR analysis of L-type Ca²⁺ channel (Cav1.2), RyR2, SR Ca²⁺-ATPase (SERCA2a), phospholamban, and Na⁺/Ca²⁺-exchanger (NCX1) revealed unaltered expression of mRNA levels when normalized to loading control L7 in R176Q/+ compared with WT mouse hearts (n = 4 and P = NS in each group) (Figure 3A). Additionally, Western blot
Spontaneous Electric Activity and Calcium Releases in Neonatal R176Q/+ Hearts

To better understand the mechanisms underlying sudden death in young R176Q/+ mice (1 to 7 days old), we developed a system to simultaneously measure cardiac action potentials and calcium transients using optical mapping in isolated neonatal mouse hearts (see supplemental Figure 2). Isolated hearts, loaded with the Ca$^{2+}$ indicator Rhod-2-AM and the voltage-sensitive dye RH237, were superfused with Tyrode solution in an experimental chamber. The cardiac preparations were paced using field stimulation, and ventricular action potentials and Ca$^{2+}$ transients were recorded simultaneously (Figure 4). Under control conditions during pacing at 3 Hz, the ventricular action potential duration (APD$_{80}$) averaged 101±3 ms in WT and 145±8 ms in R176Q/+ mice ($P=0.004$). The Ca$^{2+}$ transient duration was longer in R176Q/+ hearts (276±5 ms, n=8) than in WT hearts (211±19 ms, n=4) ($P=0.001$). The Ca$^{2+}$ transient decay (tau) was prolonged in neonatal R176Q/+ hearts (112±2 ms, n=8) compared with WT hearts (86±2 ms; $P<0.001$), consistent with diastolic leakage of Ca$^{2+}$ from the sarcoplasmic reticulum via R176Q/+ mutant RyR2 channels (Figure 4A and 4B).

Mouse hearts could be paced to a cycle length of 200±20 ms before losing 1:1 capture, and there was no significant difference comparing R176Q/+ and WT. However, there was a trend toward increased incidence of ectopic depolarizations in R176Q/+ paced hearts (3 of 8 mice) compared with WT (0 of 10 mice; $P=0.069$). The average number of ectopic beats in R176Q/+ preparations with spontaneous activity was 8±3 bpm. By comparing voltage and calcium mapping data, we observed that ventricular ectopy occurred in R176Q/+ neonatal hearts as early afterdepolarizations arising as a second depolarization during the preceding triggering Ca$^{2+}$ transient (Figure 4F through 4H). These experiments suggest that abnormal Ca$^{2+}$ releases caused by mutant RyR2 channels led to ventricular ectopy in R176Q/+ neonatal hearts, which might result in the formation of ventricular arrhythmias in vivo.

Ventricular Tachycardias in R176Q/+ Knock-in Mice

To determine whether sudden death in neonatal R176Q/+ mice might be caused by cardiac arrhythmias as suggested by optical mapping studies, we recorded surface electrocardiograms in 3- to 10-day-old mice. At baseline, there were no significant differences in heart rate, PR interval, or QRS or QTc duration, comparing R176Q/+ and WT littermates (Figure 5A and the Table), suggesting the absence of conduction disease or repolarization abnormalities. Moreover, ectopic ventricular beats were not observed in any of the R176Q/+ (n=10) or WT (n=8) mice studied.

Subsequently, an arrhythmia challenge was conducted by injecting epinephrine (2 mg/kg i.p.) and caffeine (120 mg/kg i.p.) into the pups, a protocol known to evoke catecholamine-dependent arrhythmias in adult mice with RyR2 mutations.15 Whereas spontaneous (ie, nonpaced) ectopic beats and ventricular tachycardia occurred in only 7% of WT mice (1 of 15), 41% (7 of 17) R176Q/+ knock-in mice developed multiple ectopic beats and episodes of ventricular tachycardia ($P=0.041$) (Figure 5B). Although most of these arrhythmias were self-terminating, we observed the sudden death of 3 R176Q/+ pups during or after an episode of ventricular arrhythmia. These data suggest that the increased incidence of
SIDS in R176Q/+ might be caused by cardiac arrhythmias that are induced under conditions of catecholaminergic stress.

To exclude potential confounding effects of anesthesia on arrhythmogenicity, we recorded surface ECGs in unanesthetized 1-day-old WT (n=7) and R176Q/+ (n=8) mice. There were no differences in HR, PR, QRS, or QTc intervals comparing R176Q/+ mice and WT littermates, although the averaged heart rates in awake mice (WT: 505±22 bpm; R176Q+/+: 476±18 bpm; P=0.325) were higher compared with anesthetized mice. Consistent with studies in 3- to 10-day-old mice, we did not observe episodes of bradycardia in any of the WT or R176Q/+ mice studied.

Enhanced Arrhythmogenic Susceptibility of R176Q/+ Hearts

To confirm the presence of an arrhythmogenic substrate in the hearts of young R176Q/+ mice, we performed intracardiac programmed electric stimulation in neonatal mice (Figure 6A through 6E). After preparation of the right jugular vein (panel A) in neonatal mice (electrogram B and C), a 1.1F octapolar catheter (panels D and E) was advanced into the right ventricle of (panel C) under electrogram guidance. Surface ECG and intracardiac electrograms were recorded in mice 12 to 18 days of age, weighing 6.7±0.2 g, because the catheter could not be inserted into the jugular veins of younger mice. The basal heart rates in WT mice were 426±11 ms and in R176Q/+ mice 417±20 ms (P=0.730). Under basal conditions, overdrive pacing or a pacing protocol with one extra stimulus resulted in nonsustained ventricular tachycardia and episodes of premature ventricular contractions in 17% (2 of 12) of R176Q/+ mice compared with 0% (0 of 12) of WT mice (P=0.478).

Because arrhythmias in mice and patients with genetic mutations in RyR2 are almost always triggered by stress or catecholamines, isoproterenol (0.5 mg/kg) was administered to the neonatal mice intraperitoneally. ECG analysis revealed that isoproterenol elicited equal effects on heart rates (ie, an ≈20% increase) in WT and R176Q/+ knock-in mice (data not shown). After isoproterenol administration, ventricular tachycardia was inducible in 50% (6 of 12) of R176Q/+ pups (Figure 6F through 6H), compared with 8% (1 of 12) of WT pups (P=0.069). Episodes of both monomorphic and polymorphic ventricular tachycardia were observed in R176Q/+ mice after isoproterenol. Isoproterenol did not induce conduction abnormalities or significant differences in QTc duration comparing R176Q/+ and WT mice. These findings suggest that the R176Q mutation in RyR2 enhances the susceptibility to arrhythmias in young mice and that the increased arrhythmogenic substrate in these mice might lead to the enhanced incidence of SIDS.

Discussion

This study provides the first in vivo experimental evidence in a genetic mouse model for a possible pathogenic link between an ion channel mutation and SIDS and arrhythmias. Our findings demonstrate that an inherited gain-of-function RyR2 mutation may represent a latent pathogenic substrate for sudden death during an early stage of life. In the presence of a suitable “trigger” such as catecholamines, the pathogenic substrate may lead to triggered activity in the heart of young mice, associated with enhanced arrhythmogenesis and an increased probability of sudden death. These studies extend retrospective population-based linkage studies and biophysical studies of mutant ion channels and provide evidence for a pathogenic mechanism underlying ventricular arrhythmias and sudden death.
Approximately 3000 apparently healthy infants who die each year in the United States are classified as SIDS. A number of risk factors have been identified so far, which include young maternal age with low educational levels and socioeconomic status, poor prenatal care, exposure to cigarette smoke during and after pregnancy, prematurity, low birth weight, male sex, black race, overheating, prone sleeping position, and sleeping on a soft surface. In addition, inborn errors of metabolism, respiratory dysfunction, cardiorespiratory instability, and cardiac arrhythmias have been proposed as mechanisms underlying SIDS.

Cardiac arrhythmias caused by mutations in ion channels have been proposed as mechanisms for SIDS more than 3 decades ago. Long QT syndrome was subsequently described in a “near-miss” case of SIDS. Consequently, long-QT syndrome, short-QT syndrome, and Brugada syndrome have all been implicated as causes of SIDS. Based on postmortem studies of SIDS victims, it has been estimated that up to 10% to 15% of SIDS cases might result from cardiac channelopathies. Recently, cardiac ryanodine receptors have also been added to potassium and sodium channels as possible causes of SIDS, as 2 new mutations were identified in a cohort of SIDS victims.

Inherited mutations in the RyR2 gene have also been associated with CPVT and possibly arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC/D) in older children and adults. Moreover, several CPVT-associated RyR2 mutations have been identified in cohorts of young victims (mean age, 12 to 14 years) of sudden unexplained death. These clinical findings suggest that SIDS and

### Table: Measurement of Surface ECG Parameters in WT and R176Q/+ Neonates

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT (n=5)</th>
<th>R176Q/+ (n=4)</th>
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<tr>
<td>Baseline</td>
<td></td>
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<tr>
<td>HR (bpm)</td>
<td>Mean: 425.9</td>
<td>Mean: 417.5</td>
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<td>SEM: 11.3</td>
<td>SEM: 20.0</td>
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<td>PR (ms)</td>
<td>Mean: 42.6</td>
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<td>SEM: 1.2</td>
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<td>QRS (ms)</td>
<td>Mean: 8.4</td>
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<td>SEM: 0.6</td>
<td>SEM: 0.1</td>
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<tr>
<td>QT (ms)</td>
<td>Mean: 23.3</td>
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<tr>
<td>QTc (ms)</td>
<td>Mean: 19.6</td>
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<td>SEM: 1.8</td>
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| HR indicates heart rate; PR, interval from beginning of P wave to peak of R wave; QRS, duration of interval between beginning of Q wave to peak of S wave; QT, duration of QT interval corrected for heart rate.
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Sudden Infant Death Due to RyR2 Mutation

Figure 6. A, Presence of an arrhythmogenic substrate in young R176Q/+ knock-in mice. B, The right internal jugular vein was cannulated in 12- to 18-day-old neonatal mice. C through E, A 1.1F octapolar catheter was advanced into the right ventricle, allowing for simultaneous recording of surface ECG (F), atrial (G), and ventricular electrograms (H). F and G, Example of ventricular tachycardia (VT) in R176Q/+ mouse induced with a single premature beat after isoproterenol injection.

childhood sudden unexplained death may in fact be early and delayed manifestations of the same arrhythmia syndrome, namely CPVT. Experimental studies support this hypothesis, as the gain-of-function biophysical defects in RyR2 Ca^{2+} channels caused by CPVT-linked mutations are similar to those caused by SIDS-associated RyR2 mutations (R2267H and S4564R).\(^9,10,34\) Although it is a limitation of our study that the R176Q mutation studied in this article was identified in a 15-year-old proband\(^15\) and not in a neonatal victim of SIDS, we do believe that the R176Q/+ knock-in mouse represents a suitable model to study the physiological effects of a gain-of-function defect in RyR2 channels. In future studies, we plan to generate knock-in mice carrying RyR2 mutations identified in younger victims of SIDS.

Tiso et al\(^{12}\) have suggested that mutations in RyR2 including R176Q cause ARVC/D. This study has been controversial in recent years as the association between RyR2 mutations and ARVC/D has not been confirmed by other groups.\(^{11}\) Previous studies in adult R176Q/+ mice demonstrated the absence of right ventricular dysplasia and interstitial fibrosis, although subtle right ventricular diastolic dysfunction was detected in these mice.\(^{15}\) Histological studies in neonatal R176Q/+ mice that either died unexpectedly or were electively euthanized on postnatal day 1 also revealed an absence of right ventricular dysplasia or interstitial fibrosis. Thus, it is likely that the R176Q/+ mutation in RyR2 causes an arrhythmogenic phenotype in the absence of structural heart disease.

Our arrhythmia findings in young R176Q/+ mice are similar to those made previously in adult R176Q/+ mice that develop CPVT.\(^{15}\) Unfortunately, it is not possible to perform telemetric studies in neonatal R176Q/+ mice as the radio transmitter (4 g) is about the same size as the neonatal mice (2 to 7 g). Nevertheless, our data in 2- to 10-day-old mice revealed an increased incidence of ventricular arrhythmias strongly suggesting that these are the cause of sudden death in R176Q/+ mice. Postma et al\(^{36}\) demonstrated that some carriers of RyR2 mutations exhibit bradycardia. Our data show that R176Q/+ mutant mice exhibit slightly lower heart rates, although these differences were not significant. Based on our experimental findings, ventricular tachycardias are the most likely cause of sudden unexplained death in neonatal R176Q/+ mice, although bradycardia and atrioventricular block could not be excluded as a possible cause of death.

In vitro studies using single-channel recordings of RyR2 with the 2 SIDS mutations have demonstrated an increased open probability of the mutant channels, which was enhanced by β-adrenergic stimulation.\(^9\) In addition, the electrophysiological studies in neonatal R176Q/+ mice suggest that ectopic activity and ventricular tachycardias occur almost exclusively in the presence of a triggering event (ie, β-adrenergic stimulation or cardiac pacing). These findings are consistent with clinical observations that ventricular tachycardias are almost exclusively seen in patients with CPVT after isoproterenol.\(^31\) We observed both monomorphic and polymorphic ventricular arrhythmias in young R176Q/+ mice, whereas patients with CPVT tend to exhibit bidirectional or polymorphic ventricular tachycardias.\(^31\) At present, it remains unclear if the occurrence of monomorphic ventricular tachycardias in R176Q/+ mice is due to species differences or due to the young age at which the mice are studied. Nevertheless, these electrophysiological data suggest that catecholamine-induced cardiac arrhythmias are a likely mechanism of increased sudden cardiac death incidence observed in the young R176Q/+ mice.

The triple-risk model of SIDS proposes that the simultaneous occurrence of an exogenous stressor in an infant with an intrinsic susceptibility, during a critical developmental period, may lead to sudden death.\(^{37}\) Our data are consistent with this model because the R176Q/+ mice have a genetic susceptibility due to the Ryrr2 mutation, develop arrhythmias only after catecholaminergic stimulation (stressor), and are most susceptible during first few neonatal days, which is possibly associated with developmental changes that increase the likelihood of arrhythmias. Somewhat similar evidence for sudden death during infancy was obtained in a transgenic rabbit overexpressing the HERG-G628S mutation in the heart.\(^{38}\) Moreover, Nuyens et al\(^{39}\) reported that knock-in mice with a deletion of amino acids 1505 to 1507 (KPQ) in the cardiac SCN5A Na^{+} channel develop embryonic death, whereas heterozygous mice are susceptible to ventricular...
arrhythmias. These and other animal models may enhance our understanding of the molecular and cellular pathways involved in neonatal arrhythmogenesis, which might help to identify children at risk for SIDS and to develop new preventive treatments for high-risk individuals. Future studies of R176Q/+ mutant mice in a different inbred background may identify modifiers of the phenotype that might translate in patient populations susceptible to SIDS. In conclusion, our findings demonstrate that a gain-of-function mutation in RyR2 confers an increased risk of cardiac arrhythmias and sudden death in young mice and suggest that young R176Q/+ mice may be used as a model for elucidating the complex interplay between genetic and environmental risk factors associated with SIDS.

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Disclosures
None.

References
Sudden Infant Death Due to RyR2 Mutation

**CLINICAL PERSPECTIVE**

Sudden infant death syndrome (SIDS) is the leading cause of death in infants less than 1 year of age. Most SIDS deaths occur in infants that are seemingly healthy and are not preceded by warning signs. The exact etiology of SIDS occurs remains elusive, but many experts believe that a combination of several factors is involved. The triple-risk model of SIDS proposes simultaneous occurrence of a biological susceptibility, an outside stressor, and a critical development period. It has been suggested that inherited mutations in ion channels might lead to a vulnerability to lethal cardiac arrhythmias in infants. Interestingly, mutations in the ryanodine receptor (RyR2) calcium channel gene were identified in several victims of SIDS. We used a knock-in mouse model of mutation R176Q in RyR2 to determine whether defective RyR2 calcium channel function would result in sudden infant death caused by cardiac arrhythmias. Our results revealed an increased incidence of sudden unexpected death in R176Q knock-in mice during the first weeks of life. Surface and intracardiac ECG recordings demonstrated the presence of an arrhythmogenic substrate and ventricular arrhythmias in infant R176Q mice. Using optical mapping experiments, we also found an increased incidence of spontaneous calcium release events via defective RyR2 in R176Q mouse hearts. These studies suggest that a genetic mutation in RyR2 in infant mice may predispose them to ventricular arrhythmias and sudden death.
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Supplemental Methods

**Genotyping of R176Q/+ mice**

For genotyping of mice from the R176Q colony, genomic DNA was prepared from tails of adult mice, or tails (or limb) of deceased neonatal mice using the directPCR Lysis Kit (Viagen Biotech, Los Angeles, CA) according to manufacturer’s instructions. For the genotyping PCR, 2 µl of DNA is added to 10 µl of Go-Taq Flexi Green Buffer (5x, Promega), 3 µl MgCl₂ (25mM stock), 1 µl dNTP (10mM stock, Invitrogen), 2 µl forward primer (10mM stock), 2 µl reverse primer (10mM stock), 0.5 µl G0-Taq Flexi polymerase (Promega), and 27.5 µl de-ionized water. Sequence of the forward primer is GGG AAT GAA ATC ACT CTG GCT AAC, and reverse primer TAC ATG AGG CAC AAA ACA AAG ACC. The PCR cycling conditions included 2 min at 94°C, 35 cycles of 15 seconds at 94°C, 30 seconds at 50°C, and 90 seconds at 64°C, followed by 5 min at 68°C. Following PCR, a restriction digest is performed using RsrII. The wildtype (WT) allele yields an 800 bp band, the R176Q mutant allele an 430bp band.

The results (**Supplemental Figure 1**) reveal the presence of the larger 800 bp band in wildtype (WT) adult (left) and dead neonatal mice (right), and both the larger and smaller (430 bp) bands in R176Q/+ heterozygous knockin mice. Please note that the quality of the PCR was equal for DNA isolated from adult mouse tail or tissue (tail, limb) obtained from deceased neonatal mouse.
Design of novel optical mapping setup for neonatal mouse hearts

Isolated mouse hearts were incubated with the Ca\(^{2+}\) indicator Rhod-2-acetoxymethyl ester (90 µmol/l; Invitrogen, Carlsbad, CA) dissolved in pluronic F-127 (0.1%; Invitrogen, Carlsbad, CA) and dimethyl sulfoxide for 45 min, as described\(^2,3\). Hearts were transferred to the experimental chamber and superfused with the RH237 voltage-sensitive dye (12.5 µmol/l; Invitrogen, Carlsbad, CA) dissolved in Tyrode solution for 15-30 min. Fluorescence was excited with a 532 nm laser source (see Supplemental Figure 2). Emitted fluorescence was split with a dichroic mirror and collected with two aligned electron-multiplying CCD cameras (Cascade 128+, Photometrics, Tucson, AZ). Signals were acquired from a field of 3 mm x 3 mm, at 0.6 ms per frame with a spatial resolution of 32 x 32 (~0.09 mm/pixel). Cytosolic calcium levels were assayed at an emission wavelength of 585 nm, and membrane voltage signals at an emission wavelength of 710 nm. Bipolar stimuli were delivered at the base of the heart using platinum electrodes and a Grass stimulator triggered by computer-controlled pacing sequences as described\(^2\).
Supplemental Figure Legends

**Supplemental Figure 1. Representative example of RT-PCR genotyping of R176Q/+ and WT mice.** RT-PCR of genomic DNA isolated from adult mice (left) or deceased neonatal mice (right). The larger 800 bp band indicates the presence of the wildtype (WT) allele, and the smaller (430 bp) band indicates the R176Q mutant allele. Please note that the quality of the PCR was equal for DNA isolated from adult mouse tail or tissue (tail, limb) obtained from dead neonatal mouse.

**Supplemental Figure 2.** Schematic overview of the optical mapping setup for neonatal mouse hearts. Green light (532 nm) from laser source was shone onto the tissue. Fluorescence was collected via a customized lens, and split by a dichroic mirror. Reflected fluorescence (Ca\(^{2+}\) signal) was filtered at 585 nm, and passed fluorescence (Vm signal) was filtered by a 710 nm long-pass filter, and collected by a CCD camera.
Supplemental Figures

Supplemental Figure 1

Supplemental Figure 2
Supplemental References

