Exome Sequencing and Systems Biology Converge to Identify Novel Mutations in the L-Type Calcium Channel, *CACNA1C,* Linked to Autosomal Dominant Long QT Syndrome

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Abstract

Background—Long QT syndrome (LQTS) is the most common cardiac channelopathy with 15 elucidated LQTS-susceptibility genes. Approximately 20% of LQTS cases remain genetically elusive.

Methods and Results—We combined whole exome sequencing (WES) and bioinformatic/ systems biology to identify the pathogenic substrate responsible for non-syndromic, genotype-negative, autosomal dominant LQTS in a multigenerational pedigree and established the spectrum and prevalence of variants in the elucidated gene among a cohort of 102 unrelated patients with "genotype-negative/phenotype-positive" LQTS. WES was utilized on three members within a genotype-negative/phenotype-positive family. Genomic triangulation combined with bioinformatic tools and ranking algorithms led to the identification of a *CACNA1C* mutation. This mutation, Pro857Arg-CACNA1C, co-segregated with the disease within the pedigree, was ranked by three disease-network algorithms as the most probable LQTS-susceptibility gene, and involves a conserved residue localizing to the PEST domain in the II–III linker. Functional studies reveal that Pro857Arg-CACNA1C leads to a gain-of-function with increased $I_{Ca,L}$ and increased surface

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membrane expression of the channel compared to wildtype. Subsequent mutational analysis identified 3 additional variants within *CACNA1C* in our cohort of 102 unrelated cases of genotype-negative/phenotype-positive LQTS. Two of these variants also involve conserved residues within Ca$_v$1.2's PEST domain.

Conclusions—This study provides evidence that coupling WES and bioinformatic/systems biology is an effective strategy for the identification of potential disease causing genes/mutations. The identification of a functional *CACNA1C* mutation co-segregating with disease in a single pedigree suggests that *CACNA1C* perturbations may underlie autosomal dominant LQTS in the absence of Timothy syndrome.

Keywords

arrhythmia; calcium; genetics; ion channel; long QT syndrome

Introduction

In the United States, sudden cardiac arrest (SCA) secondary to ventricular fibrillation (VF) accounts for over 300,000 deaths annually, including thousands of individuals younger than 35 years of age who die suddenly and unexpectedly.[1, 2] VF stemming from congenital long QT syndrome (LQTS) is a leading cause for autopsy-negative sudden unexplained death.[3]

LQTS has a prevalence as high as 1 in 2000 persons[4] and is most often characterized as an autosomal dominant inherited disorder of myocardial repolarization, defined by heart rate-corrected QT interval (QTc) prolongation on resting electrocardiogram and an increased predilection for syncope, seizures, and SCA in the absence of organic heart disease or other non-cardiac pathology (i.e. skeletal myopathy, dysmorphic features, or mental retardation).[5] However, extremely rare, multi-system "syndromic" disorders associated with prolonged QT intervals, like Anderson-Tawil syndrome (ATS)[6, 7] and Timothy syndrome (TS),[8–11] have also been described.

To date, 15 LQTS-susceptibility genes have been discovered, with 75% of clinically robust LQTS attributed to mutations in three genes that encode for critical potassium (*KCNQ1,* 30% to 35%, LQT1; *KCNH2,* 25% to 30%, LQT2) or sodium (*SCN5A,* LQT3, ~ 5% to 10%) ion channel α-subunits largely responsible for the cardiac action potential[12–14]. The remaining LQTS-susceptibility genes (LQT4-15) encode for either cardiac channels, channel interacting proteins, or structural membrane scaffolding proteins that modulate channel function, and collectively contribute to < 5% of LQTS[15, 16]. Consequently, 20% of patients with a clinically strong diagnosis of LQTS remain genetically elusive and are labeled as "genotype-negative" LQTS.

With exception of the three major LQTS genes originally discovered in the mid 1990's following multi-generational whole genome familial linkage studies and positional cloning, the majority of the minor LQTS-susceptibility genes have been discovered using a biological plausible, candidate gene approach. Major technological advances in DNA sequencing have emerged recently allowing for rapid whole genome or whole exome interrogation of patient samples for the identification of novel pathogenic mutations. In fact, several recent reports have utilized whole exome sequencing (WES) approaches targeting a "trio" of affected and/or unaffected members within a pedigree, to discover novel genetic substrates for a variety of non-cardiac, heritable diseases.[17–20] In this study, we performed a WES "trio" analysis approach on a large multi-generational, "genotype-negative" LQTS pedigree to identify a novel cause for classical, "non-syndromic", autosomal dominant LQTS followed by mutational analysis of the newly discovered genetic substrate in a large

cohort of unrelated patients with robust clinical evidence for LQTS but a heretofore negative genetic test (i.e. genotype-negative/phenotype-positive-LQTS).

Methods

Study Subjects

A 15–member (8 affected, 5 unaffected, 2 unknown) multigenerational family, presenting with autosomal dominant inherited LQTS without syndactyly, cognitive impairments, facial dysmorphisms, or any other non-cardiac clinical characteristics suggestive of Timothy syndrome, (Figure 1A and Table 1) that remained genotype-negative following commercially available LQTS genetic testing, was referred to the Mayo Clinic Windland Smith Rice Sudden Death Genomics Laboratory for further research-based genetic testing. Following written consent for this IRB-approved study, medical records, including 12-lead surface electrocardiograms, and peripheral blood lymphocytes were obtained for 12 family members. Genomic DNA was obtained using the Puregene DNA Isolation Kit (Qiagen, Inc, Valencia, CA). The symptomatic index case (QTc = 498 ms), unaffected father (QTc = 383 ms), and an affected maternal aunt (QTc = 479 ms) were selected for WES.

In addition, 102 unrelated patients (71 females, 98 % Caucasian, average age at diagnosis = 23 ± 16 years, and an average QTc of 516ms $\pm$ 6.6 S.E.M.) with robust clinical evidence for LQTS (QTc 480 ms and/or a Schwartz-Moss[21] score 3.5) that were referred to our laboratory previously for genetic testing were included (see Table 2 for cohort demographics). All patients signed written consent for this IRB-approved study. All 102 patients were mutation negative following LQTS mutational analysis (by DHPLC and DNA sequencing) of the three major LQTS genes: *KCNQ1, KCNH2*, and *SCN5A* and eight minor *LQTS* genes: *AKAP9, ANKB, CAV3, KCNE1, KCNE2, KCNJ2, SCN4B*, and *SNTA1*. In addition, all patients were negative for large *KCNQ1* and *KCNH2* gene rearrangements (whole single or multiple exon deletions/duplications) following gene-specific copy number variation analysis using multiplex ligation-dependent probe amplification technique.

Whole Exome Sequencing (WES)

WES and subsequent variant annotation was performed on genomic DNA derived from the symptomatic index case (III.2, Figure 1A and Table 1, ECG Supplemental Figure 1A), unaffected father (II.1, Figure 1A and Table 1, ECG Supplemental Figure 1B), and affected maternal aunt (II.5, Figure 1A and Table 1, ECG Supplemental Figure 1C) by the Mayo Clinic Advanced Genomics Technology Center and Bioinformatics Core facilities. Paired-end indexed libraries were prepared using the manufacturer's protocol (Agilent, Santa Clara, CA). The whole exome capture was carried out using SureSelect Human All Exon 50MB kit (Agilent; Santa Clara, CA). The flow cells were sequenced as 101x2 paired end reads on an Illumina HiSeq 2000 using TruSeq SBS sequencing kit version 3 and HiSeq data collection version 1.4.8 software (Illumina, Inc., San Diego, CA). Base-calling was performed using Illumina's RTA version 1.12.4.2 (Illumina, Inc., San Diego, CA).

The sequence data was processed for alignment, variant calling and annotation was completed using Targeted RE-sequencing Annotation Tool (TREAT).[22] The TREAT annotation included alignment to the human genome 37 (hg19) using BWA,[23] duplicate read removal using PICARD (http://picard.sourceforge.net/) and GATK[24] for local INDEL realignment and base quality score recalibration of aligned reads. Single nucleotide variants (SNVs) and insertion/deletions (INDELs) were then called using SNVMix[25] and GATK[24] respectively. The SNVs and INDEL annotations provided by TREAT analysis were then filtered and summarized using custom Perl and shell scripts. The custom scripts intersected the SNVs of the 2 samples in the affected group (affected index case [III.2] and affected

maternal aunt [II.5]) and intersected the SNVs of the sample in the unaffected case (unaffected father [II.1]). The SNVs in the unaffected group were then subtracted from the SNVs in the affected group to identify the novel SNVs shared by the symptomatic index case and affected maternal aunt. SNVs in the affected samples at sites with less than 5x coverage and variants present in either dbSNP,[26] the 1000 Genomes Project,[27] (http://browser.1000genomes.org/index.html) or the 200 BGI[28] (Beijing Genomics Institute) Danish exomes were excluded (See Supplemental Table 1).

Systems Biology-Aided Exome Filtering

The LQTS interactome,[29] which features 1629 genes of the human genome that are considered to be the most likely genes that encode proteins which influence or regulate cardiac repolarization, thus representing possible candidate genes for LQTS, was utilized to determine which genes (harboring variants), shared between the index case and affected maternal aunt, could be potential LQTS candidates. Next, three systems biology ranking algorithms: Endeavor[30] (http://homes.esat.kuleuven.be/~bioiuser/endeavour/tool/endeavourweb/php), SUSPECTS[31] (http://www.genetics.med.ed.ac.uk.suspects/), and ToppGene[32] (http://toppgene.cchmc.org/prioritization.jsp), were used to rank the putative disease causative genes and were accessed publicly through the internet. Default prioritization parameters were used for each algorithm. The three canonical LQTS-disease causing genes: *KCNQ1, KCNH2,* and *SCN5A* were used as the three algorithms' training genes to rank identified mutation carrying genes. Subsequently, the rare, shared variants that localized within the published LQTS interactome: *CACNA1C, DMRTB1, DSCAML1, GLTSCR, SLC26A1, MAP1A, MARCKS* and *PPP1R12C* (listed alphabetically here) were prioritized in each of these algorithms.

DNA Sanger Sequencing for Variant Confirmation and Co-Segregation Analysis

Following systems biology-aided exome filtering, all 8 candidate variants (*CACNA1C* (NM_000719) c.2570C>G, p.Pro857Arg; *DMRTB1* (NM_033067) c.248_252dupCCGCCC, p.Ala83_Ala84dup; *DSCAML1* (NM_020692) c.6307G>A, p.Ala2103Thr; *GLTSCR1* (NM_015711) c.91A>G, p.Ser31Gly; *SLC26A1* (NM_00022042) c.2007C>G, p.Asp669Glu; *MAP1A* (NM_002373) c.2842G>A, p.Val948Ile; *MARCKS* (NM_002356) c.552_557dupTGAGGC, p.Glu185_Ala186dup) and *PPP1R12C* (NM_017607) c.188A>G, p.Asp83Gly) identified by WES were confirmed and pedigree analysis for LQTS phenotype/variant co-segregation in the multigenerational pedigree was completed independently for all 8 variants using standard DNA dye terminator cycle sequencing protocols and an ABI Prism 377 automated sequencer (Applied Biosystems Inc., Foster City, CA). DNA sequence chromatograms were analyzed using Chromas version 1.45 (Queensland, Australia).

Cell Culture and Transfection

HEK293 cells were transfected with plasmids encoding either WT- or Pro857Arg-$Ca_v1.2$ with YFP fused at the N-terminus (Antzelevitch *et al.* 2007[33]), β_{2cN4}, and $\alpha_2\delta_1$ in a 1:1:1 molar ration using Lipofectamine 2000 and cultured in DMEM + 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C + 5% CO_2 as previously described (Best *et al.* 2011[34]). HEK293 cell experiments were performed 40–48 hours post-transfection.

Electrophysiology

Whole cell ruptured patch clamp experiments were performed in HEK293 cells at 21–24°C. The pipette solution consisted of (in mM) 114 CsCl, 10 EGTA, 10 HEPES, 5 Mg-ATP, and 0.1 Li-GTP (to pH 7.2 with CsOH). The recording solution consisted of (in mM) 147 CsCl,

5 $CaCl_2$, and 10 HEPES (to pH 7.4 with CsOH). Upon electrical access, capacitive transients were obtained by applying a 5-mV hyperpolarizing pulse. Whole cell Ca^{2+} currents ($I_{Ca,L}$) were elicited at 10 mV increments from a holding potential of −80 mV and recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Data were sampled at 25 kHz and filtered at 5 kHz, and leak and capacitive currents were subtracted using a P/4 protocol. Data are presented as current density by normalizing $I_{Ca,L}$ to cell capacitance. Data are plotted in Figure 2 as mean ± SEM and significance at each voltage (defined as $P<0.05$) was determined by Student's t-test using Origin 7.5 software (OriginLab, Northhampton, MA).

Cell Surface Biotinylation

HEK293 cells grown in 10 cm culture dishes were cooled to 4°C, washed three times in ice-cold PBS, and biotinylated for 30 minutes with 0.5–1.0 mg/ml EZ Link Sulfo-NHS-LC-Biotin (Thermo Scientific Pierce Protein Research Products, Rockford, IL) at 4°C. Cells were then washed twice in ice-cold Tris-buffered saline (TBS) followed by an additional wash in PBS to remove biotinylation reagent. Cells were harvested directly by scraping and lysed in buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 0.5 deoxycholic acid, and protease inhibitor cocktail containing 2 mM phenylmethylsulfonyl fluoride, 50 µg/ml aprotinin, 50 µg/ml benzamidin, 50 µg/ml leupeptin, and 5 µM pepstatin A. For capture of cell surface biotinylated proteins, 0.5–1.0 mg soluble protein lysate was incubated with 75 µl High Capacity NeutrAvidin agarose beads (Thermo Scientific Pierce Protein Research Products) for 1–2 hours at 4°C. NeutrAvidin beads were washed five times and proteins were eluted by heating for 5 minutes at 95°C with SDS-PAGE sample buffer.

SDS-PAGE and Western blotting

Protein samples were separated using SDS-PAGE and transferred to polyvinylidene difluoride membranes. Nonspecific binding sites were blocked using 5% (wt/vol) dried skim milk in TBS with 0.05% Tween-20 (TBST). Membranes were probed with the following antibodies: rabbit anti-$Ca_v1.2$ (#AAC-003, Alomone Labs, Jerusalem, Israel), mouse anti-transferrin receptor (clone H68.4, Invitrogen), and rabbit anti-GAPDH (#sc-25778, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at room temperature. Membranes were washed subsequently four times for 10 minutes before incubation in appropriate horseradish peroxidase-conjugated secondary antibody for one hour. After additional washes in TBST, immunoreactivity was visualized using ECL Plus (GE Healthcare Life Sciences, Pittsburgh, PA). Membrane stripping was performed at 55°C for 30 minutes in buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM β–mercaptoethanol, and 100 mM DTT. Densitometric analysis was achieved using ImageJ (NIH, Bethesda, MD).

CACNA1C Mutational Analysis

Comprehensive open reading frame and splice junction mutational analysis of the entire coding region (57 amplicons that encode 2139 amino acids) of *CACNA1C* (GenBank accession number NM_000719) was performed on genomic DNA derived from 102 unrelated LQTS diagnosed patients (described above) using PCR, denaturing high performance liquid chromatography (DHPLC; WAVE DNA Fragment Analysis System, Transgenomic Inc., Omaha, NE), and DNA sequencing (ABI Prism 377; Applied Biosystems Inc., Foster City, CA). Primer Sequences, PCR conditions, and DHPLC conditions are available upon request.

To be considered as a potential LQTS-causing variant, the *CACNA1C* variant had to i) be a non-synonymous (substitution of a nucleotide which leads to a residue change) variant (i.e. synonymous single-nucleotide polymorphisms were excluded), ii) involve a highly conserved residue, iii) be absent in at least 680 ethnically-matched "in house" controls (1360

reference alleles) and all publicly available databases including the 1000 Genomes Project[27] (n=1094 subjects; 381 Caucasian, 246 African-American, 286 Asians, and 181 Hispanics) the NHLBI GO Exome Sequencing Project[35] (n=5379 subjects; 3510 Caucasians and 1869 African-Americans) (http://evs.gs.washington.edu/EVS/), and the 12000 Exome Chip[36] (n=12000 subjects) (http://genome.sph.umich.edu/wiki/Exome_Chip_Design). Control genomic DNA was obtained from the European Collection of Cell Cultures (HPA Culture Collections, UK), the Human Genetic Cell Repository sponsored by the National Institute of General Medical Sciences and the Coriell Institute for Medical Research (Camden, New Jersey).

Statistical Analysis

Data in Figures 2 and 3 are plotted as mean ± SEM and significance (defined as p 0.05) was determined by Student's t-test using Origin 7.5 software (OriginLab, Northhampton, MA). The normality was confirmed for each t-test using the Sharpiro-Wilk test.

Results

Genotype-negative/Phenotype-positive Autosomal Dominant LQTS Pedigree

The index case is a 33-year-old Caucasian female (III.2 Figure 1A and Table 1) who presented at the age of 27 years with a postpartum agonal breathing event. Subsequent medical history revealed multiple startle-triggered and exercise-induced syncopal events starting at the age of 13 years. Her electrocardiogram revealed a prolonged QTc of 498 ms (Figure 1B and Supplemental Figure 1A). Her medical therapy included ICD placement and a left cardiac sympathetic denervation. Due to complications, she had a brief period in which her ICD was removed. Subsequent to ICD removal, she had one more event while sleeping that included shaking and agonal breathing, from which she spontaneously recovered. Following this event, an ICD was re-implanted. Her family history is positive for LQTS and a sudden unexplained infant death.

Screening electrocardiograms were performed on 11 of the 12 available family members, and genomic samples were gathered from 12 family members. Based on the ECG findings, 7 family members have borderline/prolonged QTc and/or a significant history of cardiac events. The 5 family members with normal ECGs were asymptomatic (Figure 1A and Table 1).

Overall, the family history and inheritance pattern indicated autosomal dominant LQTS. One of the index case's maternal aunts, II.3, suffered out of hospital cardiac arrest at the age of 44 which resulted in significant neurological damage. A second maternal aunt, II.4, died during infancy of unknown cause. A third maternal aunt, II.5, suffered one syncopal event during pregnancy (QTc = 479 ms, ECG available Supplemental Figure 1C). The index case's daughter, IV.1, has been treated with prophylactic beta block therapy since the age of 8 years (QTc = 450 ms). The index case's asymptomatic mother, II.2, has a prolonged QTc of 486 ms. The electrocardiogram of the index case's asymptomatic maternal uncle, II.6, shows sinus bradycardia, early repolarization, and a QTc of 454 ms (Table 1).

Whole Exome Sequencing (WES) for Novel Pathogenic Substrate Identification in a Large Multigenerational LQTS Pedigree

Genomic DNA samples of the "trio" of participants in the pedigree, index case (III.2), her affected maternal aunt (II.5), and unaffected father (II.1), were analyzed by WES. Between the three samples, approximately 98% of the reads were mapped, and approximately 44% of the reads were within the target region (Supplemental Table 1).

The detected single nucleotide variations (SNVs) and insertion/deletions (INDELs) were configured in a spreadsheet so subsequent filtering strategies could be utilized to identify potential pathogenic variants. After alignment, and SNV calling, 46,307 genetic variants were present in the exome of the index case (Supplemental Table 1). Following exclusion of "common" variants present in 1000 Genomes Project,[27] dbSNP,[26] and 200 Beijing Genomics Institute (BGI) exomes[28] as well as all synonymous SNVs, 263 variants remained (Supplemental Table 1). Variants shared between the index case (III.2) and the unaffected father (II.1) and unshared variants between the index case (III.2) and the affected maternal aunt (II.5) were subtracted leaving 110 rare variants present in both the index case and the maternal aunt (data not shown).

Of these 110 remaining variants, only 8 variants, 6 SNVs and 2 InDels, (*CACNA1C* c. 2570C>G, p.Pro857Arg; *DMRTB1* c.248_252dupCCGCCC, p.Ala83_Ala84dup; *DSCAML1* c.6307G>A, p.Ala2103Thr; *GLTSCR1* c.91A>G, p.Ser31Gly; *SLC26A1* c. 2007C>G, p.Asp669Glu; *MAP1A* c.2842G>A, p.Val948Ile; *MARCKS* c. 552_557dupTGAGGC, p.Glu185_Ala186dup) and *PPP1R12C* c.188A>G, p.Asp83Gly) resided in genes that were present in the previously published 1629 node LQTS interactome[29], suggesting these 8 variants as the best candidates for pathogenicity in this multi-generational LQTS pedigree.

Candidate gene priority ranking using three *in silico* bioinformatic/systems biology algorithms (ToppGene[32], Endeavour[30], and Suspects[31]) with *KCNQ1, KCNH2*, and *SCN5A* serving as the network training genes, unanimously ranked the L-type calcium channel encoded by *CACNA1C* as the number one LQTS-causing candidate gene, followed by *SLC26A1, MAP1A, DMRTB1, DSCAML1, GLTSCR1, PPP1R12C,* and then *MARCKS* (Table 3). In addition, none of the 102 shared variants that resided outside the LQTS interactome scored higher than *CACNA1C* with any of the *in silico* tools (data not shown).

In addition to the in silico analysis which ranked *CACNA1C* unanimously as the most likely LQTS-associated candidate gene within the exomes of these patients, we analyzed the genomic DNA from all 12 family members (7 affected and 5 unaffected) for the presence/absence of all 8 variants. Consistent with the *in silico* prediction, Pro857Arg-CACNA1C was the only variant with complete co-segregation with the LQTS phenotype (i.e. present in all affected and absent in all unaffected family members) in this 12 member multi-generational pedigree (Figure 1A, and Table 1).

The Pro857Arg-CACNA1C missense mutation is a single nucleotide genetic variant (c. 2570 C>G) resulting in the substitution of a neutral, non-polar amino acid (proline, pro) for a positively charged amino acid (arginine, arg) at position 857 of the L-type calcium channel (LTCC, also annotated as $Ca_v1.2$). This putative pathogenic mutation was absent among 1094 (1000 Genomes Project[27]), 5379 (NHLBI GO Exome Sequencing Project[35]), 12000 (12000 exome chip[36]), 200 individuals (200 Danish BGI exomes),[28] and an additional 680 ethnically matched controls analyzed in our laboratory. Pro857Arg localizes to $Ca_v1.2$'s II–III linker that contains the critical PEST domain, a sequence motif rich in the amino acids proline [P], glutamic acid [E], serine [S], and threonine [T]) that provides as an important signal for rapid protein degradation and LTCC protein stability[37, 38].

In Vitro Functional Analysis of the Pro857Arg-CACNA1C

To further understand the effect that Pro857Arg has on the CACNA1C channel, we used the ruptured whole cell patch clamp technique to determine if there were electrophysiological differences between Pro857Arg- and WT-CACNA1C currents. Functional characterization of the mutation utilizing whole cell patch clamp technique in a heterologous HEK293 expression system revealed a gain-of-function phenotype with a 113% increase in peak $I_{Ca,L}$

at +10 mV compared to wildtype (Figure 2), which may lengthen the cardiac action potential and prolong the QT interval.

Because the Pro857Arg-CACNA1C mutation lies within a PEST domain, we hypothesized that the Pro857Arg mutation may impair the normal degradation of $Ca_v1.2$, thus leading to an increase in surface membrane channels and functional $I_{Ca,L}$. To test this, we performed cell surface biotinylation experiments using HEK293 cells transiently expressing WT-CACNA1C or Pro857Arg-CACNA1C along with auxiliary β_{2cN4} and $\alpha_2\delta_1$ subunits. Figure 3 shows a representative western blot demonstrating that the Pro857Arg mutation leads to an increase in NeutrAvidin-isolated (and thus plasma membrane localized) $Ca_v1.2$ signal compared to WT (Figure 3A, top left panel). Detection of endogenous transferrin receptor was included to show that expressing the Pro857Arg-CACNA1C mutant did not lead to a nonspecific increase in surface membrane proteins (Figure 3A, middle left panel). Densitometric analysis of nine independent experiments showed a mean 64% increase in surface membrane expression of Pro857Arg-CACNA1C relative to WT-CACNA1C (Figure 3B, P = 0.05). No significant difference was detected in the total cellular $Ca_v1.2$ signal between Pro857Arg and WT (Figure 3A, top right panel and 3C). Probing for the cytosolic protein GAPDH ensured that the biotinylation reagent did not significantly cross the cell membrane (Figure 3A, bottom panels).

Spectrum and Prevalence of CACNA1C Variants in a Large Cohort of Unrelated Patients with "Genotype-Negative/Phenotype-Positive" LQTS

Following comprehensive mutational analysis of *CACNA1C*, we identified 3 novel, putative pathogenic missense variants in 3/102 (2.9%) unrelated "genotype-negative/phenotype-positive" LQTS patients (Table 4 and Figure 4). Two of these three *CACNA1C* variant-positive patients were females. All 3 missense variants (c.2500A>G, p.Lys834Glu; c.2570C>T, p.Pro857Leu; and c.5717G>A, p.Arg1906Gln) were absent in 680 ethnically matched (Caucasian) controls, 1094 subjects from the 1000 Genomes Project[27], 5379 subjects from the NHLBI GO Exome Sequencing Project[35] and the 12000 Exome Chip.[39] Interestingly, 2 of the missense variants (Lys834Glu and Pro857Leu) localize to the same critical PEST domain where the original mutation (Pro857Arg) discovered in the multigenerational LQTS pedigree resides (Figure 4). In fact, one of the variants (Pro857Leu) affects the same amino acid residue (Proline 857) as the LQTS-causing mutation identified in the pedigree. A third variant (Arg1906Gln) localizes very closely to the recently described STIM1 (stromal interaction molecule 1) binding domain (amino acid residues 1806 – 1905) in $Ca_v1.2$'s C-terminus, where STIM1 is a Ca^{2+} sensor of the endoplasmic reticulum, and interacts with the CACNA1C resulting in a decrease of LTCC mediated current as well as chronically triggers LTCC internalization, suppressing channel function.[40–42] Importantly, like the affected subjects within the multigenerational pedigree, none of these 3 CACNA1C variant-positive subjects exhibit any clinical characteristics suggestive of Timothy syndrome.

Instead, the Lys834Glu variant was identified in a 15-year-old Caucasian female with a personal history of syncope, QTc of 475 ms, and a negative family history. The Arg1906Gln variant was identified in a 39-year-old Caucasian female who presented with palpitations, syncope, a QTc of 513 ms, and had a negative family history.

Similar to the multigenerational pedigree that yielded our *CACNA1C* discovery with its Pro857Arg mutation, the Pro857Leu missense variant was identified in an asymptomatic 15-year-old Caucasian male who was diagnosed with LQTS (QTc of 514 ms) following the sudden unexplained death of his 12 year-old sister during sleep. His mother, maternal grandmother, maternal great uncle, and maternal great aunt all have a previous history of syncopal events during childhood. Genomic DNA was available for the 12-year-old sister's

unaffected father, affected mother, and affected maternal grandmother. Both the affected mother and affected maternal grandmother were positive for Pro857Leu while the unaffected father was negative (data not shown). DNA was not available for either the affected maternal great uncle or affected maternal great aunt.

Discussion

Whole exome sequencing (WES) is a highly efficient means of interrogating the DNA sequence of the entire protein-encoding region of virtually every gene in the human genome for a given patient sample in order to potentially elucidate the underlying genetic mechanism of disease. However, in doing so, an investigator is often faced with tens of thousands of genetic variants, per patient DNA sample, that must be sifted through in order to identify the possible disease-causative variant. In order to identify potential candidate genes, we utilized publicly available internet-based gene prioritization tools like ToppGene,[32] Endeavour,[30] and SUSPECTS[31] to bioinformatically rank candidate genes[20] by training network algorithms to search a user defined gene list and prioritize genes for disease candidacy based on similarities to a set of user defined "training" genes (i.e. known disease causing genes like *KCNQ1*, *KCNH2*, or *SCN5A* for LQTS). Specifically for LQTS analysis, the previously published 1629 LQTS interactome[29] (encompassing a set of genes with the greatest potential to regulate cardiac repolarization) proved to be a very useful tool in further filtering variants down to a very manageable number of less than 10 candidates.

Here, our study is the first to utilize a next generation WES "trio" approach[17] followed by "LQTS interactome"[29] variant filtration and disease-network analysis based gene ranking prioritization to discover a novel putative LQTS-disease causative missense mutation, Pro857Arg-CACNA1C, that properly co-segregated with the LQTS phenotype in a large, multigenerational pedigree with autosomal dominant "genotype-negative/phenotype-positive" LQTS. In addition to co-segregation, this mutation produced a gain-of-function effect on heterologously expressed Pro857Arg $Ca_v1.2$ channels with a uniform increase in $I_{Ca,L}$ density. The electrophysiological findings are consistent with the increase in surface membrane channels identified by biotinylation experiments. Because $I_{Ca,L}$ contributes to phase 2 and 3 of the action potential, a scaled increase in this depolarizing current might increase action potential duration and the associated QT interval. Transgenic overexpression of $Ca_v1.2$ channels in the mouse heart has demonstrated an increase in action potential duration, but this is accompanied by complex electrical remodeling of multiple ionic currents as well as hypertrophy.[43] Thus, the extensive feedback regulation of $Ca_v1.2$ channels and possible electrical remodeling of multiple currents in the heart limit the ability to precisely extrapolate the increase in $I_{Ca,L}$ observed in the heterologous expression studies to native human heart.

CACNA1C encodes for the α-subunit of the L-type calcium channel (LTCC, $Ca_v1.2$) which is critical for cellular excitability and the cardiac action potential, excitation-contraction coupling, and regulation of gene expression.[44, 45] Interestingly, three (Lys834Glu, Pro857Leu, and Pro587Arg) of the four variants discovered in our study localize to a key PEST domain of the II–III linker of the LTCC α-subunit. PEST sequences, rich in proline (P), glutamic acid (E), serine (S), and threonine (T), act to signal rapid protein degradation through the cell's quality control system.[37, 38] A recent study has demonstrated that Akt (also known as protein kinase B, PKB) regulates LTCC channel protein stability by preventing $Ca_v1.2$ PEST sequence recognition and hence promoting stability of the channel complex resulting in increased macroscopic currents.[46]

However, mechanisms regulating the membrane stability of the LTCC are understood poorly, and how the unmasked PEST sequence leads to channel proteolysis is unknown. Our

functional studies revealed that the mutation, Pro857Arg-CACNA1C precipitates a 113% increase in peak $I_{Ca,L}$ at +10 mV compared to wildtype. We have also shown that Pro857Arg leads to a 64% increase of surface membrane expression compared to wildtype. The combination of these findings lead to our hypothesis that Lys834Glu-, Pro857Leu- and Pro857Arg-Ca_v1.2 may disrupt the functional PEST sequence and lead to increased channel protein stability, increased number of surface membrane L-type calcium channels, as seen through biotinylation experiments, and increased $I_{Ca,L}$, as seen through our electrophysiological studies of Pro857Arg-CACNA1C. The increase in $I_{Ca,L}$ might increase the action potential duration and contribute to the genesis of early after depolarizations and potentially Ca^{2+} overload related delayed after depolarizations, resulting in an LQTS phenotype with the propensity for potentially lethal arrhythmias.

The Arg1906Gln variant, resides one amino acid away from the Ca_v1.2 C-terminus STIM1 (stromal interaction molecule 1) binding domain. STIM1, the main activator of store-operated Ca^{2+} channels, directly suppresses depolarization-induced opening of the voltage-gated Ca^{2+} channel Ca_v1.2. STIM1 binds to the C-terminus of Ca_v1.2 through its Ca^{2+} release–activated Ca^{2+} activation domain, acutely inhibits gating, and causes long-term internalization of the channel from the membrane.[40–42] Due to its proximity, Arg1906Gln could alter STIM1 binding to CACNA1C, preventing the internalization of the channel, leading to an increased number of channels on the membrane, and a gain-of-function in Ca_v1.2 currents. All of the identified variants (Pro857Arg, Pro857Leu, Lys834Glu and Arg1906Gln) appear to potentially involve channel internalization and protein degradation pathways. Further characterization of these variants may give a deeper understanding of CACNA1C protein regulation.

Interestingly, three unique *CACNA1C* gain-of-function missense mutations (Gly406Arg in *CACNA1C's* alternative exon 8a, Gly406Arg in exon 8, and Gly402Ser in exon 8) have been described in Timothy syndrome (TS), an extremely rare (fewer than 20 people world-wide have been reported), multisystem disorder associated with extreme QT prolongation, syndactyly, autism spectrum disorder, and a myriad of other organ system abnormalities which often lead to early childhood death[8–11]. Importantly, none of the *CACNA1C* variant-positive individuals in the pedigree or the cohort study exhibit syndactyly, cognitive impairments, facial dysmorphisms, autism spectrum disorders or any other non-cardiac clinical characteristics suggestive of TS. Additionally a majority of the members within the pedigree harboring the Pro857Arg-CACNA1C mutation have survived into adulthood, which is extremely unlikely in patients with Timothy syndrome.

Although previously described in TS, no *CACNA1C* mutations have been reported for non-syndromic LQTS. With our functional studies confirming Ca_v1.2 gain-of-function as the cellular basis for the *CACNA1C* mutation-positive patient's LQTS phenotype, a paradox has emerged. Namely, how can some gain-of-function mutations, like Gly406Arg in the alternatively spliced exon 8A, result in the multi-organ system phenotype of TS while other missense mutations, like Pro857Arg, yield a cardiac-only phenotype of autosomal dominant LQTS? With this being a new discovery, it is not surprising that the answer is elusive.

Several potential mechanisms have been proposed, but the most likely explanation is that *CACNA1C* is expressed as many different isoforms throughout the body. Therefore, variable splice isoforms, as well as differences in the corresponding accessory subunits (such as the β-subunit) vary between skeletal, cardiac, and neuronal tissues. In fact, a single nonsynonymous CACNA1C variant (Ala39Val), identified in patients with Brugada Syndrome, results in a trafficking defect of the channel that is apparent only in cells expressing the cardiac isoforms of *CACAN1C*, while being normal in the context of the neuronal rat isoform.[47] Therefore, it is possible that the variants identified here will perturb

only the cardiac isoforms of the channel or auxiliary subunits, and will have minimal or no effect on *CACNA1C* expressed in other tissues, thereby leading exclusively to cardiac disease.

Given that all unrelated TS patients described to date have either the same exact sporadic de novo Gly406Arg (16 cases) mutation in exon 8a, Gly406Arg in exon 8 (1 case) or a mutation residing only 4 amino acids away (Gly402Ser, 1 case), suggests that this specific amino acid residue or region of CACNA1C may have a unique functional effect on the LTCC in multiple organ systems, whereas mutations in other regions of CACNA1C may produce a cardiac-only phenotype. All three TS mutations lead to an impaired open-state voltage-dependent inactivation of the LTCC whereas Pro857Arg leads to increase in peak $I_{Ca,L}$ current presumably through an increase in cell surface membrane expression of LTCCs without perturbing either channel kinetics or gating properties.

Limitations

Only one of the four identified CACNA1C variants have been characterized functionally thus far. Further functional studies will be necessary to definitively understand the pathogenic mechanisms underlying these *CACNA1C* variants and their implications in LQTS. HEK293 cells have specific current/kinetic properties and trafficking properties that may not be reflective of the properties of cardiomyoctes. Alternative approaches such as adenoviral transduction of primary cardiac myocytes or patient specific induced pluripotent stem cell (iPS)-derived cardiomyocytes may overcome some of the limitations of the heterologous expression system, but these approaches are beyond the scope of this study.

Conclusions

The combination of the 4 identified variants (absent in nearly 15,000 non-LQTS individuals) as well as familial co-segregation with disease for two variants (Pro857Arg and Pro857Leu) suggests that perturbations in the *CACNA1C*-encoded L-type calcium channel (LTCC) are *bona fide* pathogenic substrates for classical, non-syndromic, autosomal dominant LQTS with implications for LQTS genetic testing. If this frequency is validated in subsequent studies, *CACNA1C* may fall after *KCNQ1*, *KCNH2*, *SCN5A*, and *KCNE1* and account for approximately 1% of LQTS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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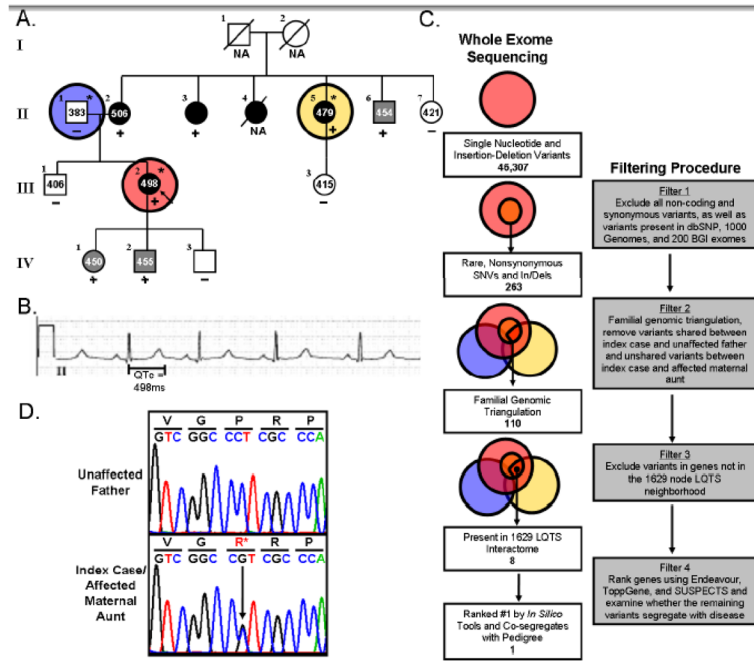
Figure 1.
Whole Exome Sequencing and Familial Genomic Triangulation for the Elucidation of a Novel Genetic Substrate for LQTS. **(A)** Black circles/squares are affected, grey are borderline, and white are unaffected with LQTS. Arrow identifies the proband. Asterisks represent those who were whole exome sequenced. Numbers in each circle/square represent QTc values (milliseconds) when available. Plus signs represent Pro857Arg-CACNA1C mutation positive family members. Minus signs represent mutation negative family members. NA denotes where DNA samples were not available. **(B)** Lead II of the electrocardiogram for the index case (III.2). The full electrocardiogram is available in Supplemental Figure 1A. **(C)** Schematic representation of our sequencing strategy. **(D)** DNA sequencing chromatogram showing the Pro857Arg mutation.
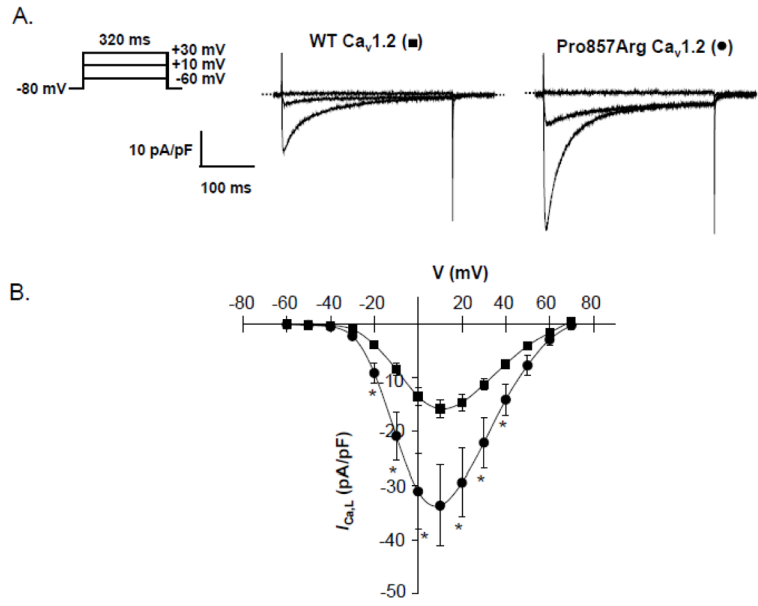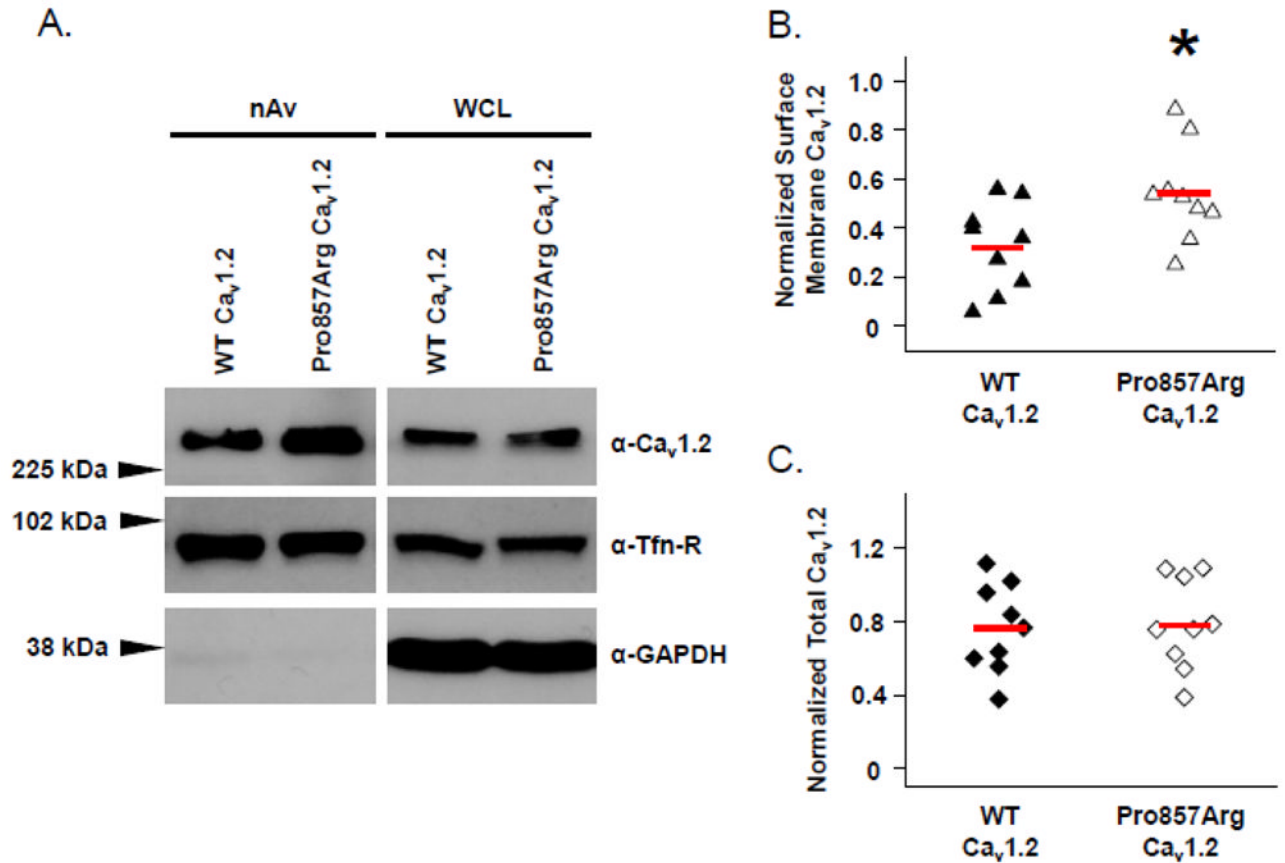
Figure 2.
Functional Analysis of Pro857Arg-Ca$_v$1.2 Mutation Using Whole Cell Patch Clamp Technique. **(A)** Representative $I_{Ca,L}$ traces recorded from HEK293 cells expressing WT- or Pro857Arg-Ca$_v$1.2 along with auxiliary β_{2cN4} and $\alpha_2\delta_1$ subunits. **(B)** Current-voltage (I–V) relations constructed from WT-Ca$_v$1.2 (■, $n = 10$) or Pro857Arg-Ca$_v$1.2 expressing cells (●, $n = 10$). Data were compared using Student's unpaired t-test and * denotes $p < 0.05$ when WT-Ca$_v$1.2 was compared with Pro857Arg-Ca$_v$1.2.

Figure 3.
Increased Surface Membrane Density of $Ca_v1.2$ Pro857Arg Channels. HEK293 cells expressing either YFP-tagged $Ca_v1.2$ WT or $Ca_v1.2$-Pro857Arg along with auxiliary β_{2cN4} and $\alpha_2\delta_1$ subunits were cell surface biotinylated at 4°C. **(A)** NeutrAvidin (nAv)-captured surface membrane proteins (left panels) and whole cell lysates (WCL, right panels) were analyzed by SDS-PAGE and western blotting. Densitometric analysis of surface membrane $Ca_v1.2$ signal **(B)** or total cellular $Ca_v1.2$ **(C)** was performed to summarize the results of nine independent experiments. In both cases, normalization of $Ca_v1.2$ signal was achieved using the corresponding signal of the endogenous surface membrane protein transferrin receptor (Tfn-R). Solid red lines through distributions indicate population means. Data were compared using Student's unpaired t-test and * denotes p 0.05 when WT-$Ca_v1.2$ was compared with Pro857Arg-$Ca_v1.2$.
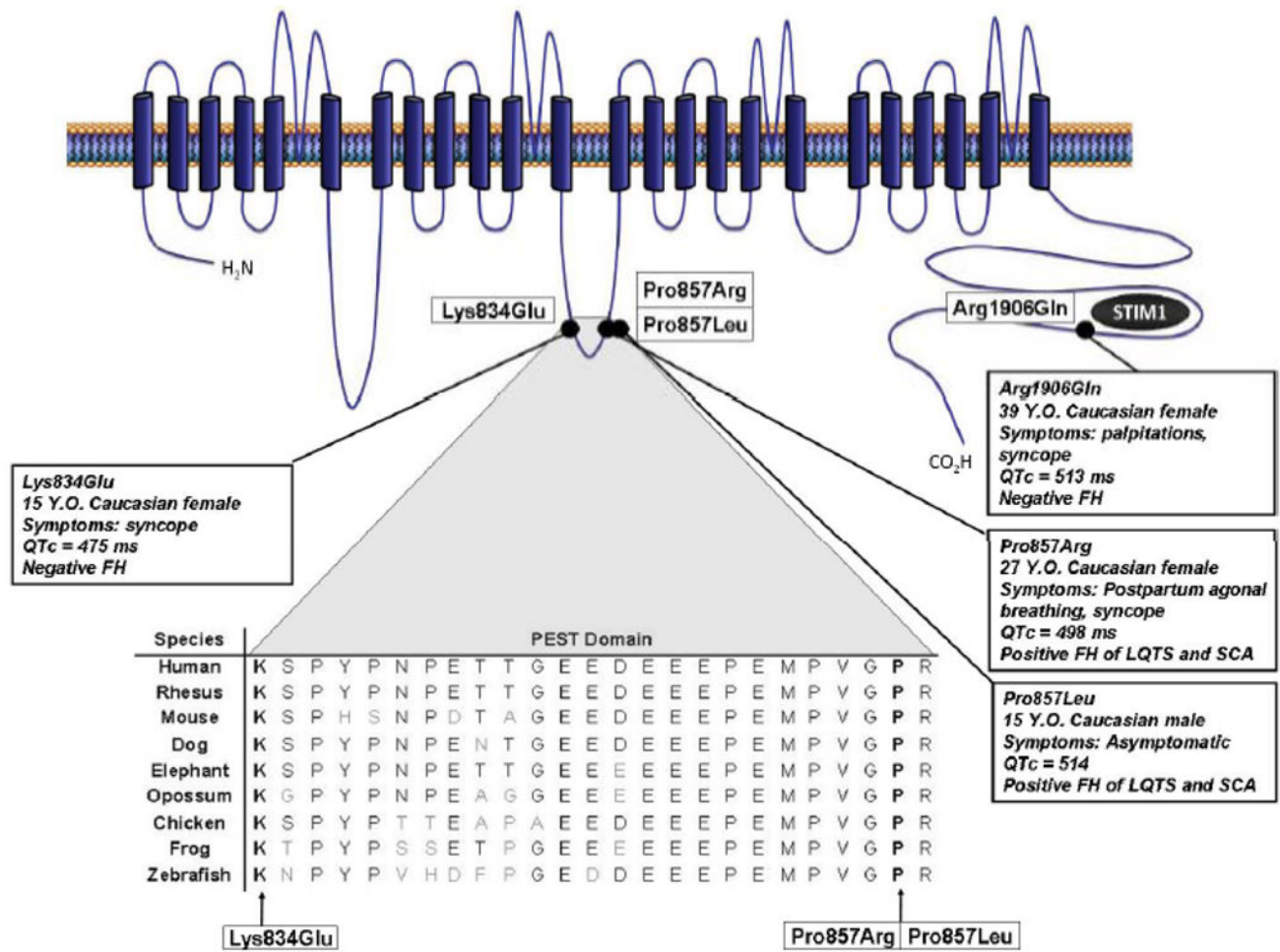
Figure 4.
Topology Diagram of the CACNA1C Channel Alpha-Subunit. Each variant is represented as a black circle followed by the amino acid position and change. Asterisk represents the mutation identified by whole exome sequencing in the pedigree (Figure 1). Patient history is displayed for each variant. The PEST domain sequence is highlighted showing the position of the three variants residing within this domain as well as the species conservation. STIM1 binding region, 1806–1905 is shown in the C-terminal tail of CACNA1C. STIM1 interacts with the LTCC to normally suppress channel function [40, 41].

Table 1

Summary of Pedigree as Shown in Figure 1

PATIENT	SYMPTOMS	QTc (ms)	THERAPY	Pro857Arg STATUS
I.1	Died in late 60s during heart surgery	NA	NA	NA
I.2	Status Unknown	NA	NA	NA
II.1 [†]	Asymptomatic	383	None	−
II.2	Asymptomatic	486	None	+
II.3	Out of hospital cardiac arrest	NA	ICD Implantation	+
II.4	Died during infancy	NA	NA	NA
II.5 [†]	Syncope during pregnancy	479	None	+
II.6	Asymptomatic	454	None	+
II.7	Asymptomatic	421	None	−
III.1	Asymptomatic	406	None	−
III.2 [*,†]	**Postpartum agonal breathing, syncope**	**498**	**ICD Implantation, sympathetic denervation**	**+**
III.3	Asymptomatic	415	None	−
IV.1	Asymptomatic	450	Prophylactic beta blocker therapy	+
IV.2	Asymptomatic	455	None	+
IV.3	Asymptomatic	NA	None	−

[*] Index Case

[†] Whole Exome Sequenced

NA Not available

+ Pro857Arg present

− Pro857Arg absent

QTc is the heart rate corrected QT interval and is shown in milliseconds

Table 2

Demographics of the LQTS Genotype-negative/Phenotype Positive Cohort

LQTS Genotype-negative/Phenotype-positive Cohort (N=102)	
Age at Diagnosis (yrs) ± standard deviation	23 ± 16
Sex (male/female)	31/71
Caucasian	98%
QTc (ms) ± S.E.M.	516 ± 6.6
Syncope	47%
Cardiac Arrest	22%
Family History of Sudden Cardiac Death	51%

Table 3

Gene Ranking Using Bioinformatic/Systems Biology Algorithms

Gene	Rank			
	Endeavour[30]	SUSPECTS[31]	ToppGene[32]	Total Rank
CACNA1C	**1**	**1**	**1**	**1**
SLC26A1	3	2	2	2
MAP1A	2	6	3	3
DMRTB1	8	3	4	4
DSCAML1	6	4	6	5
GLTSCR1	5	8	5	6
PPP1R12C	4	7	8	7
MARCKS	7	5	7	8

Table 4

Summary of *CACNA1C* Variants

Case #	Nucleotide Change	Protein Change	Location in CACNA1C	Age (years)	Sex	QTc (ms)	Symptoms	Family History LQTS	Family History SCD
1	c.2500 A>G	p.Lys834Glu	II–III	15	F	475	Syncope	No	No
2*	**c.2570 C>G**	**p.Pro857Arg**	**II–III**	**27**	**F**	**498**	**Agonal breathing**	**Yes**	**No**
3	c.2570 C>T	p.Pro857Leu	II–III	15	M	514	None	Yes	Yes
4	c.5717 G>A	p.Arg1906Gln	C-terminal	39	F	513	Syncope, palpitations	No	No

*
Case identified through whole exome sequencing, pedigree shown in Figure 1 and Table 1