Channelopathy as a SUDEP Biomarker in Dravet Syndrome Patient-Derived Cardiac Myocytes

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SUMMARY

Dravet syndrome (DS) is a severe developmental and epileptic encephalopathy with a high incidence of sudden unexpected death in epilepsy (SUDEP). Most DS patients carry de novo variants in SCN1A, resulting in Na\textsubscript{v}1.1 haploinsufficiency. Because SCN1A is expressed in heart and in brain, we proposed that cardiac arrhythmia contributes to SUDEP in DS. We generated DS patient and control induced pluripotent stem cell-derived cardiac myocytes (iPSC-CMs). We observed increased sodium current (I\textsubscript{Na}) and spontaneous contraction rates in DS patient iPSC-CMs versus controls. For the subject with the largest increase in I\textsubscript{Na}, cardiac abnormalities were revealed upon clinical evaluation. Generation of a CRISPR gene-edited heterozygous SCN1A deletion in control iPSCs increased I\textsubscript{Na} density in iPSC-CMs similar to that seen in patient cells. Thus, the high risk of SUDEP in DS may result from a predisposition to cardiac arrhythmias in addition to seizures, reflecting expression of SCN1A in heart and brain.

INTRODUCTION

Dravet syndrome (DS) is a severe and intractable developmental and epileptic encephalopathy characterized by pharmacoresistant seizures, cognitive impairment, and increased mortality. The majority of DS patients carry de novo variants in SCN1A that result in haploinsufficiency for the voltage-gated sodium channel (VGSC) Na\textsubscript{v}1.1 (Claes et al., 2001; Meisler and Kearney, 2005). Up to 20% of DS patients die from sudden unexpected death in epilepsy (SUDEP) (Cooper et al., 2016). Indirect evidence has linked SUDEP to central or obstructive apnea, pulmonary edema, dysregulation of cerebral circulation, autonomic dysfunction, or cardiac arrhythmias (Surges et al., 2009). While SUDEP is the most devastating consequence of epilepsy, very little is understood about its causes and there are no biomarkers to predict which patients are at increased risk for SUDEP. Because SCN1A is expressed in heart as well as in brain (Malhotra et al., 2001; Mishra et al., 2015), we proposed that alterations in cardiac excitability may contribute to the mechanism of SUDEP in SCN1A-linked DS. Here, we used DS patient induced pluripotent stem cell-derived cardiac myocytes (iPSC-CMs) to test this hypothesis.

The cardiac action potential (AP) is a tightly regulated process that relies on the ability of ion channels to depolarize and repolarize in synchrony to maintain proper heart rhythm (Rodens et al., 2002). Mutations in cardiac VGSC genes result in arrhythmias (Veerman et al., 2015). Tetrodotoxin (TTX)-resistant Na\textsubscript{v}1.5 channels, encoded by SCN5A, are the major cardiac VGSCs in mammals (Bao and Isom, 2014). Na\textsubscript{v}1.1, a TTX-sensitive VGSC that is highly expressed in brain (Westenbroek et al., 1989), is also expressed in human and mouse heart, although at lower levels than Na\textsubscript{v}1.5 (Malhotra et al., 2001; Mishra et al., 2015). Work in rodents indicates that TTX-sensitive VGSCs, including Na\textsubscript{v}1.1, are expressed in specific cardiac subcellular domains where they play important roles in shaping the cardiac AP and modulating excitation-contraction coupling (Frasier et al., 2016; Lin et al., 2015; Maier et al., 2002, 2004; Radwanski et al., 2015; Westenbroek et al., 2013). We showed previously that Scn1aR1407X and Scn1b null DS mouse CMs have compensatory increases in transient and persistent sodium current (I\textsubscript{Na}) and increased incidence of early afterdepolarizations in the AP waveform (Auerbach et al., 2013; Lin et al., 2015; Lopez-Santiago et al., 2007). This work demonstrated that, in addition to aberrant central and peripheral neuronal excitability (Kalume et al., 2013; Yu et al., 2006), DS mice have differences in CM ionic currents that serve as substrates for arrhythmias. Importantly, our work suggested that...
cardiac arrhythmias contribute to the mechanism of SUDEP in DS patients.

While transgenic mouse models have provided valuable insights into potential SUDEP mechanisms, mice are not small humans. As a result, no biomarkers exist to assess SUDEP risk in patients. To address this problem, we have generated CMs from SCN1A-DS patient-derived iPSCs. Importantly, iPSC-CMs retain the patient’s unique genetic background. In addition, cellular phenotypes are autonomous; changes in ionic currents are intrinsic to the cell rather than the result of remodeling in response to altered autonomic innervation or seizures, as might occur in the whole animal. Here, we generated iPSC-CMs from 4 SCN1A-linked DS subjects and two controls without epilepsy and measured contraction rates and VGSC function. Our results were predictive of altered cardiac electrophysiology in one subject before cardiac symptoms were diagnosed.

RESULTS

Skin biopsies were collected from four SCN1A-linked DS patients and two controls without epilepsy under Institutional Review Board approval. Table 1 characterizes the patient SCN1A mutations as well as their clinical parameters. Reprogramming and pluripotency analyses were described previously (Liu et al., 2013). To generate iPSC-CMs we used chemically defined, growth factor-free media containing small-molecule Wnt inhibitors (Lian et al., 2012). Following at least 60 days of maturation, iPSC-CMs had an elongated morphology (Figures 1 A–1C and 2B). Immunofluorescence staining (Figures 1 and S1) showed that both patient and control iPSC-CMs expressed α-actinin (Figures 1 A–1C and S1A–S1E), connexin 43 (Figures 1 A and S1A–S1E), Nav1.5 (Figures 1 B and S1A–S1E), and the homeobox transcription factor NKX2.5 (Figures 1 C and S1A–S1E).

iPSC-CMs spontaneously contract in monolayer culture (Zhang et al., 2009). Video recordings of the contracting monolayers showed a higher rate of contraction for DS patient iPSC-CMs compared with controls, with no differences between DS patient lines (Figures 1D and S2; Video S1). We used multi-electrode array (MEA) recordings as an independent approach to assess contraction rates. Two lines, control 3 and DS4, were tested using this method. In agreement with visually quantified rate differences, the firing rate in the MEA recordings was increased in DS4 compared with control (Figures 1E, 1F, and S2C).

We performed whole-cell voltage-clamp recordings to investigate whether iPSC-CMs from DS patients had altered INa density due to compensatory expression of other VGSC genes, as observed in DS mouse models (Auerbach et al., 2013; Lopez-Santiago et al., 2007). Transient INa density was increased at least 1.5-fold in all four DS iPSC-CM lines (1.57-fold for DS2, 1.80-fold for DS4, 1.74-fold for DS10, and 2.37-fold for DS5) over control lines (Figures 2A and 2C–2E; Table S1). Current density in the DS5 CMs was so large that proper voltage control could not be consistently maintained and voltage-dependent properties could not be accurately measured (Figure 2E, inset; Table S1), thus the value reported for INa density is likely an underestimate of the true increase. The increase in DS5 INa density was significantly greater than the average increase observed in the other three DS patient lines (p = 0.03; Figure 2D and Table S1). We found no changes in persistent INa density between control and DS lines. DS10 showed a small but significant decrease in τ for the fast component of inactivation at −30 mV, with no changes in τ for the slow phase of inactivation between control and DS lines. DS4 and DS10 showed small but significant positive shifts in the voltage dependence of inactivation, with DS4 also showing a reduced slope of the inactivation curve compared with control. DS2 and DS10 showed negative shifts in the voltage dependence of activation as well as decreases in activation slope compared with control (Table S1).

We performed qRT-PCR (Figure 2F) to assess changes in the level of VGSC mRNA expression in DS patient iPSC-CMs compared with control iPSC-CMs and non-failing

| Sex     | DS2  | DS4  | DS5  | DS10 |
|---------|------|------|------|------|
| SCNA mutation | c.975T>A (p.Y325X) | c.3982T>C (p.S1328P) | c.664C>T (p.R222X) | c.G965>T (p.R322I) |
| Sample type | fibroblasts | fibroblasts | fibroblasts | fibroblasts |
| Age at skin biopsy (years) | 7 | 7 | 2 | 10 |
| Notes | cardiac evaluation at age 4 years showed abnormal T-wave inversions and lateral T-wave flattening | after this study, was diagnosed with Wolff-Parkinson-White syndrome and underwent successful ablation | | |
adult human heart. While there were measurable differences in SCNS5A mRNA expression between the male and female control lines (1.9-fold increase in total SCNS5A in female over male, 4.5-fold increase in SCNS5A-6 in female over male, and 3.7-fold increase in SCNS5A-6A in female over male), there were no consistent differences in expression between the male and female DS lines, so the data for these lines were pooled. We observed increased total SCNS5A expression in DS iPSC-CMs over the male control iPSC-CM line (3.2-fold) and over the female control line (1.7-fold), with adult human heart showing a 9.9-fold increase compared with the male control and a 5.2-fold increase compared with the female control. We also used specific primers to differentiate between adult (SCNS5A-6) and embryonic (SCNS5A-6A) splice variants of SCNS5A. We observed a 4.6-fold increase in the combined DS iPSC-CM data over the male control but no difference compared with the female control. The DS iPSC-CM data also showed a 5.2-fold increase in the embryonic (SCNS5A-6A) splice variant over the male control and a 1.5-fold increase over the female control. As expected, non-failing adult human heart had high levels of SCNS5A-6A with almost undetectable levels of SCNS5A-6A.

Because our previous work in Scn1b null DS mice showed increased Scn3a mRNA, encoding the TTX-S VGSC Na\(_{\text{a},1.3}\), and increased \(^{3}\text{H}\)saxitoxin binding, in addition to increased Scn5a and Na\(_{\text{a},1.5}\) protein in the heart (Lin et al., 2015; Lopez-Santiago et al., 2007), we asked whether changes in SCNS5A mRNA expression could account for the observed increase in \(I_{\text{Na}}\). We found no differences in SCNS1A, SCNS3A, or SCNS8A expression in the DS lines compared with controls (Figure S3A). Thus, our results suggest that, while compensatory upregulation of SCNS5A in response to SCNS1A haploinsufficiency partially contributed to the observed increases in \(I_{\text{Na}}\) in the DS lines, changes in TTX-S VGSC expression likely did not play a role.

As expected from previous reports (Doss et al., 2012; Herron et al., 2016), the level of KCNJ2 mRNA (encoding K\(_{\text{p},2.1}\)) expression was low in all iPSC-CM lines compared with the adult human heart samples (24.4-fold higher in non-failing adult human hearts compared with control iPSC-CM lines; Figure S3A), demonstrating a comparable level of maturity of all iPSC-CM lines tested. NKX2.5, a marker of cardiac development (Reecy et al., 1997), was present in all iPSC-CM lines as well as in human heart samples (Figures 1C and S3A).

To determine whether levels of SCNS5A expression could be used as a potential biomarker for SUDEP, we performed qRT-PCR on autopsy samples obtained from two DS patients who died of SUDEP. When compared with non-failing adult human heart, there were no differences in total SCNS5A, SCNS5A-6, or SCNS5A-6A expression (Figure S3B). We also investigated several common polymorphisms in SCNS5A that are known to result in gain or loss of function, depending on the presence of other genetic mutations (Ackerman et al., 2004; Cheng et al., 2011; Hu et al., 2015; Kapplinger et al., 2015; Makielski et al., 2003; Shillapawittayatorn et al., 2011; Ye et al., 2003). We tested the four most likely polymorphisms S524Y, H558R, 1077del, or R1193Q. However, none of the polymorphisms were present in either DS5 or control 2 (Figure S3C).

The large increase in \(I_{\text{Na}}\) density in DS iPSC-CMs compared with controls led to the recommendation that this patient receive a referral to a pediatric cardiologist for evaluation. Annual electrocardiography starting at 4 years of age revealed normal sinus rhythm without evidence of QT prolongation (corrected QT interval = 436 ± 5.4 ms, \(n = 3\)) but abnormal T-wave inversions and lateral T-wave flattening (Figure 3A and Table S2). A 2D echocardiogram showed normal ventricular size and function without significant intracardiac defects (data not shown). Because of the association of autonomic dysfunction in DS patients (Delogu et al., 2011), ambulatory 24-hr Holter monitoring was performed. Annual assessment demonstrated normal mean heart rates (Table S3) but very limited heart-rate variability with decreased time-domain parameters (root-mean-square of successive differences = 16.5 ± 1.1 ms and standard deviation of NN intervals = 57.9 ± 4.3 ms, \(n = 3\)) and altered frequency domain measurements (Figure 3B) (Jarrin et al., 2015).

Because patient DS5 exhibited T-wave abnormalities on the electrocardiogram, we recorded APs from control 2
and DS5 lines. We first treated iPSC-CMs with a viral construct to increase the expression of KCNJ2 to generate a mature resting membrane potential, as described by Vaidyanathan et al. (2016). We found no differences in the resting membrane potential, threshold, AP duration (at 30%, 50%, 75%, or 90% repolarization), or amplitude...
between genotypes (Table S4). In contrast, there was an increase in the incidence of delayed afterdepolarizations (DADs) and early afterdepolarizations (EADs) in DS5 compared with the control (86% versus 25% of cells tested, respectively; p = 0.02; Figures 3C and 3D). DADs and EADs represent potential arrhythmogenic substrates that may exist at the whole-organ level. Their observed increased incidence in DS5 CMs is consistent with the electrocardiogram data as well as our previous observations in DS mouse models (Auerbach et al., 2013; Lin et al., 2015).

We used CRISPR gene editing (Tidball et al., 2017, 2018) to generate a heterozygous deletion in SCN1A (SCN1A+/−) in a control line to ask whether Nav1.1 haploinsufficiency alone was sufficient to increase I_{Na} in iPSC-CMs, or whether this result was dependent on genetic background. The mutant clone used in this study had a heterozygous deletion of a cytosine at position 23
of the SCN1A cDNA (c.23del in NM_001165963.2), resulting in a mutation with a proline changed to a histidine at position 8, and a frameshift resulting in a premature STOP codon at position 91 (p.P8fsTer91) (Figure S4). The genotypes of the mutant and control lines were confirmed with next-generation sequencing of PCR amplicons of the targeted locus (Figure S4). The iPSC clones expressed the pluripotency markers SOX2, SSEA4, and OCT4 (Figure S4). Similar to the results in DS patient iPSC-CMs shown in Figure 2, we found a 1.75-fold increase in whole-cell INa density in the CRISPR SCN1A+/− iPSC-CMs compared with SCN1A+/+ isogenic controls (Figures 3E–3G). In addition, CRISPR SCN1A+/− iPSC-CMs showed a small but significant negative shift in the voltage dependence of activation as well as a decrease in the slope of the inactivation curve compared with SCN1A+/+ isogenic control (Table S5), consistent with the shift in the voltage dependence of activation observed in DS2 and DS10, as well as the decrease in slope observed in DS4 (Tables S1 and S5).

DISCUSSION

Taken together, our DS patient-derived iPSC-CM and limited clinical data suggest that the high risk of SUDEP in DS results from a predisposition to cardiac arrhythmias in addition to neuronal hyperexcitability, reflecting haploinsufficiency of SCN1A in heart and brain and the resulting compensatory overexpression of other VGSC genes in those tissues. We observed increased transient INa density and rates of spontaneous contractions in DS patient iPSC-CMs. For the subject with the most markedly increased INa density (patient DS5), increased incidence of arrhythmogenic AP substrates was recorded from iPSC-CMs, and cardiac and autonomic abnormalities were revealed upon clinical evaluation of the patient. This work demonstrates that iPSC-CMs are a valuable model for investigating SUDEP mechanisms in genetic ion-channel epilepsies and uncovering potential biomarkers of SUDEP risk.

EXPERIMENTAL PROCEDURES

Human Subjects
Fibroblast samples were obtained from human subjects under Institutional Review Board approval as described by Liu et al. (2013).

Statistics
Results are expressed as mean ± SEM. For contraction rate and electrophysiological recordings, a one-way ANOVA followed by Fisher’s LSD post hoc test was used to compare iPSC-CM lines. Statistical significance was defined as p < 0.05.

Detailed methods for the generation of human iPSCs, CRISPR gene deletion, iPSC-CM differentiation and characterization, mRNA expression analyses, SCN5A sequencing, immunocytochemistry, MEA recording, and whole-cell voltage-clamp analysis are provided in Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, five tables, and one video and can be found with this article online at https://doi.org/10.1016/j.stemcr.2018.07.012.

AUTHOR CONTRIBUTIONS

C.R.F. designed and performed the electrophysiology experiments, analyzed the data, sequenced SCN5A, and wrote the manuscript; H.Z. performed cardiac differentiations, measurements of contraction rates, and qRT-PCR analyses of iPSC-CMs; H.S. performed cardiac differentiations; J.O. performed immunofluorescence confocal analyses; L.T.D. generated the iPSC SCN1A CRISPR deletion in iPSCs; D.S.A. designed and performed preliminary electrophysiology experiments; V.J.B. performed and interpreted the clinical cardiac workup for patient DSS; C.C. assisted with DNA isolation and sequencing of SCN5A; A.G. provided postmortem cardiac mRNA samples from SUDEP patients; L.L.E. developed the KCN2 viral construct; J.M.P. and L.L.I. designed and supervised the research and wrote the manuscript.

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