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Dantrolene rescues arrhythmogenic RYR2 defect in a patient-specific stem cell model of catecholaminergic polymorphic ventricular tachycardia

Christian B. Jung, Alessandra Moretti, Michael Mederos y Schnitzler, Laura Iop, Ursula Storch, Milena Bellin, Tatjana Dorn, Sandra Ruppenthal, Sarah Pfeiffer, Alexander Goedel, Ralf J. Dirschinger, Melchior Seyfarth, Jason T. Lam, Daniel Sinnecker, Thomas Gudermann, Peter Lipp, Karl-Ludwig Laugwitz

Corresponding author: Karl-Ludwig Laugwitz, München, Technische Universität

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1st Editorial Decision 30 August 2011

Thank you for the submission of your manuscript "Dantrolene rescues arrhythmogenic RYR2 defect in a patient-specific stem cell model of CPVT" to EMBO Molecular Medicine.

As you have seen, the reviewers find the topic of your manuscript potentially interesting. However, they also raise significant concerns on the study, which should be addressed in a major revision of the manuscript.

In particular, the reviewers feel that the study should be strengthened by a further assessment of the cardiomyocyte cell type used, protein expression data as well as data concerning the calcium handling.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can convincingly address the issues that have been raised within the time constraints outlined below.

Revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless arranged otherwise with the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

REFEREE REPORTS:

Referee #1

General Remarks:
Recently, using CPVT-type knock-in mouse models several researchers have reported that a single amino-acid mutation of RyR2 at three (N-terminal, central, c-terminal) hot regions induces aberrant local Ca2+ release (i.e. Ca2+ leak) from the RyR2, which leads to lethal arrhythmia. Although these data definitely provide valuable and straightforward evidences regarding the underlying mechanism of CPVT, it should be clarified whether human cardiomyocytes in patients with CPVT show the same Ca2+ handling abnormalities as those seen in animal models.
Using human iPS cells derived from dermal fibroblasts of a CPVT patient carrying a novel RyR2 S406L, Jung et al demonstrated that cathecholaminergic stress led to elevated diastolic concentrations, a reduced SR Ca2+ content, an increased frequency of both Ca2+ sparks and DaDs and arrhythmia, and that all of these abnormalities in Ca2+ handling could be corrected by dantrolene which has been shown to fix defective inter-domain interactions between N-terminal (1-600) and central (2000-2500) domains within the RyR2. From these findings they concluded that defective inter-domain interaction within RyR2 plays a key role in the pathomechanism of the S406L mutation.
This is an important paper in that they provided straightforward evidences as to RyR2 defects in human iPS cells from a CPVT patient. The experiments were well performed, and there are significant data to support their conclusion. I have some concerns to be addressed.

Major comments:
The authors should fully discuss the observed differences in gene expression between normal cardiomyocytes and iPS cells. In Fig 1C the expressions of RyR2 and CASQ2 are both decreased, while those of TRDN, JCTN, and PLN are increased. Such differences in the expression level of the Ca2+ handling proteins may significantly affect the local Ca2+ release property, although it does not appear to influence on the CPVT phenotype. How about the protein expression level of these Ca2+ handling proteins? Since gene expression does not always change in parallel with protein expression, the author should clarify whether there is any change in the protein expression level of these Ca2+ handling proteins among groups shown in Fig 2F. If there are any significant differences in the protein expression level between normal human cardiomyocytes and IPS cells, the authors will need to be cautious upon interpretation of data.

Minor comments:

In Fig 1G, some RyR2s do not appear to be localized along with sarcomeres; some RyR2 are detected outside of sarcomeres, and moreover no RyR2s can be seen in many sarcomeres. Also, in the same figure, RyR2s appear to exist in nucleus as well as cytoplasm. RyR2 usually locates in SR membrane or nuclear envelope, but does not in the nucleus. This is perhaps due to apparent overlapping of RyR2s on the SR or nuclear membrane. Transverse section of cell may provide important data for clarification.

In Fig3, the amplitude of Ca spark is significantly higher in CPVT myocytes at baseline, although channel gating property is leaky and SR Ca content tends to be decreased in CPVT myocytes as shown in Fig 2E. Please explain this.

Referee #2

General Remarks:
This is a rather modest manuscript. The authors have used iPSCs to examine the effect of a human ryanodine receptor (RYR) mutation on calcium handling. The use of iPSCs is not novel, although it has not been done before for this particular mutation.

The authors claim that their data 'provides valuable insights' into the pathology of CPVT. However, nothing mechanistic emerges from this study. It is all observational. The use of dantrolene for inhibiting spontaneous calcium release is not novel. The data are consistent with what is already know, rather than providing a new insight. The iPSCs would be useful for screening, but as they stand there is no new information except that the S406L mutation leads to more calcium sparks under some experimental conditions.

There are several points that need to be addressed:
1. The ryanodine receptor immunostaining in figure 1G is peculiar in that there are no obvious striations. Even in immature myocytes there is an obvious striated pattern of RYR distribution. The lack of striations suggeststst that the cells are very immature, or that RYRs are not correctly targeted within the cells.
2. Deriving cardiac myocytes from EBs usually produced ventricular, atrial and SA node cells. From what types of cells were the data obtained?
3. The effect of iso on the calcium responses is incredibly weak. The systolic calcium signal increases by ~50 - 100 nM. That is far smaller than published effects in acutely isolated cells. Furthermore, caffeine also gave a very low amplitude response. The modest effects of iso and caffeine could point to a problem with RYR expression/function in the iPSCs.

Referee #3

General Remarks:
The manuscript by Jung el al. described providing human model of catecholaminergic polymorphic ventricular tachycardia (CPVT) using reprogramming of skin cells to generate patient-specific induced pluripotent stem cells (iPSCs) with the S406L mutation in ryanodine receptor 2 (RyR2). This group has previously reported the generation and characterization of human iPSC-derived cardiomyocytes with long QT syndrome type 1, revealing that this team is one of the leading groups in the field of cardiovascular studies using stem cell technology to unveil mechanisms of arrhythmogenesis. In this study, the authors examined cellular phenotypes in calcium handling and electrical activity in iPSC-derived cardiomyocytes from a patient with a novel mutation S406L in RyR2 calcium release channels and found that patient-specific cardiomyocytes showed abnormal calcium transients and sparks particularly under catecholaminergic stress and that dantrolene could rescue these defects in the patient cells.
Overall I find the study timely and important to understand molecular basis of the cardiac arrhythmia using human model. Their approach also provides a useful platform to find drug candidates to treat the disease and the findings demonstrate that the technology using patient-specific iPSCs is widely applicable in the fields of cardiovascular studies and pharmaceutical sciences and interesting for general audience in this journal. I believe this study for CPVT is well accomplished, but to confirm their findings, it would be great if the authors could address two main concerns below.

Main concerns:

A) Cell type of cardiomyocytes:
As is widely reported, human ESC/iPSC can generate three major types of cardiomyocytes: embryonic ventricular-, atrial- and nodal-like myocytes that spontaneously contract, with the protocol that the authors used with serum for differentiation culture media. To examine ventricular-specific phenotypes associated with catecholaminergic polymorphic ventricular tachycardia (CPVT), immunocytochemistry with ventricular marker myosin light chain (MLC2v) antibody and/or single cell RT-PCR will be required to make sure that only ventricular cardiomyocytes but not other cells were examined in calcium imaging experiments. Although action potential recording

helps us know which cells were ventricular-like, single cell RT-PCR is useful to confirm that their findings are ventricular-specific deficits in the electrophysiological experiments. In addition, when atrial- and nodal-like CPVT cardiomyocytes were examined, was any defect observed in those cells? If not, why were those cells not affected by abnormal function of RyR2, which can be expressed in those cells as well as ventricular cells? Results or comments regarding this concern would be very informative for reader to understand the disease mechanisms in which tachycardia happens in the ventricles of CPVT patients under stress condition.

B) SR/ER calcium handling:
To conclude the findings, further characterization of SR/ER calcium handling under Iso (stress-induced calcium overload) using additional tools will be informative although 100 mM caffeine was used to measure SR/ER calcium content. The reason why I am interested in seeing SR/ER calcium handling is that SR/ER calcium ion alters RyR2 activity dynamically and their measurement of caffeine-evoked calcium transients is not sufficient to investigate calcium handling in the SR/ER in patient cells. Genetically encoded calcium indicators localized in SR/ER lumen or Fluo-5N AM loading with saponin will be available for the authors to address this concern. Of course, ionomycin may be used to see SR/ER calcium content to confirm reduced content of SR/ER calcium in patient cells but using the SR/ER indicators described above would enable them to further characterize more physiological and dynamic calcium handling to study the disease mechanisms; particularly how is calcium storage altered/reduced immediately after adding Iso to CPVT myocytes? Unfortunately, the authors found that the cells that they generated in vitro showed very low mRNA expression of calsequestrin, an essential calcium buffering molecule, likely suggesting that the tested cells had weak SR/ER buffering and were not mature yet and probably that human model using patient iPSC-derived cardiomyocytes (even with 2-4 month culture) is not ideal system to investigate the molecular and cellular basis of CPVTs (especially arrhythmias with calsequestrin mutants due to stress-induced calcium overload). I think the authors may have to mention about pros and cons of using human models of CPVTs with iPSCs-derived cardiomyocytes in the discussion section. Another option to address the concern is to examine the expression of calsequestrin proteins using Western blot because proteins are sometimes detectable even when the mRNA is low.

Minor points:

1) Characterization of iPSCs: What was an essential factor for selecting the three iPSC lines of each control and patient group (ex. teratoma formation assay, gene silencing or morphology)? Or, were only three lines generated from each subject in this study?

2) Variability of cell lines: Checking variability of the cell lines in patient groups would be good in order to confirm that there was no differences in phenotypes of CPVT cells among three patient lines in some key results (ex. Fig 2 A).

3) qPCR: TNNT2 and GAPDH primer information in Table S1 are missing. And, was there any difference in the data between using GAPDH and TNNT2 expression as an internal control? I thought that most of researchers had used GAPDH (or beta-actin/tubulin) as control to obtain relative expression of target transcripts because TNNT2 expression can be altered by cardiac phenotypes associated with abnormal cardiac development. The authors need to examine correlation between GAPDH and TNNT2 expression in control and patient cells for the qPCR in Fig 1F or difference in TNNT2 expression between control and patient cells.

4) RyR2 antibody in Fig 1G: Does RyR2 antibody work well on human cardiomyocytes? The pattern of RyR2 expression was not junctional SR-like alignment but punctated. Is this because of immature cardiomyocytes or antibody property? It would be good if the authors could provide negative control samples for the immunocytochemistry. The images would be helpful to make sure the specificity of RyR2 antibody that the authors used in this study.

5) Sarcomeric staining in Fig 1G: because phalloidin cannot detect only sarcomeric actin, it would be better to avoid using 'sarcomeric actin' in the figure legend.

6) Action potential: Were cells showing spontaneous action potential observed in basal condition without electrical stimulation? As the authors kept spontaneously contracting embryoid bodies for 2-4 months, I thought that still some of cells contracted spontaneously in the physiological condition

for the current-clamp recordings. Were only cells that didn't contract spontaneously examined to measure evoked action potential in the cells? And, how much current input was used to get resting potential and evoked action potential in current clamp recording?

7) Are Ca traces (Fig 2A top) in control or CPVT cells? In the traces, scale bars are needed.

8) Fig 3A-B panel name are not (a) (b) but (i) (ii) in Figure 3 legend. And I was wondering if the authors examined the effect of Iso on calcium sparks (in Fig 3A) in independent cell samples because the cell images are different in basal and Iso.

9) Fig S1A-C column and name require position adjustment.

10) How many images were taken per minute for Fura-2 AM imaging?

11) Evoked contraction/calcium transient: how much strong stimulation was used?

12) Fig 4A dashed lines seemed to be higher than control basal for Spark FWHM and Tau decay. Should the lines be at 1.0?

13) Fig 4A requires representative images or movies of calcium spark recording to make sure the effect of dantrolene on calcium sparks in patient cells.

14) This journal has more general audience compared to other cardiac-specific or calcium signal-related journals so that it would be good for reader to know how the authors analyzed calcium traces in Fig S2 for measuring diastolic and systolic intracellular calcium ion level in Fig 2C-D (ex. using arrow heads in the traces to show where are points of diastolic and systolic calcium ion level).

1st Revision - Authors' Response 15 November 2011

Responses to Referee #1:

We would like to thank the reviewer for the insightful and critical input during the initial review of the manuscript. In fact, the constructive comments addressed by the referee really improved the quality of the work, and we are grateful for the efforts of the reviewer. Accordingly, we have now addressed each of the substantive issues raised by the referees *via* an extensive set of new experiments, as well as *via* editorial revision in order to clarify key issues raised in the review. These changes have now been incorporated into the revised version of the manuscript. The new results regarding (**i**) cardiomyocytes cell types, (**ii**) differences in gene expression in control and diseased iPSC-generated myocytes and (**iii**) further Fluo-5N measurements on calcium handling have been incorporated into the revised manuscript and Supporting information.

A whole new series of experiments addressing expression levels of pivotal Ca^{2+} handling proteins by Western blotting (RYR2, CASQ2, TRDN, JCTN, and PLN) and sub-cellular localization of RYR2 by confocal immunofluorescence analysis in control and CPVT iPSC-derived cardiac myocytes has resulted in a revised Figure 1 and a new Figure 2 in the manuscript. We addressed the concerns of Referee #2 and #3 regarding the subtypes of cardiomyocytes and their disease phenotype, by analyzing Ca^{2+} spark properties in both ventricular (MLC2v positive cells) and non-ventricular (MLC2v negative cells) myocytes and comparing their susceptibility to DADs/triggered activity. These data, showing that the CPVT phenotype is present in both ventricular and atrial cell types, resulted in a new Figure 5 in the revised manuscript and in a new Figure S6 of the Supporting information. In addition, we provided data regarding the differentiation efficiency of control and CPVT-iPSC clones into the 3 distinct subtypes of cardiomyocytes (atrial-, ventricular- and nodal-like), obtained by analysis of specific molecular marker expression (MLC2v and MLC2a) and action potential recordings, as shown in Panel A of the new Figure 5. Finally, according to the suggestions of Referee #3, we addressed the question regarding the dynamic changes in luminal SR Ca^{2+} by performing experiments using a low-affinity Ca^{2+} indicator, Fluo-5N, in healthy and diseased cardiac myocytes. These data are shown in a new Figure S4 of the Supporting information.

The specific responses to each of the points are noted below:

Major Points:

1. "The authors should fully discuss the observed differences in gene expression between normal cardiomyocytes and iPS cells. In Fig 1C the expressions of RyR2 and CASQ2 are both decreased, while those of TRDN, JCTN, and PLN are increased. Such differences in the expression level of the Ca^{2+} handling proteins may significantly affect the local Ca^{2+} release property, although it does not appear to influence on the CPVT phenotype. How about the protein expression level of these Ca^{2+} handling proteins? Since gene expression does not always change in parallel with protein expression, the author should clarify whether there is any change in the protein expression level of these Ca^{2+} handling proteins among groups shown in Fig 2F. If there are any significant differences in the protein expression level between normal human cardiomyocytes and IPS cells, the authors will need to be cautious upon interpretation of data."

Following the referee's suggestion, we performed Western blotting of major Ca^{2+} handling proteins (RYR2, CASQ2, TRDN, JCTN, and PLN) in control and CPVT-iPSC-generated myocytes at 3-4 months of maturation and in adult heart tissue, to complement the mRNA expression data from 2 month-old cells. Importantly, expression levels of the tested proteins do not differ between myocyte explants derived from control and CPVT-iPSCs, as shown in the revised Figure 2, panel B. Our gene expression analysis clearly revealed that CASQ2 expression levels in

iPSC-derived cardiomyocytes at 2 months of maturation is almost undetectable compared to adult heart tissue. This result confirms already published data on human ES/iPSC-derived cardiac myocytes (Gupta et al., BMC Dev. Biol. 10, 98 (2010)). Interestingly, although very low, CASQ2 protein levels by Western blotting in iPSC-derived cardiomyocytes at 3-4 months of maturation are comparable to those detected in the purchased human adult heart lysate (Imgenex). Moreover, expression of RYR2, TRDN, JCTN, and PLN proteins is also similar among the groups. These results have been incorporated in the revised Figure 2 and discussed in the result section of the revised manuscript on page 6, first paragraph.

Although we agree with the referee that low expression of SR Ca^{2+} buffering proteins like CASQ2 is a weakness of human iPSC-derived cardiac myocytes as a model system to study SR function in CPVT, we, however, believe this feature is related to the stage of maturation within the myocytic lineage and did not influence the analysis of the CPVT disease phenotype, since both control and iPSC-CPVT-derived cardiomyocytes exhibited the same degree of CASQ2 expression at day 90-130 of differentiation, the window of time during which cells were measured.

Minor Points:

1. "In Fig 1G, some RyR2s do not appear to be localized along with sarcomeres; some RyR2 are detected outside of sarcomeres, and moreover no RyR2s can be seen in many sarcomeres. Also, in the same figure, RyR2s appear to exist in nucleus as well as cytoplasm. RyR2 usually locates in SR membrane or nuclear envelope, but does not in the nucleus. This is perhaps due to apparent overlapping of RyR2s on the SR or nuclear membrane. Transverse section of cell may provide important data for clarification."

As suggested by the referee, we exchanged the original non-confocal immunofluorescence images of RYR2 and polymerized actin with confocal ones and now show optical transverse sections of the nuclear plane in control and CPVT-derived cardiac myocytes in new Figure 2, panel C. These new images clearly show absence of RYR2 expression in the nucleus and partial co-localization with the sarcomeres as expected, since cardiac myocytes generated from pluripotent stem cells lack a well-developed T-tubule system (Itzhaki et al., Ann. N.Y. Acad. Sci. 1080: 207 (2006); Satin et al., Stem Cells 26:1961 (2008); Lieu et al., Stem Cells Dev. 18:1493 (2009)). Furthermore, we show that RYR2 cluster density is similar between healthy and diseased cells, panel D of revised Figure 2. We have included this result in the text of the revised manuscript on page 6, first paragraph.

2. "In Fig3, the amplitude of Ca spark is significantly higher in CPVT myocytes at baseline, although channel gating property is leaky and SR Ca content tends to be decreased in CPVT myocytes as shown in Fig 2E. Please explain this."

Our findings indicate that under basal, i.e. non stimulated, conditions SR Ca^{2+} content was similar between control and CPVT myocytes. Following stimulation with isoproterenol, interestingly, SR Ca^{2+} content was only increased in control cells (see Figure 3E of the revised manuscript). In a recent seminal paper by Lothar Blatter's and Don Bers groups (Zima et al, J Physiol (Lond.) 588:4743 (2010)) the authors study the relationship between SR Ca^{2+} content and spark frequency, amplitude, spread and duration and found that all those parameters are positively correlated with the SR Ca^{2+} content. In our manuscript, we could confirm all those results for the control cells (revised Figure 4C, compare black bars before and after iso-treatment), but interestingly, in CPVT

cells these straight forward relationships appear to be more complex, most likely due to the mutation-induced changes in RYR2 properties. The properties of Ca^{2+} sparks are already altered when comparing basal, i.e. non-stimulated, conditions even though SR Ca^{2+} content is the same. Since this appeared to be a very important finding, we have verified the SR Ca^{2+} content by directly measuring the loss of SR Ca^{2+} following caffeine-induced emptying with Fluo-5N loading (see new Figure S4 in the Supporting information).

The amplitude of Ca^{2+} sparks is influenced by intrinsic properties of the RYR2 channel, such as the Ca^{2+} conductance and open probability. Thus, alterations of Ca^{2+}sparks under basal conditions are likely to be attributed to mutation-induced modulation of the gating behaviour, similarly to other RYR2 mutations (Meli et al, Circ Res 109:281 (2011)). Since the difference in Ca^{2+} spark amplitude at baseline can be rescued by the application of dantrolene, which stabilizes the closed state of the RYR2 channel, it is likely that this difference is due to altered open/closed probability of the mutated protein.

Responses to Referee #2:

We would like to thank the reviewer for the insightful and critical input during the initial review of the manuscript. In fact, the constructive comments addressed by the referee really improved the quality of the work, and we are grateful for the efforts of the reviewer. Accordingly, we have now addressed each of the substantive issues raised by the referees *via* an extensive set of new experiments, as well as *via* editorial revision in order to clarify key issues raised in the review. These changes have now been incorporated into the revised version of the manuscript. The new results regarding (**i**) cardiomyocytes cell types, (**ii**) differences in gene expression in control and diseased iPSC-generated myocytes and (**iii**) further Fluo-5N measurements on calcium handling have been incorporated into the revised manuscript and Supporting information.

A whole new series of experiments addressing expression levels of pivotal Ca^{2+} handling proteins by Western blotting (RYR2, CASQ2, TRDN, JCTN, and PLN) and sub-cellular localization of RYR2 by confocal immunofluorescence analysis in control and CPVT iPSC-derived cardiac myocytes has resulted in a revised Figure 1 and a new Figure 2 in the manuscript. We addressed the concerns of Referee #2 and #3 regarding the subtypes of cardiomyocytes and their disease phenotype, by analyzing Ca^{2+} spark properties in both ventricular (MLC2v positive cells) and non-ventricular (MLC2v negative cells) myocytes and comparing their susceptibility to DADs/triggered activity. These data, showing that the CPVT phenotype is present in both ventricular and atrial cell types, resulted in a new Figure 5 in the revised manuscript and in a new Figure S6 of the Supporting information. In addition, we provided data regarding the differentiation efficiency of control and CPVT-iPSC clones into the 3 distinct subtypes of cardiomyocytes (atrial-, ventricular- and nodal-like), obtained by analysis of specific molecular marker expression (MLC2v and MLC2a) and action potential recordings, as shown in Panel A of the new Figure 5. Finally, according to the suggestions of Referee #3, we addressed the question regarding the dynamic changes in luminal SR Ca^{2+} by performing experiments using a low-affinity Ca^{2+} indicator, Fluo-5N, in healthy and diseased cardiac myocytes. These data are shown in a new Figure S4 of the Supporting information.

The specific responses to each of the points are noted below:

Major point:

1. "The authors have used iPSCs to examine the effect of a human ryanodine receptor (RYR) mutation on calcium handling. The use of iPSCs is not novel, although it has not been done before for this particular mutation. "

Modern translational medicine is based on the progressive study of pathways and principles from animal model organisms to clinical research in humans. To date, most of our knowledge of human cardiac disease is based on studies in patients themselves. However, many of the most critical and puzzling human cardiovascular disorders cannot be adequately studied *in vitro* because specific human cardiovascular cell types, such as cardiomyocytes, cannot be easily obtained and maintained in culture without loss of their physiological properties.

Disease modelling with iPSCs is a young, yet rapidly evolving field. After our first report of modelling a cardiac arrhythmogenic disorder, the long-QT syndrome 1, with iPSC technology (Moretti et al., N Engl J Med 363: 1397 (2010)), several other groups have also reported iPSC-based models for the long-QT syndrome (e.g. Itzhaki et al., Nature 471: 225 (2011); Yazawa et al., Nature 471: 230 (2011); Mats ate al., Eur Heart J 32:952 (2011); Malan et al., Circ Res 109:841 (2011)). In our current study, we model for the first time CPVT, a disease caused by defective

intracellular Ca^{2+} cycling, which takes this technology to a different and mechanistically more complex class of arrhythmogenic cardiac disorders with respect to what has been previously published.

2. "The authors claim that their data 'provides valuable insights' into the pathology of CPVT. However, nothing mechanistic emerges from this study. It is all observational. The use of dantrolene for inhibiting spontaneous calcium release is not novel. The data are consistent with what is already know, rather than providing a new insight."

It is true that no new mechanistic concepts have arisen yet from iPSC-based modeling of CPVT. However, our methodology allows for the first time to directly visualize (by means of Ca^{2+} spark imaging) molecular properties of wild-type and mutated human RYR2 in the context of an intact human cardiac myocyte. Moreover, the same patient-specific human cardiac myocytes were used to test the effect of the drug dantrolene, which, until now, has proven to be efficient in animal models only.

3. "The iPSCs would be useful for screening, but as they stand there is no new information except that the S406L mutation leads to more calcium sparks under some experimental conditions."

Drug screening is indeed a very promising future application of iPSC technology. To be able to design such screening experiments, however, it is necessary to first define basic characteristics of the used model, as provided by this current study.

Specific Concerns:

1. "The ryanodine receptor immunostaining in figure 1G is peculiar in that there are no obvious striations. Even in immature myocytes there is an obvious striated pattern of RYR distribution. The lack of striations suggeststst that the cells are very immature, or that RYRs are not correctly targeted within the cells."

In the revised version of the manuscript, we exchanged the original non-confocal immunofluorescence images of RYR2 and polymerized actin with confocal ones in control and CPVT-derived cardiac myocytes (new Figure 2, panel C). These new images clearly show partial co-localization of RYR2 with the myofilaments. Since cardiac myocytes generated from pluripotent stem cells lack a well-developed T-tubule system (Itzhaki et al., Ann. N.Y. Acad. Sci. 1080: 207 (2006); Satin et al., Stem Cells 26:1961 (2008); Lieu et al., Stem Cells Dev. 18:1493 (2009)), it is expected that the pattern of RYR2 distribution does not resemble the striated one of an adult cardiac myocyte, but is rather cytosolic and perinuclear, as several other groups have already reported for mouse and human ESC/iPSC-derived myocytes (Sauer et al., Am J Physiol Heart Circ Physiol 281:H411 (2001); Satin et al., Stem Cells 26:1961 (2008); Zhu et al., PLoS ONE 4:e5407 (2009); Itzhaki et al., PLoS ONE 6:e18037 (2011)). Important for reliable modeling of the disease phenotype is a similar RYR2 distribution and cluster density between healthy and diseased cells, as shown in revised Figure 2, panel C and D.

2. "Deriving cardiac myocytes from EBs usually produced ventricular, atrial and SA node cells. From what types of cells were the data obtained?"

Indeed, the differentiation protocol used in our work leads to the generation of all three subtypes of cardiomyocytes mentioned by the referee. These cells can be discriminated easily based on the characteristics of their action potentials. However, in our present work, most of the physiological data come from whole-cell Ca^{2+} imaging and from Ca^{2+} sparks analysis, where action potentials

from single cells were not recorded. Since tachycardia happens in the ventricles of CPVT patients under stress condition, it might be expected that CPVT is a disease of ventricular cardiomyocytes. We have performed most of our experiments using the whole population of myocytes generated by our differentiation protocol. The following points give us reason to believe that our results are valid:

1) Ventricular myocytes are the predominant cell type in our preparation. In the revised version of the manuscript (new Figure 5, panel A) we provide data on the precise distribution of the three subpopulations of myocytes based on specific molecular marker expression (MLC2v, for ventricular cells, and MLC2a, for atrial cells) and action potential recordings. Additionally, we show that there is no difference in this distribution between control and CPVT cells.
2) We now demonstrate, by retrospective MLC2v staining of the cells used for Ca^{2+} spark imaging, that the observed differences in Ca^{2+} spark properties between control and CPVT cells persist if ventricular (MLC2v positive cells) and non-ventricular (MLC2v negative cells) myocytes are analyzed individually. These results are now illustrated in panel B of the new Figure 5 and have been incorporated in the revised text on page 8, second paragraph.
3) Patch clamp experiments on non-spontaneous active myocytes show that the susceptibility to DADs/triggered activity and the protective effect of dantrolene are not constrained to CPVT cells with ventricular-like action potentials, but also affect CPVT cells with atrial phenotype. A new Figure S6 of the Supporting information, summarizing the electrophysiological data on atrial-like iPSC-derived myocytes, is now provided and these results are discussed in the revised text on page 10.

3. "The effect of iso on the calcium responses is incredibly weak. The systolic calcium signal increases by ~50 - 100 nM. That is far smaller than published effects in acutely isolated cells. Furthermore, caffeine also gave a very low amplitude response. The modest effects of iso and caffeine could point to a problem with RYR expression/function in the iPSCs."

We agree with the referee that the effects of isoproterenol and caffeine on our iPSC-derived cardiomyocytes are smaller that those measured in acutely isolated adult myocytes. However, data from several groups have demonstrated that cardiomyocytes derived from pluripotent stem cells do not behave exactly like their mature adult counterparts. Despite the modest effects following β-adrenergic stimulation, the major phenotypical hallmark of CPVT, elevated diastolic Ca^{2+} and lower SR Ca^{2+} content under stress, could successfully be reproduced only in CPVT but not in control myocytes after addition of isoproterenol. Most essential for reliable disease modeling with iPSCs is that the expression levels of critical proteins involved in the disease phenotype is comparable between control and diseased cells. We have now provided multiple independent lines of evidence that RYR2 expression is similar between control and CPVT cells (mRNA level, protein level by Western blotting, and intracellular distribution and cluster density). These results are now illustrated in the new Figure 2 of the revised manuscript.

Most of our knowledge on CPVT to date comes from rodent models. Rodent models are also far from ideal models for human arrhythmogenic disorders considering the major electrophysiological differences leading to heart rates five times faster in mice as compared to men. Since a perfect model system does not exist (as Norbert Wiener has put it: „the best material model for a cat is another, or preferably the same cat", Philos. Sci. 12:316, 1945), we believe that our iPSC-based model system provides an important complement to the well-established rodent-based models.

Responses to Referee #3:

We would like to thank the reviewer for the insightful and critical input during the initial review of the manuscript. In fact, the constructive comments addressed by the referee really improved the quality of the work, and we are grateful for the efforts of the reviewer. Accordingly, we have now addressed each of the substantive issues raised by the referees *via* an extensive set of new experiments, as well as *via* editorial revision in order to clarify key issues raised in the review. These changes have now been incorporated into the revised version of the manuscript. The new results regarding (**i**) cardiomyocytes cell types, (**ii**) differences in gene expression in control and diseased iPSC-generated myocytes and (**iii**) further Fluo-5N measurements on calcium handling have been incorporated into the revised manuscript and Supporting information.

A whole new series of experiments addressing expression levels of pivotal Ca^{2+} handling proteins by Western blotting (RYR2, CASQ2, TRDN, JCTN, and PLN) and sub-cellular localization of RYR2 by confocal immunofluorescence analysis in control and CPVT iPSC-derived cardiac myocytes has resulted in a revised Figure 1 and a new Figure 2 in the manuscript. We addressed the concerns of Referee #2 and #3 regarding the subtypes of cardiomyocytes and their disease phenotype, by analyzing Ca^{2+} spark properties in both ventricular (MLC2v positive cells) and non-ventricular (MLC2v negative cells) myocytes and comparing their susceptibility to DADs/triggered activity. These data, showing that the CPVT phenotype is present in both ventricular and atrial cell types, resulted in a new Figure 5 in the revised manuscript and in a new Figure S6 of the Supporting information. In addition, we provided data regarding the differentiation efficiency of control and CPVT-iPSC clones into the 3 distinct subtypes of cardiomyocytes (atrial-, ventricular- and nodal-like), obtained by analysis of specific molecular marker expression (MLC2v and MLC2a) and action potential recordings, as shown in Panel A of the new Figure 5. Finally, according to the suggestions of Referee #3, we addressed the question regarding the dynamic changes in luminal SR Ca^{2+} by performing experiments using a low-affinity Ca^{2+} indicator, Fluo-5N, in healthy and diseased cardiac myocytes. These data are shown in a new Figure S4 of the Supporting information.

The specific responses to each of the points are noted below:

Major Points:

1. "Cell type of cardiomyocytes: As is widely reported, human ESC/iPSC can generate three major types of cardiomyocytes: embryonic ventricular-, atrial- and nodal-like myocytes that spontaneously contract, with the protocol that the authors used with serum for differentiation culture media. To examine ventricular-specific phenotypes associated with catecholaminergic polymorphic ventricular tachycardia (CPVT), immunocytochemistry with ventricular marker myosin light chain (MLC2v) antibody and/or single cell RT-PCR will be required to make sure that only ventricular cardiomyocytes but not other cells were examined in calcium imaging experiments. Although action potential recording helps us know which cells were ventricular-like, single cell RT-PCR is useful to confirm that their findings are ventricular-specific deficits in the electrophysiological experiments. In addition, when atrial- and nodal-like CPVT cardiomyocytes were examined, was any defect observed in those cells? If not, why were those cells not affected by abnormal function of RyR2, which can be expressed in those cells as well as ventricular cells? Results or comments regarding this concern would be very informative for reader to understand the disease mechanisms in which tachycardia happens in the ventricles of CPVT patients under stress condition."

Indeed, the differentiation protocol used in our work leads to the generation of all three subtypes of cardiomyocytes mentioned by the referee. These cells can be discriminated easily based on the characteristics of their action potentials and the expression of specific markers. However, in our present work, most of the physiological data come from whole-cell Ca^{2+} imaging and from Ca^{2+} sparks analysis, where action potentials from single cells were not recorded. We agree with the referee that, based on the clinical pathophysiology, it might be expected that CPVT is a disease of ventricular cardiomyocytes. We have performed most of our experiments using the whole population of myocytes generated by our differentiation protocol. The following points give us reason to believe that our results are valid:

1) Ventricular myocytes are the predominant cell type in our preparation. In the revised version of the manuscript (new Figure 5, panel A) we provide data on the precise distribution of the three subpopulations of myocytes based on specific molecular marker expression (MLC2v, for ventricular cells, and MLC2a, for atrial cells) and action potential recordings. Additionally, we show that there is no difference in this distribution between control and CPVT cells.
2) Following the referee's suggestion, we now demonstrate, by retrospective MLC2v staining of the cells used for Ca^{2+} spark imaging, that the observed differences in Ca^{2+} spark properties between control and CPVT cells persist if ventricular (MLC2v positive cells) and non-ventricular (MLC2v negative cells) myocytes were analyzed individually. These results are now illustrated in panel B of the new Figure 5 and have been incorporated in the revised text on page 8, second paragraph.
3) Patch clamp experiments on non-spontaneous active myocytes show that the susceptibility to DADs/triggered activity and the protective effect of dantrolene are not constrained to CPVT cells with ventricular-like action potentials, but also affect CPVT cells with atrial phenotype. A new Figure S6 in the Supporting information, summarizing the electrophysiological data on atrial-like iPSC-derived myocytes, is now provided and these results are discussed in the revised text on page 10.

2. "SR/ER calcium handling: To conclude the findings, further characterization of SR/ER calcium handling under Iso (stress-induced calcium overload) using additional tools will be informative although 100 mM caffeine was used to measure SR/ER calcium content. The reason why I am interested in seeing SR/ER calcium handling is that SR/ER calcium ion alters RyR2 activity dynamically and their measurement of caffeine-evoked calcium transients is not sufficient to investigate calcium handling in the SR/ER in patient cells. Genetically encoded calcium indicators localized in SR/ER lumen or Fluo-5N AM loading with saponin will be available for the authors to address this concern. Of course, ionomycin may be used to see SR/ER calcium content to confirm reduced content of SR/ER calcium in patient cells but using the SR/ER indicators described above would enable them to further characterize more physiological and dynamic calcium handling to study the disease mechanisms; particularly how is calcium storage altered/reduced immediately after adding Iso to CPVT myocytes? Unfortunately, the authors found that the cells that they generated in vitro showed very low mRNA expression of calsequestrin, an essential calcium buffering molecule, likely suggesting that the tested cells had weak SR/ER buffering and were not mature yet and probably that human model using patient iPSC-derived cardiomyocytes (even with 2-4 month culture) is not ideal system to investigate the molecular and cellular basis of CPVTs (especially arrhythmias with calsequestrin mutants due to stress-induced calcium overload). I think the authors may have to mention about pros and cons of using human models of CPVTs with iPSCs-derived cardiomyocytes in the discussion section. Another option to address the concern is to examine the expression of calsequestrin proteins using Western blot because proteins are sometimes detectable even when the mRNA is low."

The application of brief caffeine (10 mM) has a very long history (almost 2 decades) and has proven to be both robust and informative in many seminal publications (Bers et al., J Mol Cell Cardiol 25:1047 (1993); Bassani et al., Am J Physiol 268:C1313 (1995); Diaz et al., J Physiol 501: 3 (1997)) as well as in one of our recent publications ((Hammer et al., J Mol Cell Cardiol 49:10 (2010)). It is thus regarded as a standard approach to determine relative SR Ca^{2+} content. We agree with the referee that further characterization of dynamic Ca^{2+} handling in the SR/ER lumen in control and CPVT cardiac myocytes, particularly in response to isoproterenol, by the mean of a low-affinity Ca^{2+} indicator, Fluo-5N AM, would be very informative. Unfortunately, when we loaded Fluo-5N specifically into the ER/SR following a protocol published recently ((Picht et al, Circ Res 108:847 (2011)) and tried to record the relative changes of the SR Ca^{2+} concentration after application of isoproterenol by confocal microscopy, we could not measure any clear drug effect, even in control cells. This negative result may be attributable to a lower SR Ca^{2+} concentration in iPSC-derived myocytes compared to adult cells, which likely resides in the very low range of detection by Fluo-5N, whose Kd is 90 μM, ideal for measurements of Ca^{2+} concentrations in between 1μM-1mM. Nevertheless, following the suggestions of the referee we could validate part of our caffeine results with the independent Fluo-5N approach. In agreement with the previous caffeine recordings, our new Fluo-5N traces strongly supported our finding that in both control and CPVT cells caffeine resulted in a similar unloading amplitude in SR Ca^{2+}. These data are now presented in a new Figure S4 of the Supporting information.

We have also performed a whole new set of experiments addressing expression levels of pivotal Ca^{2+} handling proteins (RYR2, CASQ2, TRDN, JCTN, and PLN) by Western blotting in cardiac explants from control and CPVT-iPSCs after 3-4 months maturation and normal human adult heart. Although very low, CASQ2 protein levels in iPSC-derived cardiomyocytes are comparable to those of adult heart and do not differ between control and CPVT cells. Moreover, expression of RYR2, TRDN, JCTN, and PLN are also similar among the groups. These results have been incorporated in the revised Figure 2, panel B, and in the result section of the revised manuscript on page 6, first paragraph. Although we agree with the referee that low expression of SR Ca^{2+} buffering proteins like CASQ2 is a weakness of human iPSC-derived cardiac myocytes as a model system to study SR function in CPVT, we, however, believe this feature is related to the stage of maturation within the myocytic lineage and did not influence the analysis of the CPVT disease phenotype, since both control and iPSC-CPVT-derived cardiomyocytes exhibited the same degree of CASQ2 expression at day 90-130 of differentiation, window of time during which cells were measured.

We are aware that cardiomyocytes derived from human pluripotent stem cells do not behave exactly like the mature adult counterparts and may not represent the ideal model system to investigate the pathomechanisms underlying adult cardiac disorders. Most of our knowledge on CPVT to date comes from rodent models. Rodent models are also far from ideal models for human arrhythmogenic disorders considering the major electrophysiological differences leading to heart rates five times faster in mice as compared to men. Since a perfect model system does not exist (as Norbert Wiener has put it: „the best material model for a cat is another, or preferably the same cat", Philos. Sci. 12:316, 1945), we believe that our patient-specific iPSC-based model of CPVT provides an important complement to the well-established rodent models.

Minor Points:

1. "Characterization of iPSCs: What was an essential factor for selecting the three iPSC lines of each control and patient group (ex. teratoma formation assay, gene silencing or morphology)? Or, were only three lines generated from each subject in this study?"

We generated iPSCs from primary fibroblasts from 1 CPVT patient and 1 healthy control. After retroviral transfection of the four reprograming factors (OCT4, SOX2, KLF4, and c-MYC) many iPSC-like colonies appeared and 10 of them were picked per each individual. After an initial characterization based on morphology, expression of the pluripotent cell markers NANOG and TRA1-81 by immunofluorescence analysis, and differentiation efficiency towards the cardiac lineage, we have chosen 3 iPSC lines of each control and patient group for further characterization (as illustrated in Figure S1 of the Supporting information) and used them to perform the current study.

2. "Variability of cell lines: Checking variability of the cell lines in patient groups would be good in order to confirm that there was no differences in phenotypes of CPVT cells among three patient lines in some key results (ex. Fig 2 A)."

We agree with the referee that checking for phenotypical homogeneity among iPSC lines from the same individual is of fundamental importance for iPSC-based disease modeling. The 3 lines we have chosen for each group have shown similar efficiency of differentiation towards myocytic lineages and generate cardiac myocytes which express comparable levels of pivotal genes involved in myocytic Ca^{2+} handling and EC coupling (revised Figure 2, panel A). Following the reviewer's suggestion, we have added a new Figure S3 in the Supporting information illustrating the contribution of each of the three control and CPVT-iPSC lines to the results shown in our original Figure 2A (now Figure 3A). The percentage of cells with normal and aberrant Ca^{2+} cycling is comparable among clones of the same individual but differ between control and CPVT lines, as described on page 7 of the revised manuscript.

3. "qPCR: TNNT2 and GAPDH primer information in Table S1 are missing. And, was there any difference in the data between using GAPDH and TNNT2 expression as an internal control? I thought that most of researchers had used GAPDH (or beta-actin/tubulin) as control to obtain relative expression of target transcripts because TNNT2 expression can be altered by cardiac phenotypes associated with abnormal cardiac development. The authors need to examine correlation between GAPDH and TNNT2 expression in control and patient cells for the qPCR in Fig 1F or difference in TNNT2 expression between control and patient cells."

We have initially examined expression of GAPDH, TUBB, and TNNT2 in cardiac explants from control and CPVT-iPSC lines to choose the most appropriate internal reference gene for normalization of gene expression. All three genes correlated quite well and showed similar expression levels in cardiac explants from each control and CPVT-iPSC lines. We have provided these data to the referee in panel A of the supplemental Figure for Referee#3. Since the purity of cardiomyocytes in micro-dissected explants may vary (80-90% purity), we decided to use TNNT2 as reference gene, which allowed us to normalize also for the proportion of myocytes.

4. "RyR2 antibody in Fig 1G: Does RyR2 antibody work well on human cardiomyocytes? The pattern of RyR2 expression was not junctional SR-like alignment but punctated. Is this because of immature cardiomyocytes or antibody property? It would be good if the authors could provide negative control samples for the immunocytochemistry. The images would be helpful to make sure the specificity of RyR2 antibody that the authors used in this study."

In the revised version of the manuscript, we exchanged the original non-confocal immunofluorescent images of RYR2 and polymerized actin with confocal ones in control and CPVT-derived cardiac myocytes (new Figure 2, panel C). These new images clearly show partial co-localization of RYR2 with the myofilaments. Since cardiac myocytes generated from pluripotent stem cells lack a well-developed T-tubule system (Itzhaki et al., Ann. N.Y. Acad. Sci. 1080: 207 (2006); Satin et al., Stem Cells 26:1961 (2008); Lieu et al., Stem Cells Dev. 18:1493 (2009)), it is expected that the pattern of RYR2 distribution does not resemble the striated one of an adult cardiac myocyte, but appears rather punctated in the cytosol and perinuclear region, as several other groups have already reported for mouse and human ESC/iPSC-derived myocytes using different RYR2 antibodies (Sauer et al., Am J Physiol Heart Circ Physiol 281:H411 (2001); Satin et al., Stem Cells 26:1961 (2008); Zhu et al., PLoS ONE 4:e5407 (2009); Itzhaki et al., PLoS ONE 6:e18037 (2011)).
Therefore, we believe it is not necessary to show negative control samples for the immuno-histochemistry to prove the specificity of the RYR2 antibody used in our study. However, we are happy to provide the referee with such negative control in panel B of the supplemental Figure for Referee#3.

5. "Sarcomeric staining in Fig 1G: because phalloidin cannot detect only sarcomeric actin, it would be better to avoid using 'sarcomeric actin' in the figure legend."

Following the referee's suggestions, we changed the text accordingly.

6. "Action potential: Were cells showing spontaneous action potential observed in basal condition without electrical stimulation? As the authors kept spontaneously contracting embryoid bodies for 2-4 months, I thought that still some of cells contracted spontaneously in the physiological condition for the current-clamp recordings. Were only cells that didn't contract spontaneously examined to measure evoked action potential in the cells? And, how much current input was used to get resting potential and evoked action potential in current clamp recording?"

After dissociation of cardiac explants to single cells for physiological studies, a proportion of cells spontaneously contracts and is able to generate spontaneous action potential. However, we examined only cells that were spontaneously inactive but capable of responding to electrical pacing. Evoked action potentials were obtained by injection of 1nA current for 1.5 ms. No current imput was used to generate resting potential.

7. "Are Ca traces (Fig 2A top) in control or CPVT cells? In the traces, scale bars are needed."

According to the referee's suggestions, we specified in the Figure 3A legend of the revised manuscript that the traces were recorded in a CPVT myocyte. Since these traces are just illustrative of what we define as normal Ca^{2+} cycling (R) and abnormal Ca^{2+} cycling type AR1, AR2 and AR3, we think the addition of a scale bars, that will be different in each trace for the Ca^{2+} concentration, can be misleading and will not introduce more information than what we show in the representative complete Ca^{2+} recording examples in Figure S2 of the Supporting information. Thus, we exchanged "Representative Fura-2 Ca^{2+} recordings illustrating normal (R) and aberrant (AR1, AR2, and AR3) Ca^{2+} cycling" with "Images of Fura-2 Ca^{2+} recordings depicting normal (R) and aberrant (AR1, AR2, and AR3) Ca^{2+} cycling …" in Figure 3A legend.

8. "Fig 3A-B panel name are not (a) (b) but (i) (ii) in Figure 3 legend. And I was wondering if the authors examined the effect of Iso on calcium sparks (in Fig 3A) in independent cell samples because the cell images are different in basal and Iso."

As described in the Material and Method section spark measurements were performed on the same cell population but not necessarily on the same cells. Initially, sparks were recorded in approximately 15 field of views to determine basal parameters. Following this, isoproterenol was administered for 10 minutes after which the spark recordings were again performed on 15 field of views (not identical with the first ones). In independent control experiments we have verified our approach in performing 2x 15 field of views separated by a 10 minute gap with iso-free tyrode and did not find any significant differences in the sparks recorded before and after the 10 minute resting period. Although we agree with the referee that control and iso recordings would have been desirable, the fact that isoproterenol experiments are one-off experiments (the coverslip can only used once) has encouraged us to switch to the experimental regime described above.

We have corrected the labels in the legend of Figure 4.

9. "Fig S1A-C column and name require position adjustment. "

The position of the labels has been adjusted with the column position in Figure S1A-C.

10. "How many images were taken per minute for Fura-2 AM imaging? "

We recorded Fura-2 AM Ca^{2+} imaging at 22-24 Hz. This information has been added to the Materials and Methods section of the revised manuscript on page 15, second paragraph.

11. "Evoked contraction/calcium transient: how much strong stimulation was used? "

We evoked Ca^{2+} transients using 5msec pulses of 90V. This information has also been added to the Materials and Methods section of the revised manuscript on page 15, second paragraph.

12. "Fig 4A dashed lines seemed to be higher than control basal for Spark FWHM and Tau decay. Should the lines be at 1.0?"

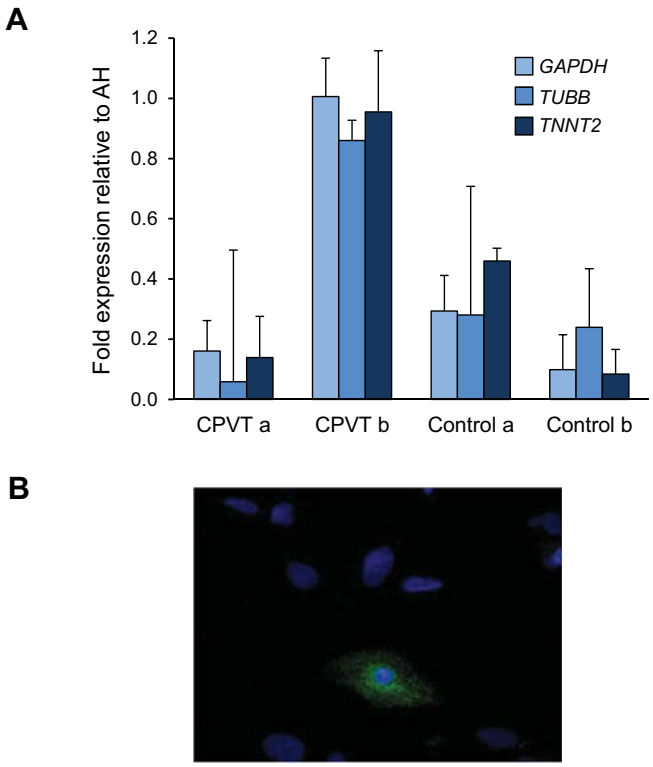
We are sorry and grateful to the referee hinting to this error. We have corrected the position of the dotted line to be at 1.0

13. "Fig 4A requires representative images or movies of calcium spark recording to make sure the effect of dantrolene on calcium sparks in patient cells."

After supporting all our bar graphs in Figure 6A of the revised manuscript with original data, we believe that additional panels of typical data depicting what can be deducted from the bar graphs in Figure 6A would not add additional information to the reader justifying the inclusion. However, we are happy to provide two videos of Ca^{2+} sparks in CPVT cardiomyocytes, one in presence 1 μM isoproterenol alone and one with 1 μM isoproterenol + 10 μM dantrolene, for your reference.

14. "This journal has more general audience compared to other cardiac-specific or calcium signal-related journals so that it would be good for reader to know how the authors analyzed calcium traces in Fig S2 for measuring diastolic and systolic intracellular calcium ion level in Fig 2C-D (ex. using arrow heads in the traces to show where are points of diastolic and systolic calcium ion level)."

As the referee suggested, Figure S2 and legend have been changed accordingly.



Supplementary Figure for Referee #3.

(A) Comparison of GAPDH, TUBB, and TNNT2 gene expression in cardiac explants from different control and CPVT-iPSC clones, relative to human adult heart (AH).

(B) Representative immunofluorescence image of cells dissociated from 30 day old human iPSC-EBs after staining with RYR2 antibody (green) and Hoechst 33258 (blue).

2nd Editorial Decision 30 November 2011

Thank you for the submission of your revised manuscript "Dantrolene rescues arrhythmogenic RYR2 defect in a patient-specific stem cell model of catecholaminergic polymorphic ventricular tachycardia" to EMBO Molecular Medicine. We have now received the reports from the reviewers who were asked to re-review your manuscript.

You will be glad to see that they are now globally supportive and we can proceed with official acceptance of your manuscript pending the minor changes detailed below.

Please clarify the point raised by Reviewer #1 regarding the correct concentration of caffeine used in the imaging experiments. The problem with the format of Fig 3A (0.5 Hz) mentioned by this reviewer seems to have only been caused during file conversion, the original file shows the expected trace.

On a more editorial note, please see below for information regarding EMBO Molecular Medicine guidelines for statistical analysis of data. Please mention the actual p value in each case.

In addition, we noted that your point-by-point response contains a figure. Since this would be published in the Review Process File, please let us known whether you agree with its publication, if you would like to delete the figure or whether you would like to opt out (more information below).

I look forward to seeing a revised form of your manuscript as soon as possible.

Statistical analysis
The description of all reported data that includes statistical testing must state the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the actual P value for each test (not merely 'significant' or 'P < 0.05').

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

Overall I find the study timely and important to understand molecular basis of the cardiac arrhythmia using human model. Their approach also provides a useful platform to find drug candidates to treat the disease and the findings demonstrate that the technology using patient-specific iPSCs is widely applicable in the fields of cardiovascular studies and pharmaceutical sciences and interesting for general audience in this journal.

Referee #1 (Other Remarks):

I found the rebuttal and the changes adequate. The authors have done everything they were asked for. I find the revision quite satisfactory and a well-written rebuttal addressing most of my concerns.

Minor points:

1) Was 10 mM or 100 mM caffeine used in calcium imaging? I found 'caffeine (100 mM)' in Method section but they mentioned 10 mM in the rebuttal letter.

2) Fig 3A trace (0.5 Hz) looks strange in the current figure although the former version was fine.

Referee #2:

The authors responded well to my concerns. I have no further comments.

2nd Revision - Authors' Response 01 December 2011

Please find below our answers addressing the remaining minor points.

1. Caffeine concentration
Indeed, two concentrations of caffeine were used throughout the experiments. A concentration of 100mM was used for the fluo-4 experiments detailed in Fig.3, while 10mM were used in the Fluo-5N experiments shown in FigS4.This was due to different technical set-ups and the different time durations needed to reach saturating concentration of the drug, necessary for complete Ca2+ release from the SR. We apologize for any confusion that this may have caused.

2. Statistical analysis/p-value
We have now stated the exact p-values, unless they were inferior to 0.001. In Fig3, p-values are specified in the figure legend, for better readability of the figure.

3. Authorization of publication
We have no objections against publishing the figure contained in our point-by-point response.

4. Figures
We have uploaded print-quality pdfs of our figures onto the EMBO Molecular Medicine server.

Thank you again for your kind assistance in the review process.

