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Gene Therapy for Catecholaminergic Polymorphic Ventricular Tachycardia by Inhibition of Ca^{2+}/Calmodulin-Dependent Kinase II

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BACKGROUND: Catecholaminergic polymorphic ventricular tachycardia (CPVT), an inherited cardiac arrhythmia characterized by adrenergically triggered arrhythmias, is inadequately treated by current standard of care. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), an adrenergically activated kinase that contributes to arrhythmogenesis in heart disease models, is a candidate therapeutic target in CPVT. However, translation of CaMKII inhibition has been limited by the need for selective CaMKII inhibition in cardiomyocytes. Here, we tested the hypothesis that CaMKII inhibition with a cardiomyocyte-targeted gene therapy strategy would suppress arrhythmia in CPVT mouse models.

METHODS: We developed AAV9-GFP-AIP, an adeno-associated viral vector in which a potent CaMKII inhibitory peptide, autocamtide-2–related inhibitory peptide [AIP], is fused to green fluorescent protein (GFP) and expressed from a cardiomyocyte selective promoter. The vector was delivered systemically. Arrhythmia burden was evaluated with invasive electrophysiology testing in adult mice. AIP was also tested on induced pluripotent stem cells derived from patients with CPVT with different disease-causing mutations to determine the effectiveness of our proposed therapy on human induced pluripotent stem cell–derived cardiomyocytes and different pathogenic genotypes.

RESULTS: AAV9-GFP-AIP was robustly expressed in the heart without significant expression in extracardiac tissues, including the brain. Administration of AAV9-GFP-AIP to neonatal mice with a known CPVT mutation (*RYR2*$^{R176Q/+}$) effectively suppressed ventricular arrhythmias induced by either β-adrenergic stimulation or programmed ventricular pacing, without significant proarrhythmic effect. Intravascular delivery of AAV9-GFP-AIP to adolescent mice transduced ≈50% of cardiomyocytes and was effective in suppressing arrhythmia in CPVT mice. Induced pluripotent stem cell–derived cardiomyocytes derived from 2 different patients with CPVT with different pathogenic mutations demonstrated increased frequency of abnormal calcium release events, which was suppressed by a cell-permeable form of AIP.

CONCLUSIONS: This proof-of-concept study showed that AAV-mediated delivery of a CaMKII peptide inhibitor to the heart was effective in suppressing arrhythmias in a murine model of CPVT. CaMKII inhibition also reversed the arrhythmia phenotype in human CPVT induced pluripotent stem cell–derived cardiomyocyte models with different pathogenic mutations.

Key Words: arrhythmias, cardiac ■ calcium-calmodulin-dependent protein kinase type 2 ■ death, sudden ■ gene therapy ■ polymorphic catecholergic ventricular tachycardia

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Clinical Perspective

What Is New?

- We selectively expressed the specific Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) inhibitory peptide autocamtide-2–related inhibitory peptide in cardiomyocytes without significant extracardiac expression.
- Inhibition of CaMKII effectively suppressed ventricular arrhythmias in a murine model of catecholaminergic polymorphic ventricular tachycardia after a single therapeutic dose.
- Autocamtide-2-related inhibitory peptide suppressed the arrhythmia phenotype of catecholaminergic polymorphic ventricular tachycardia patient-derived induced pluripotent stem cell–derived cardiomyocytes harboring diverse pathogenic ryanodine receptor 2 (*RYR2*) mutations.

What Are the Clinical Implications?

- Delivery of a CaMKII inhibitory peptide by adeno-associated virus represents a novel single-dose gene therapy for catecholaminergic polymorphic ventricular tachycardia.
- CaMKII inhibition is potentially translatable and effective therapy for patients with catecholaminergic polymorphic ventricular tachycardia with diverse *RYR2* mutations.
- Adeno-associated virus–mediated, cardiomyocyte-targeted delivery of CaMKII inhibitors may be used to treat other cardiac disorders associated with CaMKII dysregulation.

Among the most malignant and difficult-to-treat channelopathies is catecholaminergic polymorphic ventricular tachycardia (CPVT), which has an estimated frequency of 1:10000. CPVT is the underlying diagnosis in at least 12% of pediatric patients who present with unheralded cardiac arrest.[1] More than 30% of patients with CPVT present with a cardiac arrest, usually before 10 years of age.[2]

Ventricular arrhythmia precipitated by emotional stress or exercise is a hallmark of CPVT.[3] Progressive physical exertion typically elicits increasingly frequent premature ventricular contractions (PVCs) followed by bursts of monomorphic, polymorphic, or bidirectional ventricular tachycardia. Dominant mutations in ryanodine receptor 2 (*RYR2*) are found in 60% to 70% of patients with CPVT.[4] There are >150 different reported *RYR2* mutations that cause CPVT, which largely fall within 4 mutational hot spots within the gene.[4] These mutations cause enhanced diastolic Ca^{2+} release through the RYR2 channel from the sarcoplasmic reticulum into the cytosol.[3] Increased cytosolic Ca^{2+} then drives the activity of the Na^{+}/Ca^{2+} exchanger in reverse, which creates a depolarizing current that can trigger early or delayed afterdepolarizations, which are thought to be the underlying cause of ventricular arrhythmia.

Current treatments for CPVT include exercise restriction, β-blockade, cardiac sympathetic denervation, and implantable cardiac defibrillators.[5] Despite therapy, over an 8-year period, 25% to 27% of patients experienced breakthrough arrhythmias, with fatal or near-fatal events in 13%.[6] Cardiac defibrillators are frequently implanted but are problematic because of increased complication rates in pediatric patients,[7] failure to convert ventricular arrhythmias, and the risk of fatal implantable cardiac defibrillator–induced electric storm.[8] Treatment with the class 1c sodium channel blocker flecainide has demonstrated efficacy in patients with CPVT,[9] although some patients are incompletely protected.[10] Clearly, more effective therapy is needed.

Arrhythmia in RYR2-mediated CPVT requires both a pathogenic mutation and adrenergic stimulation, which activates downstream kinases that enhance cardiomyocyte Ca^{2+} handling by phosphorylating numerous targets. Among these kinases is Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which has been intensively studied for its deleterious roles in experimental heart disease models.[11,12] In human induced pluripotent stem cell (iPSC)–derived cardiomyocytes (iPSC-CMs) and a mouse model of CPVT, pharmacological inhibition of CaMKII with the small-molecule KN-93 reduced calcium handling abnormalities and arrhythmia susceptibility. Moreover, using a human engineered tissue model of CPVT, we demonstrated that CaMKII phosphorylation of RYR2 at serine 2814 is required to unmask the latent arrhythmic potential of CPVT-causing *RYR2* mutations.[13] These results suggest that CaMKII activity is critical for CPVT arrhythmogenesis.[14,15] Although CaMKII deletion in the heart is well tolerated and indeed protective in experimental models of heart failure,[16] CaMKII is essential in other tissues, most notably the brain.[17] The need to selectively inhibit CaMKII in the heart has hampered development of therapeutically useful small-molecule CaMKII inhibitors.

One strategy to overcome this barrier to use adeno-associated viral (AAV) gene therapy, which has proved to be a safe and efficient vector for sustained gene transfer into many cell types, to selectively inhibit CaMKII in cardiomyocytes. Cardiotropic AAV serotypes such as AAV9[18] in conjunction with a cardiac promoter permit cardiomyocyte-targeted gene transfer. Here, we tested the hypothesis that cardiomyocyte-selective inhibition of CaMKII through the AAV-mediated delivery of a potent CaMKII inhibitory peptide would normalize cardiomyocyte Ca^{2+} handling and prevent arrhythmias in CPVT models with pathogenic *RYR2* mutations. We tested this hypothesis in both human iPSC-CMs from patients with CPVT and a CPVT mouse model. Our studies provide proof of concept that AAV-mediated CaMKII inhibition represents a promising potential therapy for CPVT.

METHODS

The extended Methods section in the online-only Data Supplement provides additional information. All data and materials that support the findings of this study are available from the corresponding author on reasonable request.

Mice

Animal procedures were performed under protocols approved by the Boston Children's Hospital Institutional Animal Care and Use Committee and in accordance with established institutional guidelines. *Ryr2*$^{R176Q/+}$ CPVT mice were described previously.[19] The investigators performing mouse assays and data analysis were blinded to genotype and treatment group.

Cloning and Production of AAV Viruses

AAV-cTNT-GFP (Addgene 105543) was modified to express autocamtide-2–related inhibitory peptide (AIP) fused to the C terminus of green fluorescent protein (GFP). The backbone vector AAV-cTNT-GFP was used as a control. AAV was prepared as described[20] and injected into postnatal day 3 pups subcutaneously at a dose of 5×10^9 to 5×10^{11} viral genomes per 1 g body weight. For adolescent mice, AAV was delivered by retro-orbital injection at 1×10^{11} viral genomes per 1 g body weight.

Electrophysiology Studies

Animals underwent electrophysiology studies at 2 to 4 months of age. Under isoflurane anesthesia, animals were instrumented to acquire a 2-lead (leads I and II) ECG. A 1.1F octapolar catheter (ADInstruments) was inserted into the right ventricle via a right jugular vein cut down. Isoproterenol (2 mg/kg) and epinephrine (3 mg/kg) were given intraperitoneally, and surface and intracardiac ECGs were recorded for 3 minutes. More than 3 PVCs or a single couplet during this recording period was considered a positive response. We performed programmed ventricular stimulation using a digital stimulator (ADInstruments) by delivering 8 stimuli (S1 8 times) followed by 2 early extrastimuli (S2 and S3). Ventricular arrhythmia was defined at ≥3 induced ventricular beats. The duration of arrhythmia was measured from the last paced beat. QT dispersion was measured by comparing the absolute difference in the corrected QT interval between ECG leads I and II. The heart rate–corrected QT (QTc) interval was calculated with an automated algorithm in LabChart.

iPSC Generation and iPSC-CM Differentiation

Patients with CPVT with pathogenic *RYR2* mutations provided informed consent to participate in this study under protocols approved by the Boston Children's Hospital Institutional Review Board. Peripheral blood mononuclear cells were reprogrammed to pluripotency with the CytoTune Sendai reprogramming kit (ThermoFisher). iPSCs were differentiated to iPSC-CMs as shown in Figure IA in the online-only Data Supplement. iPSC-CM purity was assessed by flow cytometry (Figure IB in the online-only Data Supplement).

Ca^{2+} Imaging of Single Cells

iPSC-CMs seeded on 0.1% gelatin, and Geltrex-coated coverslips were transduced with adenovirus expressing the GCaMP6f-Junctin nanosensor (JT-G6F).[21] After 48 hours, the samples were imaged on an Olympus FV3000 with the line scan mode. When indicated, myristoylated AIP (SCP0001, Sigma), myristoylated protein kinase A inhibitory peptide (47648, Millipore), or isoproterenol was added to final concentrations of 600 nmol/L, 600 nmol/L, or 1 μmol/L, respectively. The percent of iPSC-CMs with abnormal Ca^{2+} release events was defined as >1 event per 20 seconds of recording and at least 5% of the nearest Ca^{2+} transient. Spatially localized Ca^{2+} release events, or "sparks," were recorded for each spontaneously active cell and reported as the number of sparks per $(s\cdot\mu m^{-1})\times100$.

Isolation of Ventricular Cardiomyocytes

Ventricular cardiomyocytes were isolated from 8- to 12-week-old mice by retrograde perfusion and plated on laminin-coated coverslips. Cardiomyocytes were incubated with 4 μmol/L Rhod-2 for 30 minutes before imaging.

Gene Expression and Immunofluorescent Staining

Immunoblots were probed with primary antibodies (Table I in the online-only Data Supplement). Imaging and quantification were performed with a digital imager (ImageQuant LAS 4000, GE). Phospholamban phosphorylation was measured by capillary Western blot of whole-heart lysates (Wes, Protein Simple).

Transcript quantification was performed on total RNA by reverse transcription–quantitative polymerase chain reaction. Primer sequences are provided in Table I in the online-only Data Supplement.

Immunostaining was performed with primary antibodies (Table II in the online-only Data Supplement). After washing, dishes were incubated with Alexa Donkey secondary antibodies (ThermoFisher). Imaging was performed on an Olympus FV3000 confocal microscope.

Statistics

Statistical analyses were performed with JMP 14 (SAS). For nominal data, the χ^2 test was used. For continuous data, either the Student *t* test or the nonparametric Steel-Dwass test was used as indicated. For violin plots and box plots, the box and whiskers define the quartile ranges with the center line or circle as the median. For bar plots, error bars show the SEM. Values of $P\leq0.05$ were considered significant. Values of $P>0.05$ were considered nonsignificant.

RESULTS

Targeted CaMKII Inhibition in the Heart

We designed an AAV-based strategy to express a CaMKII inhibitory peptide. We selected AAV9 and the cardiac troponin T promoter because of their well-documented ability to drive strong, cardiomyocyte-selective

transgene expression. To inhibit CaMKII, we chose AIP,[22] an engineered peptide derived from the autoinhibitory domain of CaMKII itself that is both highly selective and highly potent (IC_{50}=40 nmol/L).[23] We fused AIP to the C terminus of GFP so that we could easily visualize transgene expression. We used GFP alone as a negative control transgene. We produced AAV9-GFP-AIP and AAV9-GFP viral particles and injected them subcutaneously in postnatal day 3 mice (Figure 1A). Whole-mount and tissue sections demonstrated that both AAV9-GFP-AIP and AAV9-GFP robustly expressed their transgenes in cardiomyocytes (Figure 1B and 1C). By both imaging of tissue sections and reverse transcription–quantitative polymerase chain reaction, we observed minimal GFP expression in liver, lung, skeletal muscle, and brain (Figure 1C and 1D).

We next determined whether GFP-AIP effectively inhibited CaMKII activation. CaMKII activation requires autophosphorylation of threonine 286; therefore CaMKII activation can be assessed by measuring phosphorylated threonine 286 with a phospho-specific antibody. β-Adrenergic stimulation with isoproterenol-activated CaMKII in hearts treated with AAV-GFP (Figure 1E and 1F). This activation was suppressed in mice treated with AAV-GFP-AIP (Figure 1E and 1F). To further confirm that AIP effectively suppressed CaMKII activation, we examined phosphorylation of phospholamban at threonine-17, a known target of CaMKII.[24] A phospho-specific antibody (threonine-17) demonstrated increased CaMKII-directed phosphorylation with isoproterenol stimulation that was effectively suppressed with AAV-GFP-AIP (Figure 1G and 1H).

Together, these data confirm that AAV-GFP-AIP selectively inhibits CaMKII in cardiomyocytes.

Suppression of Arrhythmias in a Murine Model of CPVT

Murine models of CPVT secondary to knock-in *RYR2* mutations recapitulate many of the clinical features of the human disease.[25] To determine whether cardiac-restricted CaMKII inhibition suppresses arrhythmias in a murine CPVT model, we administered either AAV9-GFP or AAV9-GFP-AIP to neonatal (postnatal day 3) *Ryr2*$^{R176Q/+}$ mice, an established CPVT model that harbors a known human RYR2 mutation.[19] *Ryr2*$^{R176Q/+}$ and wild-type (WT) littermates underwent electrophysiology testing at 2 to 4 months of age in which an octapolar intracardiac catheter and surface electrodes were used to measure electric responses to provocative stimuli.[26] Although not used in the clinical assessment of patients with CPVT, programmed stimulation is routinely performed in murine models of CPVT.[19]

First, we characterized the effect of CaMKII inhibition on electrocardiographic parameters of normally conducted heartbeats measured at baseline or after stimulation with either epinephrine (3 mg/kg) or isoproterenol (2 mg/kg), which produced stereotypical increases in heart rate from baseline (Figure IIA in the online-only Data Supplement). We also assessed the potential impact of AAV9-GFP-AIP on ventricular function by echocardiography. There was no significant change in the fractional shortening of animals treated with AAV9-GFP-AIP (Figure IIB in the online-only Data Supplement). QTc, a measure of cardiac repolarization that correlates with myocardial instability and risk for arrhythmia,[27] was not significantly different in WT treated with AAV9-GFP or AAV9-GFP-AIP at baseline or after β-adrenergic stimulation (Figure 2A). However, AAV9-GFP-AIP did induce a small increase in the QTc interval in *Ryr2*$^{R176Q/+}$ animals at baseline (47.8±1.5 milliseconds versus 43.8±1.5 milliseconds; $P<0.05$) and with epinephrine (55.9±1.9 vs. 61.4±2.2; $P<0.05$; Figure 2A). Although statistically significant, these small increases were within the normal range for mice under anesthesia.[28] We also examined QT dispersion, a known marker of arrhythmogenic instability,[29] by measuring the QTc interval in 2 different ECG leads. QT dispersion did not differ significantly in WT animals treated with AAV9-GFP-AIP, and QT dispersion was normalized by treatment with AAV9-GFP-AIP in *Ryr2*$^{R176Q/+}$ animals after adrenergic stimulation (Figure 2B). These data suggest that AAV9-GFP-AIP does not dramatically increase the risk for arrhythmia or impair normal cardiac physiology.

Next, we measured the effect of CaMKII inhibition on ventricular arrhythmias induced by β-adrenergic stimulation, which has been shown previously to stimulate ventricular ectopy in murine CPVT models. With AAV9-GFP expression, 9 of 18 (50%) *Ryr2*$^{R176Q/+}$ animals demonstrated significant ventricular ectopy, defined as ≥3 PVCs per minute or nonsustained ventricular tachycardia (Figure 2F and 2G). Treatment with AAV9-GFP-AIP dramatically reduced the frequency of spontaneous ventricular ectopy in *Ryr2*$^{R176Q/+}$ mice (interquartile range, 5.35–0.93 PVCs/min versus 0.52–0.03 PVCs/min; $P<0.05$: Figure 2F and 2H) to a frequency comparable to that of WT.

To further assess the ability of AAV-GFP-AIP to reduce CPVT arrhythmia vulnerability, we performed programmed ventricular stimulation, a technique used in both humans and animal models to assess arrhythmia vulnerability.[19] After control AAV-GFP treatment, a protocol of 2 extrastimuli (S2 and S3) after steady-rate pacing produced ventricular arrhythmias in 14 of 18 (78%) *Ryr2*$^{R176Q/+}$ mice compared with only 2 of 10 (20%) WT mice (Figure 2C and 2D). In contrast, AAV9-GFP-AIP treatment suppressed the frequency of induced arrhythmia in Ryr2$^{R176Q/+}$ mice (77.8% versus 17.6%; $P<0.01$; Figure 2C and 2D) and reduced the length of induced arrhythmias (interquartile range,1.47–0.11 seconds versus 0.2–0 seconds; $P<0.05$; Figure 2C and 2E). Indeed, the frequency and length of induced

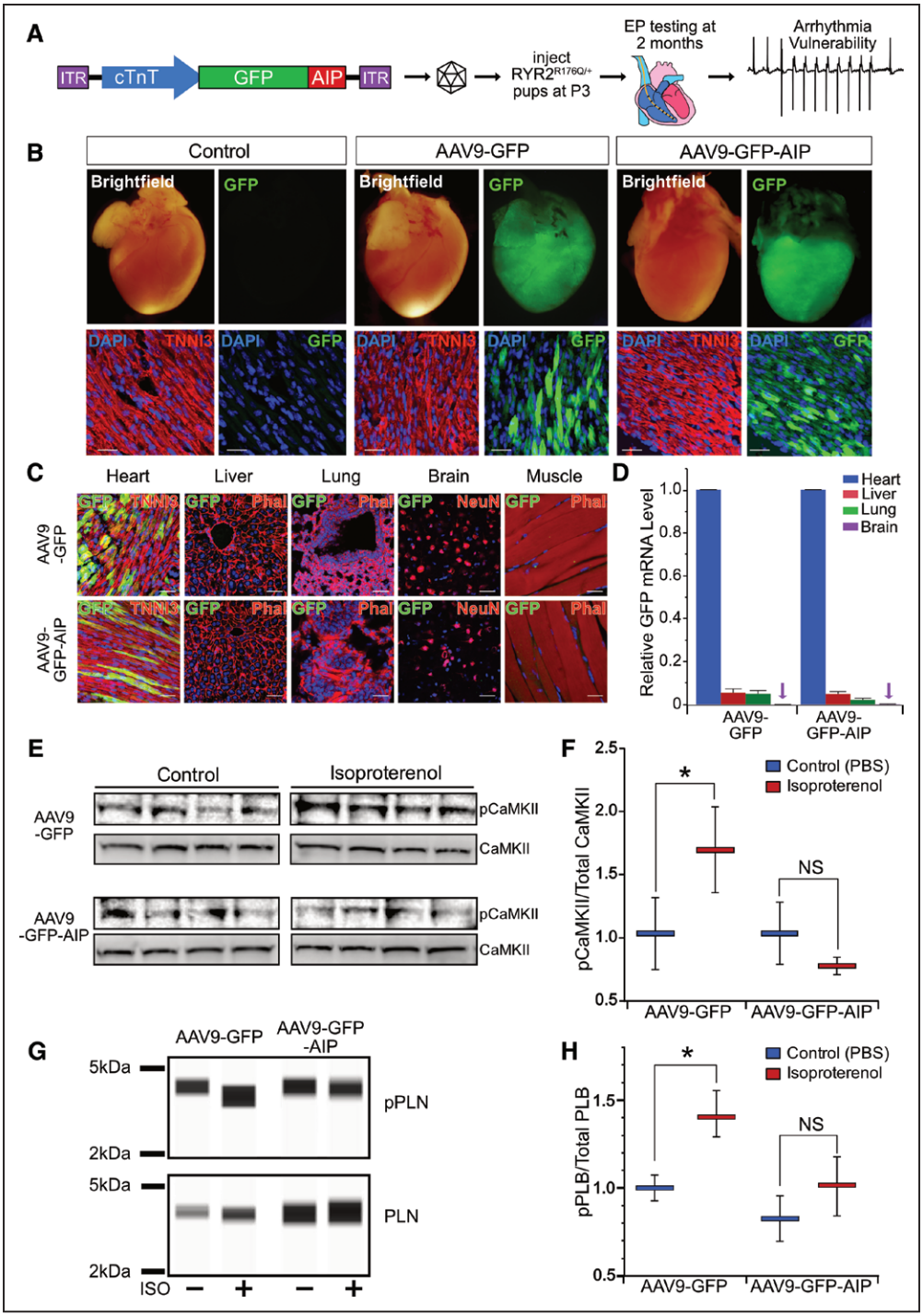

Figure 1. Cardiac-restricted expression of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) inhibitory peptide by adeno-associated virus (AAV) 9. **A**, Schematic depicting AAV vector and overall experimental design. **B**, Cardiac transduction by AAV vectors. **Top** row, Paired bright-field and epifluorescent images of hearts 2 weeks after indicated treatment. **Bottom** row, Heart sections show robust cardiomyocyte expression of green fluorescent protein (GFP). **C**, Sections of indicated tissues from mice injected with AAV9-GFP or AAV9-GFP-AIP. GFP was detected only in cardiomyocytes. Sections were stained with DAPI and cardiac troponin I (cTNT; TNNI3) antibody (heart), phallodin (Phal; liver, lung, skeletal muscle), or NeuN antibody (brain). Scale bars, 40 μm. **D**, Reverse transcription–quantitative polymerase chain reaction measurement of relative GFP transcript level in indicated tissues, normalized to GAPDH. Cardiac expression level was defined as 1. Purple arrows indicate ×1000 lower expression in brain. n=4 per group. **E** and **F**, Western blot of whole-heart lysates from mice treated with isoproterenol (ISO). Blots were probed with antibody specific to total or activated CaMKII. Quantification showed that AAV9-GFP-AIP inhibited ISO-induced CaMKII activation (n=4 samples per condition). **G** and **H**, Capillary Western blot analysis of whole-heart lysates probed with antibodies for total phospholamban (PLN) or phosphorylated-phospholamban (pPLN; threonine-17) demonstrated suppression of CaMKII-mediated PLN phosphorylation by autocamtide-2–related inhibitory peptide (AIP; n=3 samples per condition). ITR indicates inverted terminal repeat; NS, nonsignificant; and pCaMKII, CaMKII phosphorylated on threonine 286. *P<0.05 (2-tailed Student t test).

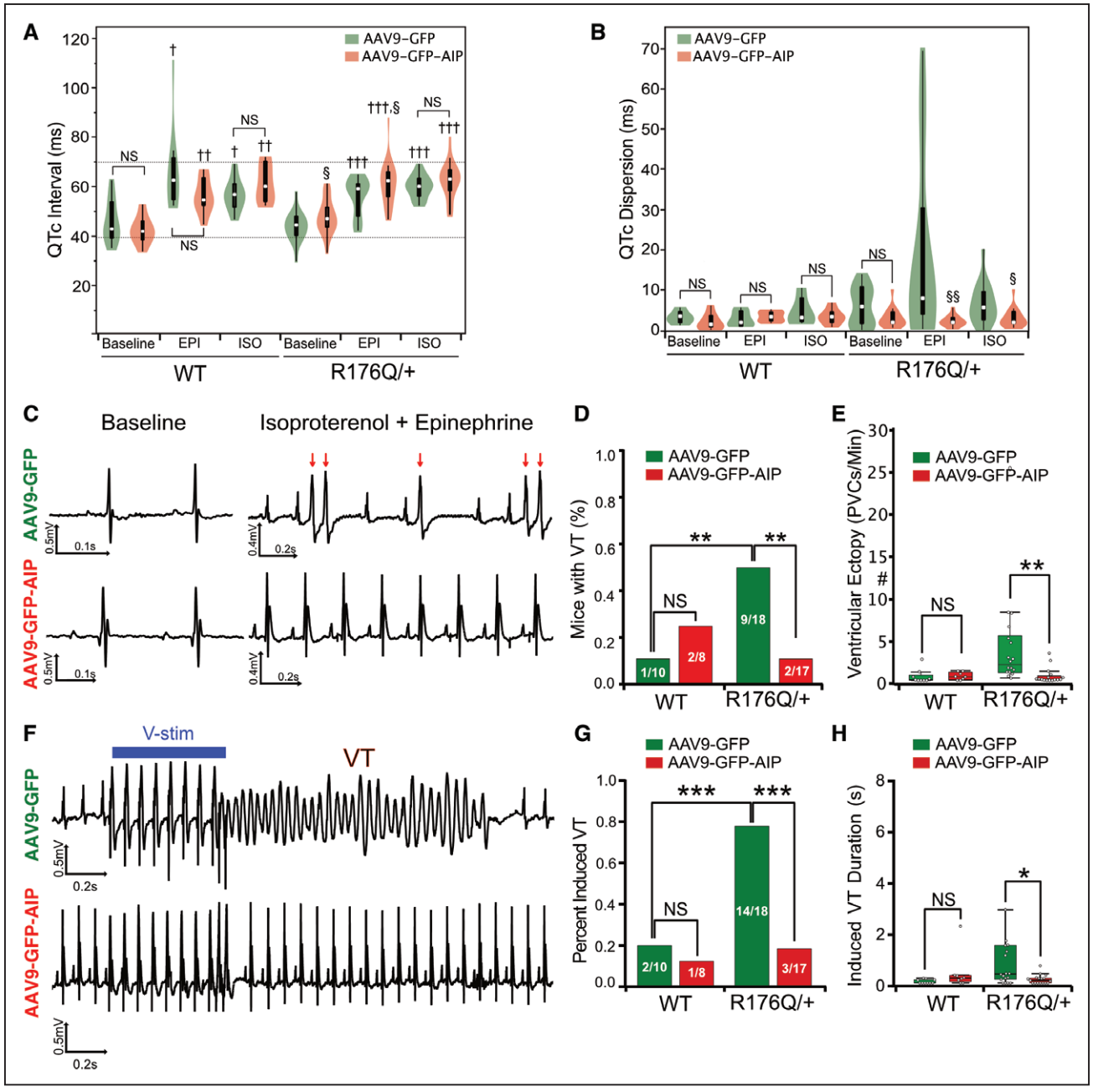

Figure 2. AAV9-GFP-AIP suppresses ventricular arrhythmias in *Ryr2*$^{R176Q/+}$ mice.

A, Corrected QT interval (QTc) in wild-type (WT) or *Ryr2*$^{R176Q/+}$ mice treated as indicated. Dashed lines indicate the normal QTc interval range. Neonatal treatment of *Ryr2*$^{R176Q/+}$ mice with AAV9-GFP-AIP slightly increased the QTc interval under β-adrenergic stimulation compared with AAV9-GFP, but the QTc interval remained within the normal range. Significance testing by Steel-Dwass. †Epinephrine (EPI) or isoproterenol (ISO) vs baseline with corresponding virus. §AVV9-GFP vs AAV9-GFP-AIP with corresponding condition. †§*P*<0.05. ††§§*P*<0.01. †††*P*<0.001. **B**, QTc dispersion, the difference in QTc between leads I and II, in WT or *Ryr2*$^{R176Q/+}$ mice treated as indicated. Dispersion increased with adrenergic stimulation in *Ryr2*$^{R176Q/+}$ animals and normalized with AAV9-GFP-AIP treatment. Significance testing by Steel-Dwass. §AVV9-GFP vs AAV9-GFP-AIP with corresponding condition. §*P*<0.05. §§*P*<0.01. **C**, Representative surface ECGs of *Ryr2*$^{R176Q/+}$ mice showed spontaneous arrhythmia (arrows) after administration of ISO and EPI in AAV9-GFP that was suppressed by AAV9-GFP-AIP. **D** and **E**, Quantification of the fraction of mice with ventricular tachycardia (VT; **D**) and the frequency of ventricular ectopy (**E**) provoked by ISO+EPI. **F**, Surface electrocardiographic recordings of ventricular stimulation (V-stim) studies of *Ryr2*$^{R176Q/+}$ mice treated with AAV9-GFP or AAV9-GFP-AIP. Blue line designates programmed stimulation. **G** and **H**, Quantification of percent of mice with induced arrhythmias (**G**) and induced VT duration (**H**). Significance testing for **D** through **G**, χ^2; for **E** and **H**, Student *t* test. For **A** and **B**: WT+GFP, n=9; WT+AIP, n=8, R176Q+GFP, n=17; R176Q+AIP, n=18. For **C** through **H**, the number per group is as listed. AAV indicates adeno-associated virus; AIP, autocamtide-2–related inhibitory peptide; GFP, green fluorescent protein; NS, nonsignificant; and PVC, premature ventricular contraction. **P*<0.05. ***P*<0.01. ****P*<0.001.

arrhythmia were not significantly different between AAV9-GFP-AIP–treated *Ryr2*$^{R176Q/+}$ mice and their WT littermates (Figure 2C and 2E). There was also no increase in the frequency or duration of significant ventricular arrhythmias in WT animals, suggesting the absence of proarrhythmic effects.

These data collectively indicate that treatment with AAV9-GFP-AIP suppresses ventricular arrhythmia vul-

nerability in a murine model of CPVT to levels comparable to those in WT controls.

CaMKII Inhibition Normalizes Ca^{2+} Handling of Isolated CPVT Cardiomyocytes

At a cellular level, CPVT cardiomyocytes exhibit increased diastolic Ca^{2+} and early or delayed afterdepolarizations that lead to triggered activity. To determine whether expression of CaMKII inhibition by AIP is sufficient to suppress abnormal Ca^{2+} handling at the cellular level, we isolated adult cardiomyocytes from animals treated with AAV9-GFP-AIP or AAV9-GFP control. After a brief (2 hours) culture period to allow cardiomyocytes to recover from isolation and loading with the Ca^{2+} indicator Rhod-2, we performed field pacing at 1 Hz for 1 minute and recorded Ca^{2+} transients in the postpacing period (Figure 3A and 3B). After cessation of pacing, WT ventricular cardiomyocytes treated with either AAV9-GFP or AAV9-GFP-AIP do not have spontaneous Ca^{2+} transients for >30 seconds (Figure 3B). In contrast, *Ryr2*$^{R176Q/+}$ cardiomyocytes treated with AAV9-GFP exhibited abnormal postpacing events, including spontaneous transients, waves, and discrete episodes of triggered activity not present in WT cardiomyocytes (Figure 3B). AAV9-GFP-AIP expression nearly completely suppressed abnormal postpacing events (Figure 3B and 3C) and dramatically reduced the duration of triggered Ca^{2+} release events (Figure 3B and 3D). There was no increase in either spontaneous or triggered Ca^{2+} events in AIP-expressing WT cardiomyocytes, suggesting the absence of a proarrhythmic effect. Quantification of paced Ca^{2+} transients demonstrated that AAV9-GFP-AIP had no significant effect on Ca^{2+} transient duration in WT ventricular cardiomyocytes but normalized the significant Ca^{2+} transient duration prolongation in *Ryr2*$^{R176Q/+}$ cardiomyocytes with isoproterenol treatment (Figure 3E and 3F). AAV9-GFP-AIP treatment also shortens the rise time and normalized the prolonged rise time in *Ryr2*$^{R176Q/+}$ cardiomyocytes (Figure 3E and 3G).

To examine whether the effects of AIP are through CaMKII inhibition or are nonspecific, we used modified mRNA[30] to express CN19o, a potent CaMKII inhibitory peptide that differs from AIP in both sequence and mechanism of CaMKII inhibition.[31] Transfection of modRNA encoding CN19o-P2A-mCherry (Table III in the online-only Data Supplement) into *Ryr2*$^{R176Q/+}$ cardiomyocytes demonstrated robust mCherry expression within 24 hours (Figure 4A and 4B). Expression of CN19o-P2A-mCherry suppressed postpacing events in *Ryr2*$^{R176Q/+}$ cardiomyocytes compared with mCherry control and produced similar effects on Ca^{2+} transients as AIP (Figure 4C–4E). These data suggest that CaMKII inhibition with AIP suppresses abnormal Ca^{2+} signaling on a single-cell level, which correlates to the therapeutic responses that we observed at the whole-organ level.

AAV9-GFP-AIP Treats CPVT in a Translationally Relevant Context

The preceding experiments showed that AAV9-GFP-AIP is effective when administered to neonatal mice, but patients with CPVT typically present between 9 and 12 years of age.[3] To determine whether AAV9-GFP-AIP was effective for treating CPVT in a more clinically relevant context, we systemically administered AAV9-GFP-AIP or AAV9-GFP control to adolescent mice (4–6 weeks of age). Quantification of GFP expression in cardiac sections after at least 2 weeks of expression demonstrated transduction of ≈50% of ventricular cardiomyocytes with both treatment and control viruses (Figure 5B and 5C).

Baseline electrocardiographic analysis revealed no significant change in the PR interval, QRS duration, or QRS amplitude (Figure 5D and Figure IV in the online-only Data Supplement). Isoproterenol administration increased heart rate in *Ryr2*$^{R176Q/+}$ animals to a greater degree in the AAV9-GFP-AIP group compared with the AAV9-GFP group (Figure 5E). In contrast to neonatal treatment, adolescent *Ryr2*$^{R176Q/+}$ treatment did not lead to significant differences in QTc interval at baseline or after β-adrenergic stimulation (Figure 5F).

We again performed electrophysiology testing to assess arrhythmia burden and vulnerability in CPVT mice treated with AAV9-GFP-AIP versus AAV-GFP. After administration of isoproterenol plus epinephrine, 5 of 6 AAV9-GFP–treated animals developed nonsustained ventricular tachycardia, which was substantially reduced in the AAV9-GFP-AIP treatment group (1 of 6 animals; P=0.08; Figure 5G and 5H). Overall ventricular ectopy burden was significantly reduced in AAV9-GFP-AIP compared with control (P<0.05; Figure 5G and5H). We further assessed arrhythmia vulnerability by administering 2 sequential ventricular extrastimuli in the context of β-adrenergic stimulation with isoproterenol plus epinephrine. This protocol induced prolonged episodes of monomorphic and polymorphic ventricular arrhythmias in 6 of 6 *Ryr2*$^{R176Q/+}$ mice treated with AAV9-GFP (Figure 5I and 5J). In comparison, treatment with AAV9-GFP-AIP nearly completely suppressed induced ventricular tachycardia in *Ryr2*$^{R176Q/+}$ mice (observed in 1 of 6 mice; P<0.05; Figure 5I and 5J).

These data collectively demonstrate that AAV9-GFP-AIP administered at a translationally relevant age effectively suppresses ventricular arrhythmias in CPVT mice without evidence of proarrhythmia.

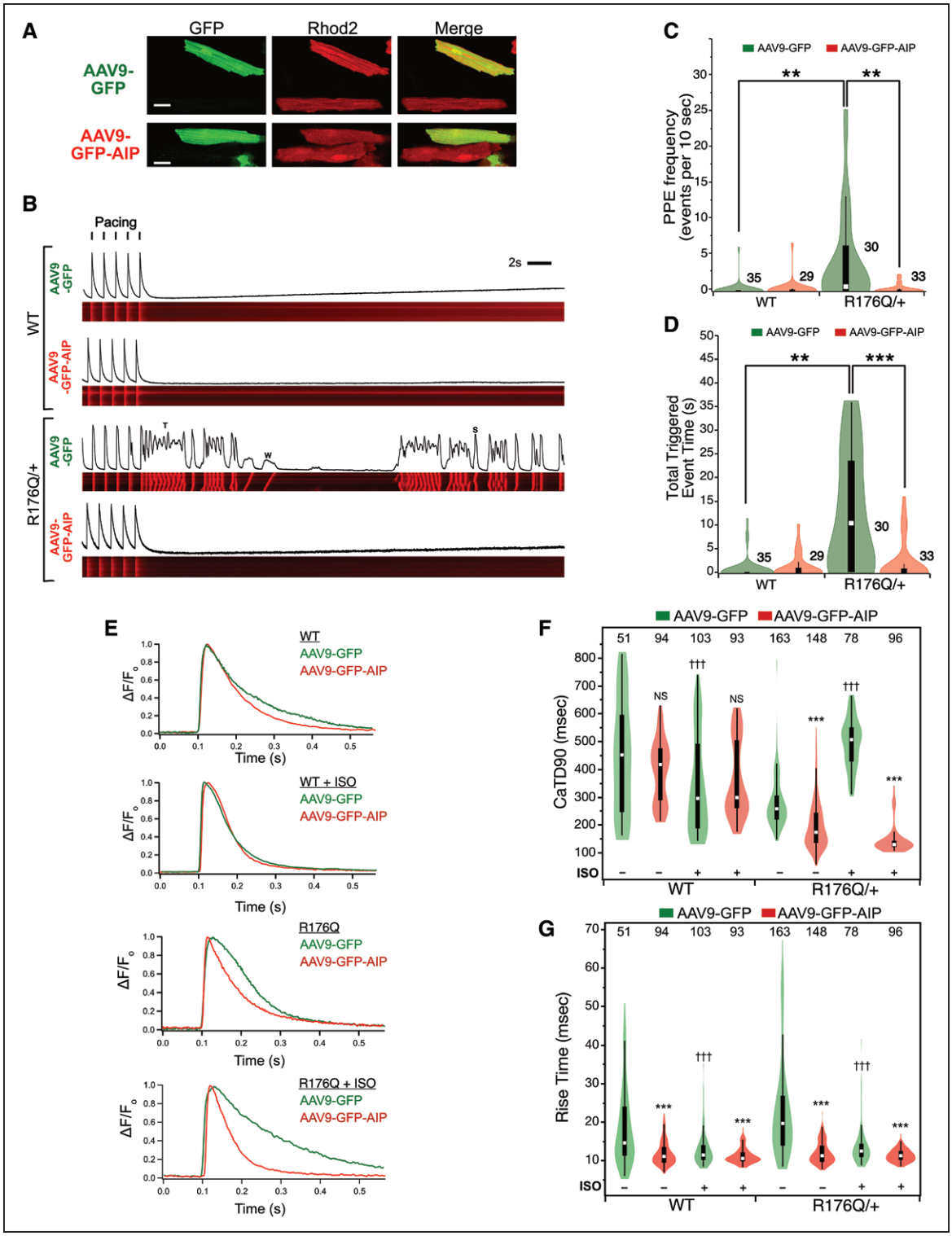

Figure 3. Normalization of abnormal Ca^{2+} handling in isolated cardiomyocytes by AAV9-GFP-AIP.
A, Confocal micrographs of isolated adult cardiomyocytes transduced with AAV9-GFP or AAV9-GFP-AIP. Cells were loaded with Ca^{2+}-sensitive dye Rhod2. **B**, Isolated cardiomyocytes from *Ryr2*$^{R176Q/+}$ mice were loaded with Rhod2 and paced for 1 minute at 1 Hz. Cardiomyocyte Ca^{2+} transients were recorded after pacing by confocal line scan imaging. Wild-type (WT) cells rarely exhibited postpacing calcium release events (PPEs), whereas *Ryr2*$^{R176Q/+}$ cells frequently displayed PPEs consisting of triggered activity (T), waves (W), or spontaneous transients (T). Expression of AAV9-GFP-AIP suppressed abnormal PPEs without inducing instability of WT cardiomyocytes. **C** and **D**, Quantification of postpacing Ca^{2+} release event frequency and duration. Number of cardiomyocytes studied is indicated next to density plots. *P* values by Student *t* test. ***P*<0.01. ****P*<0.001. **E**, Representative Ca^{2+} transients during 1-Hz pacing of WT and *Ryr2*$^{R176Q/+}$ cardiomyocytes at baseline and after adrenergic stimulation with isoproterenol (ISO). **F**, Quantification of Ca^{2+} transient duration at 90% repolarization (CaTD90) demonstrated significant prolongation in *Ryr2*$^{R176Q/+}$ cardiomyocytes with ISO treatment that was normalized by AAV9-GFP-AIP. **G**, Ca^{2+} transient rise times were also decreased by AAV9-GFP-AIP. Number of transients quantified is indicated. Data from at least 3 separate mice were pooled. AAV indicates adeno-associated virus; AIP, autocamtide-2–related inhibitory peptide; GFP, green fluorescent protein; and NS, nonsignificant. ***Comparison of ISO treatment within mouse genotype and AAV treatment group. †††Comparison of AAV treatment group within mouse genotype and ISO treatment group. Significance testing by Student *t* test: †††*P*<0.001. ****P*<0.001.

CaMKII Inhibition Effectively Reverses the CPVT Phenotype in Different Pathogenic Genotypes

There are >150 different *RYR2* mutations that cause CPVT.[3] To determine whether CaMKII inhibition is likely to be applicable to a range of CPVT-causing *RYR2* mutations, we recruited 2 patients with a clinical diagnosis of CPVT and documented *RYR2* mutation in distinct regions of the gene (Figure IV in the online-only Data Supplement). One patient had a novel mutation in the 5′ end of the gene that resulted in the substitution of arginine for serine at position 404 (S404R). This patient and iPSC line also contained a second variant of uncertain significance, N658S.

Another patient harbored a previously described CPVT mutation, G3946S,[32] as well as a known CPVT modifier, G1885E.[33] Patient-derived peripheral mononuclear blood cells were reprogrammed into iPSCs, designated CPVT-S404R and CPVT-G3946S, respectively (Figure IV in the online-only Data Supplement), which were then differentiated into functional cardiomyocytes (iPSC-CMs; Figure I in the online-only Data Supplement).[34] Immunostaining revealed expression of the cardiomyocyte markers troponin I and RYR2 in both CPVT and WT iPSC-CMs (Figure 6A). High-resolution confocal microscopy of CPVT and WT iPSC-CMs demonstrated regular sarcomeric structure highlighted by troponin I staining with localization of RYR2 "puncta" at the Z lines (Figure 6A, arrowheads in insets).

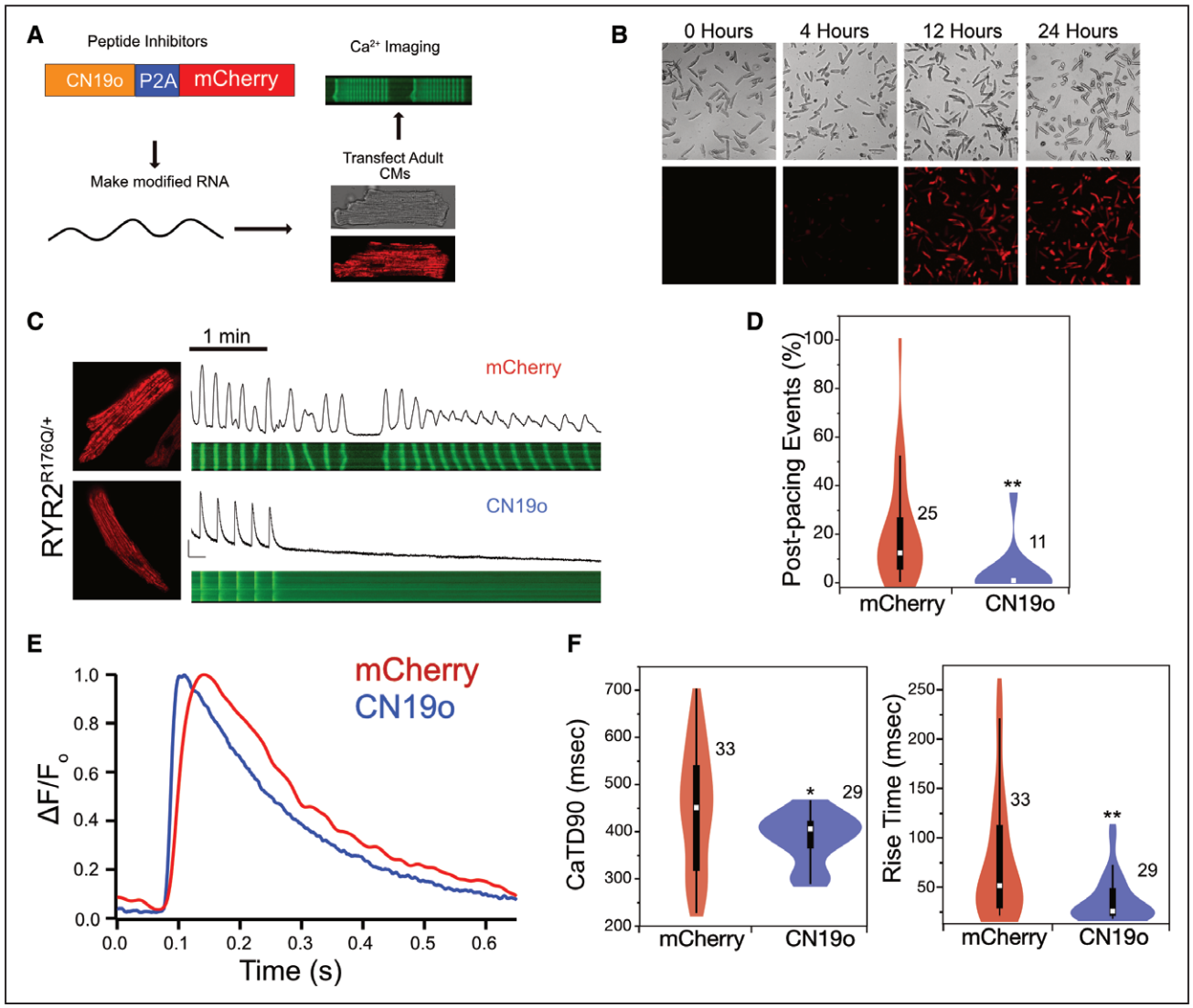

Figure 4. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) inhibitory peptide CN19o had effects comparable to those of autocamtide-2–related inhibitory peptide (AIP).

A, Schematic of experimental design. CaMKII inhibitory peptide CN19o was expressed in isolated adult cardiomyocytes by modified mRNA (modRNA) transfection. **B**, Time course of expression of modRNA in isolated RYR2-R176Q$^{+/-}$ adult cardiomyocytes (CMs) after transfection. **C**, Abrupt cessation of pacing after 1 minute at 1 Hz induced spontaneous Ca^{2+} release events in *RYR2*$^{R176Q/+}$ cardiomyocytes, as indicated by confocal line scan recording of Fluo4 fluorescence. **D**, Percentage of CMs with abnormal postpacing events. CN19o suppressed abnormal postpacing events compared with mCherry. **E** and **F**, Representative Ca^{2+} transients from adult CMs expressing mCherry or CN19o. CN19o decreased Ca^{2+} transient duration at 90% repolarization (CaTD90) and reduced Ca^{2+} transient rise time. Numbers next to distribution profiles indicate sample sizes. Significance testing for **D** and **F** by Student *t* test. *P<0.05. **P<0.01.

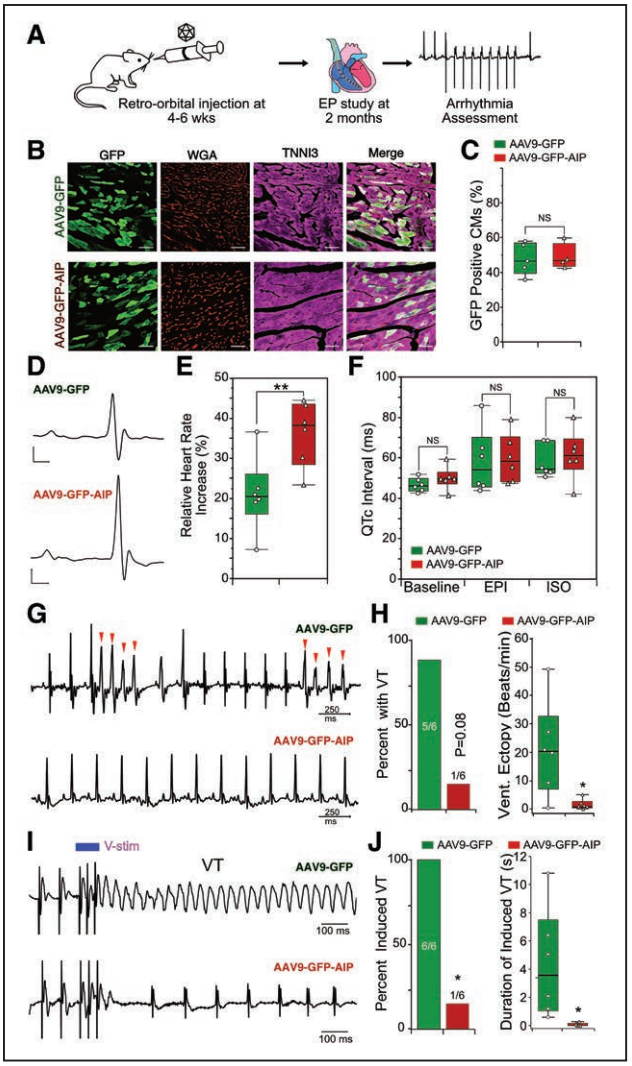

Figure 5. AAV9-GFP-AIP treatment effectively suppresses ventricular arrhythmias in adolescent catecholaminergic polymorphic ventricular tachycardia (VT) animals.

A, Experimental design. AAV9-GFP-AIP or AAV9-GFP was administered retro-orbitally to 4- to 6-week-old *RYR2*$^{R176Q/+}$ mice. Electrophysiology (EP) testing was performed 2 weeks later. **B**, Confocal micrographs of cardiac sections demonstrated robust expression of either AAV9-GFP or AAV9-GFP-AIP. Scale bar, 50 μm. **C**, Quantification of green fluorescent protein (GFP)–expressing cardiomyocytes. AAV9-GFP and AAV9-GFP-AIP transduced a similar fraction of cardiomyocytes (47.7±4.1% [n=5] vs 48.8±3.8% [n=4], respectively). **D**, Representative surface electrocardiographic tracings of *RYR2*$^{R176Q/+}$ mice treated with AAV9-GFP or AAV9-GFP-AIP. **E**, Isoproterenol (ISO) stimulation increased heart rate more in *RYR2*$^{R176Q/+}$ mice treated with AAV9-GFP-AIP compared with AAV9-GFP. $^{**}P<0.01$. **F**, Effect of AAV9-GFP or AAV9-GFP-AIP on corrected QT (QTc) interval. **G**, Ventricular ectopy provoked by β-adrenergic stimulation (ISO+epinephrine [EPI]). Red arrowheads denote nonsustained VT. **H**, Quantification of the percentage of animals with nonsustained and sustained VT (**left**) and the rate of ventricular ectopy (**right**). $^{*}P<0.05$. **I**, Representative surface electrocardiographic tracings from *RYR2*$^{R176Q/+}$ mice undergoing programmed ventricular stimulation after treatment with EPI+ISO. **J**, Quantification of mice with inducible VT (**left**) and duration of VT (**right**). AAV indicates adeno-associated virus; AIP, autocamtide-2–related inhibitory peptide; NS, nonsignificant; TNNI3, troponin I; and WGA, wheat germ agglutinin. $^{*}P<0.05$.

To measure Ca^{2+} activity, we transduced single iPSC-CMs with an adenovirus that expresses a recently developed Ca^{2+} nanosensor, JT-G6F, in which the genetically encoded Ca^{2+} sensor GCaMP6f is fused to the RYR2-associated molecule junctin.[21] This nanosensor dramatically increases signal-to-noise ratio during imaging of Ca^{2+} release events.[21] Confocal line scanning of single WT iPSC-CMs expressing JT-G6F revealed spontaneous Ca^{2+} transients, which increased in frequency with isoproterenol stimulation (1 μmol/L; Figure 6B, top). In contrast, CPVT-R1 and CPVT-R3 iPSC-CMs consistently displayed abnormal spontaneous Ca^{2+} release events consisting of early afterdepolarizations, delayed afterdepolarizations, and waves (Figure 6B and 6C).

To determine whether CaMKII inhibition was sufficient to suppress the arrhythmogenic phenotype in our iPSC-CM models of CPVT, we preincubated iPSC-CMs with isoproterenol or isoproterenol plus a myristoylated, cell-permeable form of AIP. At baseline or with isoproterenol, the majority of iPSC-CMs derived from patients with CPVT demonstrated frequent, abnormal Ca^{2+} release events (Figure 6C, red arrows, and Figure 6D). Administration of AIP (600 nmol/L) significantly reduced the percentage of CPVT-R1 and CPVT-R3 iPSC-CMs with abnormal Ca^{2+} transients (Figure 6C and 6D). In addition to whole-cell changes in Ca^{2+} transients, we observed small discrete Ca^{2+} release events or sparks (Figure VA and VB in the online-only Data Supplement). The frequency of Ca^{2+} sparks was higher in CPVT-R1 and CPVT-R3 iPSC-CMs compared with WT cells, and this was further exacerbated by treatment with isoproterenol (Figure 6E and 6F). Treatment with AIP dramatically reduced the frequency of Ca^{2+} sparks in CPVT-R1 and CPVT-R3 iPSC-CMs and restored regular, spontaneous Ca^{2+} transients (Figure 6E and 6F). In contrast, there was no change in spark frequency in CPVT-R1 iPSC-CMs preincubated with a myristoylated peptide inhibitor of protein kinase A, suggesting that the reversal of abnormal Ca^{2+} signaling is from CaMKII inhibition (Figure VC in the online-only Data Supplement).

Together, these data demonstrate that targeted inhibition of CaMKII ameliorates abnormal Ca^{2+} handling in CPVT patient-derived iPSC-CMs containing at least 2 distinct pathogenic *RYR2* mutations, suggesting that targeted CaMKII inhibition may be a broadly effective treatment for CPVT.

DISCUSSION

In this study, we provide proof of concept that AAV-mediated delivery of a CaMKII inhibitory peptide has promise as a translatable therapy for CPVT. We provide 3 critical lines of evidence. First, we demonstrate that CaMKII inhibitory peptide is highly effective in suppressing the CPVT arrhythmia phenotype in both murine and human iPSC disease models. The inhibitory peptide effectively suppressed ventricular arrhythmias in a well-established mouse model of CPVT in the face of provocative stimulation by β-adrenergic agonists

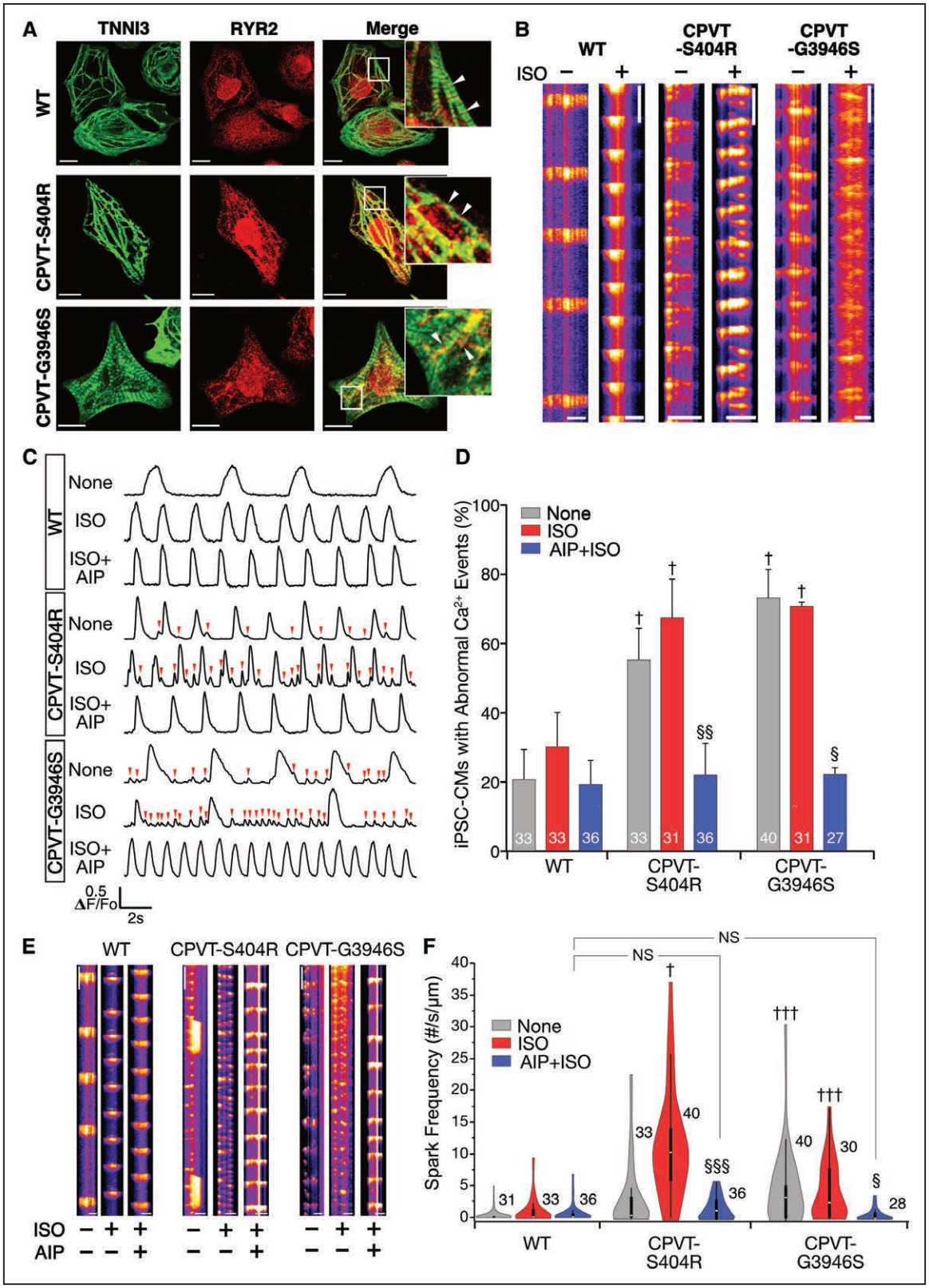

Figure 6. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) inhibition suppresses abnormal Ca^{2+} responses in induced pluripotent stem cell–derived cardiomyocyte (iPSC-CM) models of catecholaminergic polymorphic ventricular tachycardia (CPVT) with different pathogenic genotypes. **A**, Confocal micrographs of iPSC-CMs demonstrate expression of cardiac markers TNNI3 and ryanodine receptor 2 (RYR2). High-magnification inlay reveals localization of a subset of RYR2 (arrows) at z lines. Scale bar, 20 μm. **B**, Representative confocal line scans of iPSC-CMs expressing Ca^{2+}nanosensor. Addition of isoproterenol (ISO) increased beating frequency and abnormal Ca^{2+} release events in CPVT iPSC-CMs. Vertical bar, 20 μm; horizontal bar, 0.5 second. **C** and **D**, Representative Ca^{2+} transients recorded by confocal line scan. Incubation with autocamtide-2–related inhibitory peptide (AIP) suppressed abnormal Ca^{2+} release events (early [EADs] and delayed afterdepolarizations [DADs]) in CPVT iPSC-CMs. Red arrowheads denote DADs and EADs. (*Continued*)

Figure 6 Continued. Quantification of iPSC-CMs with at least 1 abnormal Ca^{2+} release events during the record period. **E** and **F**, Ca^{2+} sparks were recorded by confocal line scanning. Vertical bar, 20 μm; horizontal bar, 1 second. Quantification of spark frequency showed that sparks were significantly increased in CPVT iPSC-CMs and were effectively suppressed by incubation with AIP. Numbers within or adjacent to bars indicate sample size. NS indicates not significant. Steel-Dwass *P* values: †vs wild-type (WT) with the matching ISO treatment. §vs matching cell line+ISO-AIP. †*P*<0.05. §*P*<0.05. §§*P*<0.01. †††*P*<0.005. §§§*P*<0.005.

with or without ventricular extrasystoles. The treatment was successful when administered to neonatal animals and in a more clinically relevant scenario in which it was delivered to adolescent mice with established disease via an intravascular (retro-orbital) route. We provide further evidence of the efficacy of the CaMKII inhibitory peptide in human iPSC-CMs derived from patients with 2 different pathogenic *RYR2* mutations affecting distinct regions of the protein. Second, we show that AAV-mediated expression of the CaMKII inhibitory peptide was not proarrhythmic in WT or CPVT mice or in human iPSC-CMs. Third, we demonstrate that the inhibitory peptide can be delivered to cardiomyocytes using an AAV9 gene therapy vector and a cardiomyocyte-specific promoter while avoiding expression in other tissues, most notably the brain. A single dose of AAV9-GFP-AIP yielded robust cardiomyocyte expression of the inhibitory peptide with minimal extracardiac expression, both necessary requirements for effective translation. Moreover, treatment was not dependent on transducing all cardiomyocytes because we achieved effective arrhythmia suppression with 40% to 50% cardiomyocyte transduction. Further dose-finding experiments are required to define the minimum transduction efficiency required for successful arrhythmia suppression.

Although we did not observe proarrhythmic effects of AAV9-GFP-AIP, we did observe some effects on cardiac repolarization and heart rate. After neonatal treatment with AAV9-GFP-AIP, the QTc interval in β-agonist–stimulated *Ryr2*$^{R176Q/+}$ animals was ≈10% higher than AAV9-GFP controls. Although this difference was significant, the QTc interval did not become elevated outside the range of values reported in comparable studies of normal mice.[35] Furthermore, this difference was absent in the adolescent treatment cohort and was not observed in WT mice. For these reasons, it is unlikely that this effect will represent a source for proarrhythmia. We also observed a significant increase in the heart rate response to isoproterenol of *RYR2*$^{176Q/+}$ mice treated in adolescence with AAV9-GFP-AIP. Data from both isolated sinoatrial nodal cells and mouse models of CaMKII inhibition have demonstrated a role for CaMKII activity in the heart rate response to β-adrenergic stimulation but not the maintenance of the basal heart rate.[36] Patients with CPVT display baseline bradycardia,[37] possibly from alternations in the Ca^{2+} clock within pacemaker cells through negative regulation of the L-type Ca^{2+} channel.[38] Therefore, it is reasonable to hypothesize that CaMKII inhibition with AIP in *Ryr2*$^{R176Q/+}$ animals restores the Ca^{2+} clock within the sinoatrial node, leading to the recovery of a normal isoproterenol response. Excessive diastolic Ca^{2+} may also cause microfibrosis or changes in gene expression within sinoatrial nodal cells, leading to sinus node dysfunction in CPVT.[39] Treatment with AAV9-GFP-AIP may prevent these changes, increasing heart rate and effectively reversing sinus node dysfunction.

Effective clinical gene replacement by single-dose AAV therapy for loss-of-function mutations has recently been demonstrated for hemophilia[40] and spinal muscular atrophy type I.[41] These studies highlight the effectiveness and safety of AAV-directed therapy to stably transfer and express a WT gene that replaces the function of the mutant gene. Gene replacement for *RYR2* mutations that cause CPVT is not possible because the large size of RYR2 (>15 kbp) exceeds the limited cargo capacity of AAV (≈3.5 kbp). Furthermore, CPVT-causing *RYR2* mutations are dominant. To overcome these limitations, we targeted CaMKII, which functions in a molecular signaling pathway that is essential to unmask the CPVT phenotype during β-adrenergic receptor stimulation.[13] Previous studies in both mice and iPSC-CMs demonstrated that Ca^{2+} handling and arrhythmias were ameliorated by treatment with KN-93, a small molecule that inhibits CaMKII.[15] However, KN-93 and other currently available small molecules that inhibit CaMKII are not clinically viable because they inhibit CaMKII in other tissues such as the brain, where CaMKII is essential for learning and memory. Our gene therapy strategy overcomes this hurdle by selectively targeting expression of a potent CaMKII inhibitory peptide to cardiomyocytes.

Other gene therapy approaches have been proposed for CPVT. Autosomal recessive mutations in calsequestrin (*CASQ2*) cause a rare form of CPVT that accounts for ≈1% to 3% of patients with CPVT.[42] Because of its small size (≈1.2 kb), *CASQ2* is easily packaged into AAV viral particles and can be delivered to the heart for direct gene replacement.[43] Consistent with our study, effective suppression of arrhythmias in *Casq2*-mutant mice was achieved with an AAV-CASQ2 dose that transduced 40% to 50% of cardiomyocytes.[43] Although effective for CPVT caused by CASQ2 deficiency, this condition is rare and would not benefit the large majority of patients with CPVT with dominant *RYR2* mutations.[44] Other efforts to treat CPVT caused by *RYR2* mutations have used allele-specific silencing[45] or knockout of the mutant allele. Although effective in animal or cell models, this allele-specific strategy will be challenging to translate because a different gene therapy product would need to be developed for each of >150 different *RYR2* mutations. Another proposed strategy is the expression of modified forms of calmodulin with reduced Ca^{2+} binding, which may treat CPVT by reducing the activity of CaMKII and other

Ca^{2+} handling proteins within the cardiomyocyte.[46] This strategy has the potential to be effective in treating a board range of pathogenic *RYR2* and *CASQ2* genotypes, but the potential for proarrhythmic effects has not been evaluated. Mutations in calmodulin genes (*CALM1* and *CALM2*) have been shown to cause both CPVT and long-QT phenotypes with life-threatening ventricular arrhythmias,[47] raising considerable concern that the introduction of modified calmodulin proteins may have unpredictable proarrhythmic effects. In contrast, inhibition or loss of function of CaMKII within the heart does not have deleterious effects and indeed has been associated with cardioprotective effects in multiple disease models, including heart failure[48] and arrhythmia.[48,49] Therefore, targeted inhibition of CaMKII not only is likely to be safe but also may be beneficial in other forms of acquired heart disease. However, large-animal studies to evaluate the risk of proarrhythmia are necessary to confirm that targeted inhibition of CaMKII is safe for clinical translation.

Effective translation of our gene therapy strategy to a first-in-human clinical trial will require optimization of the viral capsid, cardiac promoter, and peptide inhibitor. To minimize the inhibition of CaMKII outside of cardiomyocytes, the ideal viral delivery system would detarget the brain and other noncardiomyocyte cell types while maintaining efficient targeting of cardiomyocytes. Although our study with AAV9 and a cardiomyocyte-selective promoter showed no significant expression in brain, AAV9 exhibits sufficient transduction of neurons within the central nervous system that it is in use for clinical treatment of spinal muscular atrophy.[41] Therefore, AAV9 may not be the ideal capsid for treatment of CPVT. Capsid engineering efforts have already yielded variants that reduce viral transduction of the brain and liver and improve delivery to muscle, including the heart.[50] Further optimization of the cardiac promoter may increase cardiomyocyte expression or reduce ectopic expression in noncardiomyocytes. The gene product expressed by the clinical candidate will need to eliminate GFP, and the inhibitor potentially can be engineered to optimize its potency, specificity, expression, and half-life. One intriguing candidate is CN19o, an engineered peptide derived from naturally occurring CaMKII inhibitors in the brain that has been reported to be >40-fold more potent than AIP,[51] which we showed has effects to similar to those of AIP.

Conclusions

Here, we provide proof of concept that the inherited arrhythmia disorder CPVT could be effectively treated by an AAV gene therapy vector that selectively delivers a CaMKII inhibitory peptide to cardiomyocytes. We demonstrate that CaMKII inhibition effectively suppresses ventricular arrhythmias in both mouse and human iPSC-CM models, that AAV-mediated delivery can achieve cardiomyocyte-specific effect and avoid expression in the brain, and that the gene therapy strategy did not have significant observed toxicity. Although additional research and refinement are necessary to effectively translate this therapy to the treatment of patients, it represents a promising strategy with distinct advantages over previous approaches.

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Disclosures

None.

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