Altered Cardiac Electrophysiology and SUDEP in a Model of Dravet Syndrome

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Abstract

Objective: Dravet syndrome is a severe form of intractable pediatric epilepsy with a high incidence of SUDEP: Sudden Unexpected Death in epilepsy. Cardiac arrhythmias are a proposed cause for some cases of SUDEP, yet the susceptibility and potential mechanism of arrhythmogenesis in Dravet syndrome remain unknown. The majority of Dravet syndrome patients have de novo mutations in SCN1A, resulting in haploinsufficiency. We propose that, in addition to neuronal hyperexcitability, SCN1A haploinsufficiency alters cardiac electrical function and produces arrhythmias, providing a potential mechanism for SUDEP.

Methods: Postnatal day 15-21 heterozygous SCN1A-R1407X knock-in mice, expressing a human Dravet syndrome mutation, were used to investigate a possible cardiac phenotype. A combination of single cell electrophysiology and in vivo electrocardiogram (ECG) recordings were performed.

Results: We observed a 2-fold increase in both transient and persistent Na+ current density in isolated Dravet syndrome ventricular myocytes that resulted from increased activity of a tetrodotoxin-resistant Na+ current, likely Na1.5. Dravet syndrome myocytes exhibited increased excitability, action potential duration prolongation, and triggered activity. Continuous radiotelemetric ECG recordings showed QT prolongation, ventricular ectopic foci, idioventricular rhythms, beat-to-beat variability, ventricular fibrillation, and focal bradycardia. Spontaneous deaths were recorded in 2 DS mice, and a third became moribund and required euthanasia.

Interpretation: These data from single cell and whole animal experiments suggest that altered cardiac electrical function in Dravet syndrome may contribute to the susceptibility for arrhythmogenesis and SUDEP. These mechanistic insights may lead to critical risk assessment and intervention in human patients.

Introduction

Dravet Syndrome (DS, previously known as Severe Myoclonic Epilepsy of Infancy) is a devastating, intractable pediatric epileptic encephalopathy [1]. Patients exhibit developmental regression during early childhood and frequent pharmacoresistant seizures [1-3]. Up to 15% of DS subjects die during early childhood or adolescence, and most of these cases are thought to represent SUDEP [2], defined as Sudden Unexpected, witnessed or unwitnessed, nontraumatic and nondrowning Death in patients with Epilepsy [4], excluding cases of documented status epilepticus. In the most widely used definition, death may occur with or without evidence of a seizure, and postmortem examination does not reveal a toxicological or anatomical cause of death. SUDEP accounts for 7.5-17% of all deaths in epilepsy [5,6]. Indirect evidence has linked SUDEP to seizure-induced apnea, pulmonary edema, dyregulation of cerebral circulation, and cardiac arrhythmias [5-7]. Arrhythmias may also occur secondary to hormonal or metabolic changes or autonomic discharges [6-8].

More than 80% of DS patients carry de novo mutations in SCN1A [9,10] that result in haploinsufficiency. SCN1A encodes...
the voltage-gated Na⁺ channel (VGSC) α subunit Na,1.1. The tetrodotoxin–resistant (TTX-R) Na,1.5 channel, encoded by SCN5A, is the predominant VGSC in the mammalian heart [11]. TTX-sensitive (TTX-S) VGSCs, including Na,1.1, Na,1.3, and Na,1.6, are also expressed in areas of the heart that include the ventricles and sino-atrial node, although their function is not well understood [11-16]. More recently, Kaufmann and colleagues [17] showed that, in addition to Na,1.5, human atrial myocytes express TTX-S VGSCs Na,1.1, Na,1.2, Na,1.4, and Na,1.6. VGSCs provide a pore for the movement of Na⁺ into the cell, resulting in a rapidly activating and inactivating transmembrane current (Iₜ₉₉) responsible for the action potential (AP) upstroke and impulse propagation. The level of functional expression and biophysical properties of ion channels give the cardiac AP its characteristic shape. The balance between depolarizing currents (e.g., Iᵢₛ and Iᵢᵢₛ) and repolarizing currents (e.g., Iᵢᵢ, Iᵢₓ, Iᵢᵢᵧ, and Iᵢₜ) determines the level of excitability, AP morphology, AP duration (APD), and dynamics of impulse propagation [18]. Channelopathies disrupt this balance, leading to AP changes and increased susceptibility to arrhythmias and sudden death [10,19]. Cardiac arrhythmogenic diseases can result from gain-of-function (e.g., Long QT Syndrome-3, LQTS-3) or loss-of-function (e.g., Brugada Syndrome) mutations in SCN5A [20]. LQTS-3 mutations result in increased persistent Iᵢₛ during the AP plateau, leading to triggered activity in the form of early after-depolarizations (EADs), and providing a substrate for arrhythmogenesis [19]. Homozygous deletion of Scn1a, Scn2a, Scn5a, or Scn8a in mice is lethal, revealing their non-redundant functions [10,21-23]. Blockade of TTX-S VGSCs in the heart results in altered heart rate (HR) and cardiac contractility [11,12,14,17,24]. Despite many studies examining the effects of SCN1A mutations in the nervous system [3], the influence of SCN1A abnormalities on cardiac function remains unknown.

We propose that the strong association between epilepsy and SUDEP in DS subjects is a consequence of expression of mutant SCN1A in both brain and heart. Recent work, using a Scn1a<sup>−/−</sup> DS mouse model as well as an inhibitory neuron-specific Scn1a<sup>−/−</sup> line, suggested that SUDEP may be caused by parasympathetic hyperactivity immediately following seizures, leading to atrioventricular nodal block and lethal bradycardia [25]. While this study implicated cardiac dysfunction in DS-linked SUDEP, the excitability of individual cardiac myocytes was not investigated. Further, instead of studying the effects of spontaneous seizures, as proposed to occur in SUDEP patients, this study utilized acute hyperthermia-induced seizures. Our objective here was to fill a critical gap in the literature by determining whether cardiac myocytes isolated from mice expressing a human SCN1A DS mutation [26] have altered excitability and whether DS mice exhibit cardiac dysfunction following spontaneous seizures. We propose that, in addition to neuronal dysfunction, Scn1a haploinsufficiency produces altered cardiac electrical function and arrhythmias, providing a cardiac contribution to the mechanism of SUDEP. We report that Scn1a<sup>R1407X/+</sup> heterozygous mice have increased TTX-R, but not TTX-S, cardiac Iᵢₛ, as well as altered AP and ECG properties, EADs, and arrhythmias that produce SUDEP-like events. Our results provide novel insights into an ion channelopathy that provides critical conditions for arrhythmogenesis, and suggest a mechanism for SUDEP that includes changes in cardiac Iᵢₛ.

Materials and Methods

Animals

SCN1A<sup>R1407X/+</sup> mice, previously maintained on the C57BL/6J background [26], were backcrossed to C3HFeB/HeJ (Jackson Laboratory, Bar Harbor, ME) to increase litter size. Heterozygous mutant mice of both genders from the N3 and N4 generations were studied at postnatal day (P)15-21. Whenever possible, all data analysis was conducted blinded to genotype. Heterozygous Scn1a<sup>R1407X/+</sup> mice are designated DS throughout the manuscript.

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the University Committee on the Use and Care of Animals at the University of Michigan (Approval Numbers: 04695 and 09790). All efforts were made to minimize suffering.

Genotyping

The R1407X mutation abolishes a Hpall restriction site in the wildtype Scn1a sequence. DS mice were genotyped by PCR amplification of a 518 bp genomic fragment with the primers DS-F (5’ CAATGATCCCTAGGGGATGTC 3’) and DS-R (5’ GTTCTGTGCACATTACGATTCCAC 3’). Digestion of the PCR product with Hpall generated 2 fragments, 295 and 223 bp, from the wildtype allele and an uncut 518 bp fragment from the mutant allele. Genomic DNA was amplified in a 25 µl reaction containing 1X GoTaq Buffer, 0.2 mM dNTPs, 0.5 µM each primer, 1 unit GoTaq DNA Polymerase (Promega). Incubation at 94°C for 3 min was followed by 31 cycles of 94°C for 30 sec, 62°C for 1 min, 72°C for 1 min followed by incubation at 72°C for 6 min. After digestion with Hpall, PCR products were separated on 2% agarose gels containing 0.15 µg/ml ethidium bromide.

Acutely Isolated Adult Mouse Ventricular Myocytes

WT and DS cardiac myocytes were acutely isolated from P15–21 mice using a protocol modified from Cerrone et al. [27]. In brief, hearts were isolated from WT and DS mice, and placed in ice-cold perfusion buffer. The Ca²⁺ free perfusion buffer consisted of (mM): 10 HEPES, 0.6 Na₄HPO₄, 113 NaCl, 4.7 KCl, 12 NaHCO₃, 0.6 KH₂PO₄, 1.2 MgSO₄, 10 KHCO₃, 30 Taurine, 5.5 glucose, and 10 butaneodione monoxime. The hearts were cannulated and cleared with perfusion buffer (37°C, 3 ml/min). Next, type-II collagenase (0.87 mg/ml, Worthington Biochemical), trypsin (0.14 mg/ml), and 12 μM CaCl₂ were added to the perfusion buffer for the enzymatic digestion. The lower two-thirds of the hearts were isolated and minced into small pieces in digestion buffer. The digestion...
reaction was stopped by resuspension in stopping buffer, which included perfusion buffer plus 10% fetal bovine serum and 12.5 μM CaCl₂. The solution was then incrementally brought up to 1 mM CaCl₂. Healthy ventricular cardiac myocytes were defined as those that were Ca²⁺ tolerant, rod shaped, striated, and quiescent, with a resting membrane potential less than or equal to -65 mV. All myocyte recordings were acquired within 8 h of the cell isolation.

### Single Cell Electrophysiology

Standard voltage and current clamp techniques were used to assess the effects of DS mutations on cardiac Iₙa and AP properties, respectively [28,29]. Single cell cardiac electrophysiological properties were acquired from healthy cardiac myocytes. Experiments were performed using borosilicate glass pipettes with resistance of <3 MΩ for Iₙa and 4-5 MΩ for AP recordings. Data were acquired using an Axopatch 200B amplifier (Molecular Devices, USA). The data were acquired and analyzed using pCLAMP9-10 (Molecular Devices, USA) and custom AP analysis software (National Instruments LabView, USA).

### Voltage Clamp Recordings

Voltage clamp Iₙa recordings were performed at room temperature (21–22°C) with 5 mM [Na⁺]o. The extracellular solution contained (in mM): 5 NaCl, 1 MgCl₂, 1 CaCl₂, 0.1 CdCl₂, 11 Glucose, 132.5 CsCl, and 20 HEPES. The filling solution contained (in mM): 5 NaCl, 135 CsF, 10 EGTA, 5 MgATP, and 5 HEPES. Upon gaining access to the cell, appropriate whole cell and series resistance compensation (<70%) and leak subtraction were applied. Whole cell Iₙa, TTX-R Iₙa, TTX-S Iₙa density and biophysical properties were assessed. Assessment of transient and persistent Iₙa density, Iₙa inactivation time, and the voltage dependence of Iₙa conductance were obtained by holding the cell at -120 mV, followed by stepping to voltages between -100 and +30 mV, in 5 mV steps, for 200 ms, with 2800 ms interpulse intervals. The voltage dependence of Iₙa availability was determined by holding at various voltages (-160 mV to 0 mV, 5 mV increments, 200 ms duration) and stepping to -40 mV (30 ms), with 2770 ms interpulse intervals at -120 mV. The normalized voltage dependence of Iₙa availability and conductance (based upon each cells’ reversal potential) were fit to a Boltzmann function, and differences in the Vₐ₅₀ and slope factor were compared between groups. The time dependence of Iₙa recovery was assessed by holding at -120 mV and stepping to -30 mV for 20 ms (P1), followed by a 1–40 ms (1 ms increments) interpulse interval at -120 mV, and a second step to -30 mV for 20 ms (P2). The time dependence of Iₙa recovery was calculated by P2/P1 at each timepoint, and these results were fit to a single exponential function. The rate of Iₙa inactivation was fit to a double exponential function. 100 nM TTX was added to block only the TTX-S Iₙa, and therefore pharmacologically separate the TTX-R and TTX-S Iₙa. TTX (30 μM) was then given to block all Iₙa, and used for measuring the persistent Iₙa (pre- minus post-30 μM TTX). The persistent current was measured 30-35 ms after the voltage step, which was a time when the current amplitude was stable. These persistent Iₙa results were also confirmed using the P/4 method, yielding similar results.

### Current Clamp Recordings

Current clamp AP recordings were acquired at 37°C in standard Tyrodes solution (in mM): 148 NaCl, 0.4 NaH₂PO₄, 1 MgCl₂, 5.4 KCl, 1 CaCl₂, 5.5 glucose, 15 HEPES. The internal solution included (in mM): 148 KCl, 1 MgCl₂, 5 EGTA, 5 HEPES, 2 creatine, 5 Kₐ₅-ATP, 5 phosphocreatine. Incremental amounts of current (0.1 nA steps, 0.3 ms) and pacing cycle lengths (2000 ms, 1000 ms, and up to the fastest pacing cycle length indicated, in 1 hertz (Hz) increments) were used to assess changes in excitability, AP morphology, and susceptibility to triggered activity (i.e. EADs). Only cells with a diastolic membrane potential more negative than -65 mV were used for analysis.

### In Vivo ECG Recordings

P15-17 WT (N = 8) and DS (N = 13) mice were implanted with radiotelemetry ECG devices (DSI ETA-F10) at the University of Michigan Phenotyping Core. Animals were anesthetized (isoflurane) and the unit was implanted on the dorsal surface via a small 1 cm incision. Next, a small midline incision was made from the xiphoid process to the manubrium. The leads were passed over the shoulder subcutaneously and sutured onto the intercostal muscles of the rib cage, for ECG lead configuration II. The incisions were sutured closed, the animals were treated with prophylactic antibiotics, and all mice successfully recovered from the procedure without any signs of complications. The first WT mouse with a non-working test unit remained viable without any pathologies (>180 days). ECG (1 KHz sampling), temperature, activity, and running wheel activity were acquired continuously until P70 to provide mechanistic insights into the DS in vivo cardiac phenotype and the events precipitating SUDEP. Mice were housed in a temperature controlled room (21°C) in separate cages on a 12 h light-dark cycle (6 AM - 6 PM). Recordings were monitored and analyzed remotely. The alterations in the ECG waveform were documented during pentylenetetrazole-induced convulsive seizures (PTZ, 40 mg/kg loading and then 20 mg/kg repeated every 20 min intraperitoneally) and the period leading up to spontaneous death in non-PTZ treated mice. All surviving animals were euthanized at the end of the study.

### Quantitative Reverse Transcriptase-Polymerase Chain Reactions (qRT-PCR)

Total RNA was isolated from individual hearts using Trizol Reagent (Abion/RNA). Aliquots (1.5 μg) of total RNA were treated with DNase I (Invitrogen) and cDNA was prepared using the SuperScript II First Strand Synthesis System for RT-PCR (Invitrogen). The Scn5a transcript was quantified using TaqMan gene expression assays Mm01342518_m1 and Mm00451971_m1 (ABI) which span introns 7 and 12, respectively. As an internal control, the 18S transcript was quantified using TaqMan assay Mm03928990_g1. Fluorescence was measured on a Step 1 Real Time PCR System (ABI) at the Microarray Core at the University of Michigan. The mean Ct value was determined from...
quadruplicate assays of each sample. The value of \( \Delta C_T \) was calculated by subtracting the \( C_T \) for 18S from the \( C_T \) for Scn5a.

**Western Blot Analysis**

Western blot analysis was performed as previously described [30]. Anti-Na\(_{1.5}\) antibody was provided by Dr. Peter Mohler [31] and used at a dilution of 1:1000. Secondary antibody was goat anti-rabbit antibody conjugated to horseradish peroxidase used at a dilution of 1:800 (Thermo Scientific). Signals were visualized using a chemiluminescence system (Supersignal West Femto Maximum Sensitivity Substrate, Thermo Scientific), detected on a Li-Cor Odyssey using ImageStudio software.(Li-Cor).

**Statistical Analyses**

Results are expressed as mean ± standard error of the mean. Unpaired t test with Welch’s correction, \( \chi^2 \) Test and Log-rank, Mantel-Cox, Survival Test were used as appropriate to test for significance between genotypes, and significance was considered as \( p \leq 0.05 \).

**Results**

**DS mice have increased TTX-R transient and persistent \( I_{Na} \) density**

To determine whether DS mice have altered cardiac excitability, we examined the \( I_{Na} \) properties of acutely isolated ventricular myocytes. We reported previously that Scn1a haploinsufficiency results in increased, rather than decreased, \( I_{Na} \) density and hyperexcitability in DS patient-specific induced pluripotent stem cell (iPSC)-derived neurons [32]. Consistent with this, we observed a 2-fold increase in the peak transient (\( p < 0.0001 \)) and persistent (\( p \leq 0.05 \)) \( I_{Na} \) density in cardiac myocytes from DS vs. WT littermates (Figure 1, A and B), with a hyperpolarizing shift in the voltage dependence of \( I_{Na} \) availability and conductance (Figure 1 C, \( p \leq 0.05 \)). Since the voltage dependence for pure or predominantly TTX-R Na\(_{1.5}\) expressing cells is more negative compared to TTX-S (e.g. Na\(_{1.1}\), Na\(_{1.3}\), or Na\(_{1.6}\)) VGSC expressing cells, these results suggested a change in the proportion of total cellular \( I_{Na} \) carried by Na\(_{1.5}\) [28,32-41]. We administered 100 nM TTX to test for potential changes in functional TTX-S \( I_{Na} \) density in the DS cardiac myocytes. We observed a similar reduction in DS and WT cells (Figure 1 D), consistent with previous reports [42,43]. These data suggest that the observed increase in \( I_{Na} \) in DS cardiac myocytes was not due to increased functional TTX-S VGSC expression and was instead due to an increase in activity of the predominant cardiac TTX-R VGSC, Na\(_{1.5}\).

We used 100 nM TTX to pharmacologically isolate the TTX-R and TTX-S \( I_{Na} \). Regardless of genotype, and as expected, the \( V_n \) values for TTX-R \( I_{Na} \) (following blockade of TTX-S \( I_{Na} \) with 100 nM TTX) vs. TTX-S \( I_{Na} \) (defined as total \( I_{Na} \) minus TTX-R \( I_{Na} \)) were significantly different. Figure 2 illustrates that pharmacological isolation of each current led to the expected shifts between TTX-R vs. TTX-S \( I_{Na} \) voltage dependent properties (\( p \leq 0.05 \)), with \( V_n \) values for TTX-R \( I_{Na} \) being more negative than TTX-S \( I_{Na} \) availability and conductance. However, a comparison of \( V_n \) values for the pharmacologically separated currents between genotypes showed no differences, suggesting that the observed increases in total \( I_{Na} \) were due to increases in the level of functional channel expression rather than changes in voltage-dependence.

Table 1 shows a detailed biophysical characterization of \( I_{Na} \) properties. No changes were observed in the voltage of peak \( I_{Na} \), \( I_{Na} \) reversal potential, or normalized \( I_{Na}\)-voltage relationships between genotypes. While the slope factors for the voltage dependence of the total \( I_{Na} \) availability and conductance did not differ between genotypes (Figure 1C, Table 1), when we pharmacologically separated the TTX-S and TTX-R \( I_{Na} \) (Figure 2, Table 1), we observed slope factor differences. The slope factors for TTX-R Na\(^+\) conductance and TTX-S \( I_{Na} \) availability were significantly increased in DS, suggesting changes in the sensitivity of these channels to changes in voltage.

**Differences in Scn5a transcription and translation are undetectable**

DS cardiac myocytes exhibited a 2-fold increase in TTX-R transient and persistent \( I_{Na} \) density compared to WT myocytes. In an effort to understand the molecular mechanism for this difference, we performed quantitative RT-PCR (qRT-PCR) to ascertain whether we could detect differences in the level of Scn5a transcripts between genotypes. Using two different Scn5a primer pairs and two independent cDNAs per animal (n=4-5), we observed no change in the amount of Scn5a transcript (Figure 3A). To assess differences in channel protein expression, we quantified Na\(_{1.5}\) polypeptide levels in membrane enriched ventricular myocyte fractions from each genotype. As Figure 3B-C indicate, we found no measurable changes in Na\(_{1.5}\) expression, suggesting a post-translational mechanism, e.g. altered channel trafficking, phosphorylation, or association with cytoskeletal proteins in specific subcellular domains.

**DS cardiac myocytes are hyperexcitable**

To determine whether the observed changes in \( I_{Na} \) resulted in altered cardiac myocyte excitability, we recorded APs from isolated WT and DS ventricular cardiac myocytes. DS cardiac myocytes were hyperexcitable compared to WT. DS cells required significantly less current to initiate AP firing (\( p \leq 0.05 \), Figure 4A). At all pacing cycle lengths, WT and DS mice had similar diastolic membrane potentials (ranging between -75 mV to -72 mV). At each pacing cycle length, we observed non-significant trends for increased AP upstroke velocity and APD in DS vs. WT cardiac myocytes (Figure 4B and C). Despite these values not reaching statistical significance, we observed a significant increase in the incidence of EADs in DS (67%) vs. WT cardiac myocytes (18%, \( p \leq 0.05 \), Figure 4D), which provides a substrate for the initiation of cardiac arrhythmias [19].

**DS leads to changes in cardiac excitability and sudden death**

We observed that 21% of DS mice die by P150, with 38% of these deaths occurring before P25 (similar to [25]), and 69% of
the deaths by P52 (n = 75 for each group, Figure 5A) [26]. A similar incidence of SUDEP was observed in DS mice that were implanted with radiotelemetry devices (Figure 5B). At the termination of the in vivo radiotelemetry ECG study a subset of WT and DS mice were tested for the susceptibility to PTZ-induced convulsive seizures (rated on the Racine Scale). Administration of PTZ led to marked bradycardia, which may be similar to previously observed seizure-induced bradycardia [44] and similar to that observed in Scn1a+/- mice following acute hyperthermia induced seizures [25]. Interestingly, the initial injection of PTZ resulted in a sudden 32 ± 5% decrease in the HR, which was further diminished with increasing doses of PTZ (58 ± 3% of pre-drug). Consistent with the increased persistent I\textsubscript{Na} and AP changes reported above, DS mice exhibited significant alterations in the ECG that may provide insights into a potential human DS cardiac phenotype. While there were no differences in HR, PQ interval, PR interval, or QRS duration, we observed a significant prolongation of the QT interval (HR corrected and uncorrected QT\textsubscript{50,0201}, Table 2 and Figure 7A). We were able to record the events preceding spontaneous SUDEP (or near-SUDEP) in 3 of the 13 DS mice at P41, P45, and P51 (referred to as DS-1, DS-2, and DS-3, respectively. All 8 WT mice were alive at the end of the study (Figure 5B). DS-1 and DS-2 died suddenly subsequent to ventricular fibrillation (VF), while DS-3

Figure 1. DS Mice Have Altered Cardiac I\textsubscript{Na} Properties. A. Current-voltage (I-V) relationship of transient I\textsubscript{Na}. Peak transient I\textsubscript{Na} density is increased 2-fold in the DS (N = 6, n = 14) vs WT cardiac myocytes (N = 8, n = 20, p < 0.0001). Inset: Representative traces from each group. B. I-V relationship for persistent I\textsubscript{Na} (pre- minus post-30 µM TTX) also shows a 2-fold increase in peak persistent I\textsubscript{Na} in the DS vs. WT groups. To further confirm these results we employed the P/4 method to measure the persistent I\textsubscript{Na}, yielding similar results (-60 mV, WT, -1.72 ± 0.50; DS, -3.88 ± 0.72, N = 2, n = 5-9, p = 0.02). C. Leftward shift (V\textsubscript{1/2} of Boltzman fit, p = 0.04) in the voltage dependence of I\textsubscript{Na} availability and conductance in the DS group. D. Similar percent change in peak transient I\textsubscript{Na} density upon administration of 100 nM TTX in the WT and DS groups. Unpaired t-test with Welch’s correction. doi: 10.1371/journal.pone.0077843.g001

In vivo ECG recordings suggest a cardiac mechanism for SUDEP in DS

Consistent with the increased persistent I\textsubscript{Na} and AP changes reported above, DS mice exhibited significant alterations in the ECG that may provide insights into a potential human DS cardiac phenotype. While there were no differences in HR, PQ interval, PR interval, or QRS duration, we observed a significant prolongation of the QT interval (HR corrected and uncorrected QT\textsubscript{50,0201}, Table 2 and Figure 7A). We were able to record the events preceding spontaneous SUDEP (or near-SUDEP) in 3 of the 13 DS mice at P41, P45, and P51 (referred to as DS-1, DS-2, and DS-3, respectively. All 8 WT mice were alive at the end of the study (Figure 5B). DS-1 and DS-2 died suddenly subsequent to ventricular fibrillation (VF), while DS-3
became moribund, with lack of movement, severe bradycardia, and hypothermia, and was euthanized for ethical reasons. All 3 DS mice that died exhibited R-R variability. Focal and idioventricular rhythms, conduction abnormalities, and

Figure 2. Isolation of TTX-R and TTX-S $I_{\text{Na}}$ Biophysical Properties. A. Boltzman curves for the voltage dependence of $I_{\text{Na}}$ availability and conductance for the total cardiac $I_{\text{Na}}$ (TTX-S + TTX-R $I_{\text{Na}}$; reproduction of the curve-fits from Figure 1C). In both WT and DS myocytes the $V_{\text{1/2}}$ values of TTX-R $I_{\text{Na}}$ (closed circles, following blockade of TTX-S $I_{\text{Na}}$ with 100 nM TTX) and TTX-S $I_{\text{Na}}$ (open circles, defined as total $I_{\text{Na}}$ minus TTX-R $I_{\text{Na}}$) are plotted. Pharmacological separation of TTX-S and TTX-R $I_{\text{Na}}$ was confirmed by the loss of difference in the $V_{\text{1/2}}$ values between WT vs DS, and the development of a significant difference between the TTX-S vs. TTX-R $V_{\text{1/2}}$ values for $I_{\text{Na}}$ availability and conductance. B. Zoom-in of the boxed region in A.
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Figure 3. mScn5a and Nav1.5 levels are unchanged in DS mutant hearts. A. Heart RNA from biological replicates (DS mice, n = 4; WT mice, n = 5) were used to generate two independent cDNAs per animal. The cDNAs were assayed using qPCR in quadruplicate with two independent Scn5a TaqMan primer sets and normalized to 18s RNA. B. Western blots of membrane proteins isolated from DS and WT ventricular CMs. 50 µg of protein was loaded in each lane, and probed with anti-Na,v1.5 (Mohler 1:1000), and anti-α-actin (Sigma 1:500), which served as the loading control. C. Quantification of Na,v1.5 expression normalized to α-actin expression.
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SUDEP, which occurred at 7:46 PM and 10:16 PM in DS-1 and DS-2, respectively, the animals became bradycardic, followed by large HR fluctuations and progressive HR slowing (Figure 7C). To further investigate the DS ECG phenotype preceding putative SUDEP, we analyzed the R-R interval. One day prior to death in DS-1 and DS-2 the R-R interval was regular. In contrast, 1 hour preceding death in both animals the R-R interval became irregular, with more frequent episodes of R-R variability preceding the fatal cardiac arrhythmia and sudden death (Figure 7D and E). DS-3 developed increased R-R variability in the 48 hours preceding euthanasia, with the most pronounced R-R variability in the hour preceding death (Figure 7F).

Cardiac arrhythmias in DS mice often preceded apparent spontaneous convulsive seizures observed as high frequency muscle artifacts (Figure 8). These artifacts were not observed in untreated WT mice, but arose during PTZ-induced convulsive seizures in both WT and DS mice (Figures 9 D). DS-1 and DS-2 exhibited periods of premature ventricular complexes (PVCs) and bundle branch block (BBB) that often preceded or occurred during apparent convulsive seizures (Figures 8, A and B). In contrast, the incidence of PVCs and BBB during the PTZ study was low (3 of 8 mice with 1-3 PVCs per mouse over a 3 hour period), was not correlated with PTZ induced convulsive seizures, and was not closely coupled to the catastrophic event (occurring more than 30-60 minutes prior to death from status epilepticus).

All 3 DS mice developed large R-R variability and idioventricular arrhythmias with changing QRS morphology, which were closely coupled with high frequency electrical activity, consistent with the signal during an apparent convulsive seizure (Figure 8, A-C). Other than a few PVCs in WT-2, time matched ECG recordings from WT-1, WT-2, and WT-3 did not exhibit any of these ECG manifestations and arrhythmias that preceded sudden death in DS mice. In contrast to the ECG changes preceding death in DS-1 and DS-2, the R-R and QRS changes recorded after PTZ administration did not directly precede the convulsive seizures. Ultimately, DS-1 and DS-2 underwent a catastrophic event with high frequency electrical activity without any discernible QRS complexes. A contribution from muscle artifact cannot be entirely excluded, but the ECGs from both mice are consistent with VF (Figure 8, D and E), as discussed below.

Figure 9A illustrates sinus activity with the corresponding Fast Fourier Transform (FFT) spectrum showing a dominant frequency peak at ~12 Hz that is consistent with the measured HR of 728 bpm. Figure 9B is the FFT spectrum of an ECG pattern consistent with a convulsive seizure with the maintenance of sinus activity. There were not any clear dominant frequency peaks (ECG signal from Figure 8C). Figure 9C shows the FFT spectrum during the apparent VF ECG pattern. The dominant frequency of the complex electrical activity was ~25 Hz, which is consistent with previously reported frequencies of mouse VF [49,50]. To confirm that the muscle artifact from a spontaneous lethal convulsive seizure would not yield a similar FFT spectrum as in Figure 9C, we assessed the FFT spectrum during a PTZ-induced convulsive seizure (Racine Scale 5, Figure 9D). Unlike the dominant frequency peak at ~25 Hz in Figure 9C, the ECG signal during a PTZ induced seizure was ~10 Hz (Figure 9D). In summary, neither putative spontaneous seizures nor PTZ-induced seizures in DS mice, with and without identifiable QRS

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**Table 1.** Iₙa biophysical Properties in Each Group.

| Parameter              | Mouse |WT | DS |
|------------------------|-------|----|----|
| Capacitance (pF)       |       | 84.0 ± 6.4 | 61.0 ± 3.5 | p=0.005 |
| Peak Iₙa (pA/pF)       |       | -48.6 ± 4.0 | -94.8 ± 6.3 | p<0.0001 |
| V of Peak Iₙa (mV)     |       | -40.4 ± 1.0 | -42.5 ± 1.2 | p=0.17 |
| Iₙa Rev. Pot. (mV)     |       | 5.1 ± 1.9 | 9.2 ± 1.5 | p=0.09 |
| Iₙa Availability       |       |                |                |        |
| V ½                    |       | -86.5 ± 1.8 | -92.6 ± 1.8 | p=0.02 |
| Slope Factor           |       | 6.3 ± 0.2 | 6.4 ± 0.1 | p=0.81 |
| Iₙa Conductance        |       |                |                |        |
| V ½                    |       | -50.4 ± 1.3 | -55.0 ± 1.4 | p=0.02 |
| Slope Factor           |       | 4.9 ± 0.2 | 5.3 ± 0.3 | p=0.19 |
| Iₙa Decay (τ_fast)     |       | 2.4 ± 0.3 | 1.7 ± 0.1 | p=0.02 |
| -50                    |       | 4.1 ± 0.5 | 4.5 ± 0.4 | p=0.59 |
| -45                    |       | 4.1 ± 0.5 | 3.8 ± 0.4 | p=0.59 |
| -40                    |       | 2.5 ± 0.3 | 3.0 ± 0.3 | p=0.32 |
| -35                    |       | 2.1 ± 0.3 | 2.6 ± 0.3 | p=0.28 |
| -30                    |       | 1.1 ± 0.1 | 0.8 ± 0.1 | p=0.01 |
| Iₙa Decay (τ_slow)     |       | 5.2 ± 0.8 | 5.9 ± 0.7 | p=0.50 |
| -50                    |       | 4.1 ± 0.5 | 4.5 ± 0.4 | p=0.59 |
| -45                    |       | 4.1 ± 0.5 | 3.8 ± 0.4 | p=0.59 |
| -40                    |       | 2.5 ± 0.3 | 3.0 ± 0.3 | p=0.32 |
| -35                    |       | 2.1 ± 0.3 | 2.6 ± 0.3 | p=0.28 |
| -30                    |       | 1.1 ± 0.1 | 0.8 ± 0.1 | p=0.01 |
| Persistent Iₙa @.........60 milivolts (pA/pF) |       | -2.0 ± 0.7 | -4.3 ± 0.6 | p=0.04 |
|                        | (N=2; n=7) | (N=1; n=5) |                |        |
| TTX Resistant Iₙa (%)  |       | 86.7 ± 3.1 | 87.0 ± 2.1 | p=0.94 |
|                        | (N=2; n=7) | (N=1; n=5) |                |        |
| TTX-Resistant Iₙa Availability |       |                |                |        |
| V ½                    |       | -89.3 ± 1.4 | -90.0 ± 2.6 | p=0.81 |
| Slope Factor           |       | 6.2 ± 0.3 | 7.2 ± 0.5 | p=0.11 |
| TTX Resistant Iₙa Conductance |       | -55.0 ± 1.7 | -52.1 ± 1.5 | p=0.24 |
| V ½                    |       | 4.7 ± 0.4 | 6.1 ± 0.4 | p=0.04 |
| Slope Factor           |       |                |                |        |
| TTX-Sensitive Iₙa Availability |       | -76.9 ± 1.6 | -78.1 ± 1.9 | p=0.40 |
| V ½                    |       | 4.1 ± 0.3 | 5.2 ± 0.2 | p=0.01 |
| Slope Factor           |       |                |                |        |
| TTX Sensitive Iₙa Conductance |       | -44.5 ± 4.6 | -40.2 ± 2.5 | p=0.43 |
| V ½                    |       | 4.7 ± 1.0 | 6.5 ± 0.5 | p=0.15 |

Significant differences between the WT vs. DS myocytes are defined as p ≤ 0.05. doi: 10.1371/journal.pone.0077843.s001
complexes embedded within the high frequency muscle artifact, respectively, yielded a peak at 25 Hz on the ECG FFT spectrum (Figure 9B and D). Therefore, PTZ-induced seizures and death did not phenocopy the cardiac ECG phenotype of putative SUDEP in DS-1 and DS-2. Figure 9 demonstrates the sensitivity of FFT analysis to isolate the multiple frequency components and provides further evidence that one mechanism for SUDEP in DS may be cardiac arrhythmia.

Ultimately, DS-1 and DS-2 developed wide complex, low amplitude, and bradycardic focal discharges with a BBB morphology that progressively decreased in rate to eventual asystole (Figure 8F). In contrast to DS-1 and DS-2, DS-3 did not have the opportunity to undergo a lethal cardiac arrhythmia, as it was euthanized for ethical reasons. Yet, during the final hour of ECG recordings DS-3, but not WT-3, became bradycardic (<180 bpm) and exhibited abrupt changes in the QRS morphology and amplitude. Some aspects of the spontaneous bradycardia and periods of bradycardia followed by tachycardia, observed here, are similar to effects reported in the Scn1a^-/- DS mouse model following acute hyperthermic seizures [25], although there are key differences, for example, the absence of atrioventricular nodal block and the initiation of VF in our model. Nevertheless, taken together, these data support the hypothesis that significant cardiac pathophysiological changes occur in DS mice.

Discussion

SUDEP is a catastrophic, multi-system failure that involves seizures, changes in autonomic tone, respiratory dysregulation, and cardiac arrhythmias [5,7,51]. Recent work suggested that parasympathetic hyperactivity following hyperthermia induced...
Figure 5. DS Mice Undergo SUDEP. A. Kaplan-Meier survival curves for WT and DS mice (N = 75 for each group, p < 0.0001, Log-rank, Mantel-Cox, Survival Test). B. Percent survival in WT (N = 8) and DS (N = 13) mice implanted with radiotelemetry units. SUDEP or near-SUDEP in 3 DS mice (at P41, P45, and P51, respectively).
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Figure 6. Decreased Threshold for PTZ Induced Seizures in DS Mice. WT and DS mice were administered incremental doses of pentylenetetrazole (PTZ), monitored for observable seizures, and classified on the Racine Scale.
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Table 2. Telemetry ECG Measurements of various parameters illustrating a significant prolongation of the QT interval and the corrected QT interval (QTc). QTc calculated by the Bazett formula.

| Parameter | WT          | DS          | Significance |
|-----------|-------------|-------------|--------------|
| N = # mice | N = 5       | N = 13      | p = 0.93     |
| RR        | 87.3 ± 2.9  | 86.9 ± 2.2  | p = 0.46     |
| PQ        | 29.1 ± 1.3  | 28.0 ± 0.6  | p = 0.41     |
| PR        | 33.2 ± 0.9  | 32.4 ± 0.4  | p = 0.53     |
| QRS       | 7.1 ± 0.5   | 7.5 ± 0.3   | p = 0.53     |
| QT50      | 30.6 ± 1.4  | 35.6 ± 1.9  | p = 0.05     |
| QT75      | 33.6 ± 1.2  | 39.3 ± 2.2  | p = 0.04     |
| QT90      | 35.6 ± 1.0  | 42.2 ± 2.5  | p = 0.03     |
| QT50c     | 3.6 ± 0.2   | 4.2 ± 0.2   | p = 0.04     |
| QT75c     | 3.3 ± 0.2   | 3.8 ± 0.2   | p = 0.06     |
| QT90c     | 3.8 ± 0.2   | 4.5 ± 0.2   | p = 0.03     |

Significant differences between the WT vs. DS myocytes are defined as p ≤ 0.05.

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tonic-clonic seizures resulted in severe bradycardia and death in the Scn1a-/- mouse model of DS [25]. This study, however, left open important gaps in our knowledge: first, whether cardiac myocytes isolated from a mouse model of DS have altered excitability and second, whether DS mice exhibit cardiac dysfunction following spontaneous seizures. Here, we used a multilevel approach to investigate the DS cardiac phenotype in a global knockin mouse model expressing a human DS mutation. Electrophysiological recordings of acutely dissociated ventricular myocytes demonstrated that Scn1a haploinsufficiency leads to a 2-fold increase in transient and persistent I_{Na} density. The pharmacological and biophysical properties of I_{Na} in the DS myocytes suggest that the observed increase in I_{Na} is the result of an increased number of functional Na_1.5 channels at the plasma membrane. DS ventricular myocytes exhibited alterations in AP morphology and incidences of triggered activity. In vivo, DS mice developed spontaneous seizures and pathological ECG manifestations, including bradycardia, idioventricular rhythms, RR variability, PVCs, BBB, and VF. Ultimately, these results provide mechanistic insights into how alterations in cardiac electrical function establish ideal conditions for arrhythmogenesis and
SUDEP. Thus, DS mutations in *Scn1a* lead not only to alterations in neuronal excitability, but also to cardiac electrophysiological abnormalities in isolated ventricular myocytes, contributing to the mechanism underlying SUDEP.

**DS ventricular myocytes have increased I\(\text{Na}\) density with AP changes**

Neuronal hyperexcitability in DS mouse models has been proposed to occur through a selective decrease in \(I_{\text{Na}}\) in inhibitory neurons [25,26,34,52]. Interestingly, more recent results demonstrate that at P21-24, an age older than previously investigated, \(I_{\text{Na}}\) is increased in excitatory pyramidal neurons in *Scn1a*\(^{-/-}\) mice [53]. Further, DS patient-specific forebrain-like neurons generated from iPSCs have increased \(I_{\text{Na}}\) in both excitatory and inhibitory neuronal cell types [32]. Here, we observed a significant increase in TTX-R \(I_{\text{Na}}\) density and hyperpolarizing shifts in the voltage dependence of \(I_{\text{Na}}\) conductance and availability in DS cardiac myocytes. Our data are consistent with previous reports showing that increased Na\(_v\)1.5 expression leads to hyperpolarizing shifts in the voltage dependence of \(I_{\text{Na}}\) [28,29]. These results suggest that the increased \(I_{\text{Na}}\) in DS can be explained by increased functional TTX-R Na\(_v\)1.5 activity. In another mouse model of DS (Scn1b null mice [54]) there was a 2-fold increase in Na\(_v\)1.5-mediated transient and persistent \(I_{\text{Na}}\). Furthermore, in Scn1b null mice and in ventricular myocytes over-expressing human SCN5A [28] there was action potential prolongation, with prolonged QT and RR intervals in the Scn1b mice [54]. Increased transient \(I_{\text{Na}}\) is predicted to provide more depolarizing current during the AP upstroke, resulting in a lower threshold of current injection to elicit an AP, and increased AP upstroke velocity. Increased transient \(I_{\text{Na}}\) alone would increase the safety factor and preserve the stability of impulse propagation [18]. However, as observed in models of LQTS-3 and Na\(_v\)1.5 overexpression, increased persistent \(I_{\text{Na}}\) disrupts the balance between depolarizing and repolarizing currents, leading to APD/QT prolongation, EADs, arrhythmias, and sudden cardiac death [28,55].

**DS Mice Exhibit LQTS Phenotype**

Similar to the \(I_{\text{Na}}\) recordings from DS mice presented here, our previous work using the Scn1b null mouse model of DS demonstrated proportional increases in transient and persistent \(I_{\text{Na}}\) [54]. In contrast, genetic and pharmacological models of increased persistent \(I_{\text{Na}}\) leading to LQTS3 exhibit a disproportionate increase in persistent \(I_{\text{Na}}\), with little change in transient \(I_{\text{Na}}\) [55-58]. As we previously demonstrated however [28], regardless of whether the increase in persistent \(I_{\text{Na}}\) scales with the transient current, an absolute increase in persistent \(I_{\text{Na}}\) disturbs the balance of inward and outward currents during the AP plateau (a time of high membrane resistance). Subsequently, this leads to APD prolongation and EAD.

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**Figure 8. Cardiac Arrhythmias Precede SUDEP in DS.** Lead II ECG traces illustrating cardiac arrhythmias preceding death. A-C. In mouse DS-2, muscle artifact consistent with convulsive seizures was preceded by idioventricular rhythms, including premature ventricular complexes (PVCs), bundle branch block (BBB), altered QRS morphology, and R-R variability. D and E. Initiation of high frequency electrical activity without any discernible sinus activity, consistent with VF. F. Low amplitude wide complex focal bradycardia with a BBB morphology, and eventual asystole.

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formation, which is a known trigger for the initiation of arrhythmias and sudden cardiac death [19,59]. It remains incompletely understood whether the QT interval is prolonged in DS patients. QT prolongation has been shown to be a predisposing interictal and peri-ictal factor that ultimately leads to SUDEP in some (non-DS) epilepsy patients [7]. QT prolongation has been observed in children and adult patients with chronic epilepsy, during epileptic discharges, and the QT prolongation is even more prevalent in SUDEP vs. without SUDEP epileptic patients [4,60-64]. Interestingly, 33-44% of epilepsy patients and mouse models of epilepsy are prone to cardiac arrhythmias and LQTS, and conversely, one-third of LQTS patients have a history of seizures [8,65,66]. In two studies that examined ECG parameters in patients with DS, increased QT dispersion [67] and decreased heart rate variability [67,68] were observed, with no changes in the QT interval [67,68]. Studies that have examined the QT interval in DS mouse models have yielded contrasting results, with QT prolongation observed in the Scn1b null model and no change in the Scn1a+/- model [25,54]. The present study, using a human SCN1A knockin mutant mouse model, is the first to record ECG properties in conscious, unrestrained, and telemetered DS mice that have regained circadian heart rate, temperature, and activity cycling (e.g., Figure 7C).

**DS mutations lead to alterations in the expression of other ion channels**

Changes in the expression of a single ion channel gene often result in changes in the expression levels of other ion channel genes [29,69,70]. Scn1a null mice have increased Na,1.3 expression in the hippocampus [34]. Scn1b null mice have decreased Na,1.1 and increased Na,1.3 expression in the hippocampus, increased Scn5a/Na,1.5 expression and 3H-saxitoxin binding (suggesting increased TTX-S VGSC expression) in the heart [54], as well as altered TTX-R and TTX-S Ina biophysical properties and decreased Na,1.9 expression in dorsal-root-ganglia (DRG) [48,69]. DS patient-specific iPSC excitatory and inhibitory neurons have increased Ina in spite of SCN1A haploinsufficiency [32]. These compensatory changes in ion channel expression are not limited to VGSCs, as there is also a reduction in K+ current in Scn1b null DRGs and cortical pyramidal neurons, contributing...

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**Figure 9. Dominant Frequency Analysis.** A. Sinus rhythm 1 day prior to SUDEP, which is consistent with heart rate (728 bpm) analysis. B. Muscle artifact embedded in the sinus ECG (same as Figure 9 C) without any clear frequency peaks. C. High frequency electrical activity without any discernible sinus activity, consistent with VF (~25 Hz, same as Figure 9, D and E). D. PTZ induced seizures lead to a lower frequency electrical signal (~10 - 20 Hz). Inset: Representative snapshots of the ECG signal included in the fast-fourier transformation.

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to hyperexcitability [69,71]. Dissociated DRG neurons from Scn1b null mice have a reduction in not only I_{Na} density, but also in K_{Ca} expression and I_{K} density [74]. Furthermore, Na_{v}1.15 has been shown to interact with K_{Ca}2.2 [71]. Cortical neurons from Scn1b null mice exhibited reduced I_{Na} current, and cortical pyramidal neurons exhibited APD prolongation with repetitive firing [71]. Additionally, Na_{v}1.5 and Kir2.1 have been shown to be part of a macromolecular complex, with reciprocal interactions between Na_{v}1.5 and Kir2.1 expression, and subsequent changes in I_{Na} and I_{K} density [29]. Here, we show that Scn1a haploinsufficiency leads to increased functional expression of TTX-R I_{Na} in the heart. Taken together, these data suggest a homeostatic-like mechanism in response to VGSC subunit gene mutation that overcompensates by increased activity of a different VGSC subunit.

It is not surprising that we were unable to detect a measurable decrease in TTX-S I_{Na} in our experiments. Na_{v}1.1 is one of at least three known TTX-S VGSCs expressed in cardiac myocytes, and TTX-S I_{Na} contributes only ~10% of the global I_{Na} [11,14,17,42,43]. Thus, it is unlikely that small decreases in TTX-S I_{Na} due to Scn1a haploinsufficiency, would be resolvable using the whole cell voltage clamp technique. In future studies, the use of super-resolution imaging, as in [72], will allow positioning of the recording electrode directly at the T-tubules and in the triad, where TTX-S VGSCs are localized, thus enabling more precise measurement of small, localized changes in cardiac TTX-S I_{Na}. In spite of this, it is possible that decreases in Na_{v}1.1 expression in the T-tubules may disturb the macromolecular complex that includes the Na_{Ca}2-exchanger, Na_{v}1.5-K_{v}1.2-ATPase, inositol triphosphate receptor, Ca_{2+}-calmodulin dependent protein kinase-II, and ankyrin-B [73,74]. Mutations in ankyrin-B are known to lead to altered Ca_{2+} handling and pathological ECG changes, including QT prolongation, bradycardia, sinus arrhythmia, idiopathic ventricular fibrillation, catecholaminergic polymorphic ventricular tachycardia, and risk of sudden death [75,76]. These results show that microdomain changes at the T-tubules can lead to whole cell implications. Here, in our DS model, altered Ca_{2+} handling downstream of reductions in Na_{v}1.1 expression at the T-tubules may indirectly affect Na_{v}1.5 function via changes in the binding of calmodulin to the channel or altered Ca_{2+}-calmodulin dependent protein kinase-II-mediated channel phosphorylation, ultimately resulting in increased I_{Na} and arrhythmia [77,78]. Testing this hypothesis will be the focus of future studies.

Cardiac arrhythmias provide a mechanism for SUDEP in DS

Ion channelopathies are multi-organ diseases. Simultaneous electroencephalogram (EEG) and ECG studies confirm the high prevalence (33-44%) of arrhythmias in epileptic patients [8]. Conversely, approximately one-third of LQTS patients have a history of seizures [65]. We propose that SUDEP may be caused by mutations in ion channel genes that are expressed in both the brain and the heart (e.g., KCNQ1 [8], KCNH2 [65], SCN1B [54], SCN5A [79], SCN8A [15], and SCN1A [this study and [25]]). Just prior to death, some SUDEP patients [5] and LQTS mutant mice [8] exhibit loss of EEG activity, cardiorespiratory changes, and ultimately fatal cardiac arrhythmias. Sudden death due to cardiac arrhythmias in the mice shown here is consistent with published factors that precipitated and accompanied SUDEP [7]. Our mouse data mirror a human case of near-SUDEP in which the patient developed VF following a generalized seizure and ventricular tachycardia [66]. This study is the first to reveal the implications of DS mutations in Scn1a on the incidence and mechanisms of arrhythmias and SUDEP due to changes in cardiac myocyte excitability, and suggest targets for risk assessment and intervention to prevent SUDEP in DS and perhaps other epileptic channelopathies.

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Author Contributions

Conceived and designed the experiments: DSA MHM JMP LLI. Performed the experiments: DSA JJ BCC JO GML. Analyzed the data: DSA JJ BCC JO GML. Contributed reagents/materials/analysis tools: IO KY. Wrote the manuscript: DSA LLI JMP.

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