BRIEF ULTRARAPID COMMUNICATION

Thyroid and Glucocorticoid Hormones Promote Functional T-tubule Development in Human-Induced Pluripotent Stem Cell Derived Cardiomyocytes

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Running title: T3+Dex Generates Functional T-Tubules in hiPSC-CM

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In September 2017, the average time from submission to first decision for all original research papers submitted to Circulation Research was 13 days.
ABSTRACT

**Rationale:** Human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CM) are increasingly being used for modeling heart disease and are under development for regeneration of the injured heart. However, incomplete structural and functional maturation of hiPSC-CM including lack of t-tubules, immature excitation-contraction (EC) coupling, and inefficient Ca-induced Ca release (CICR) remain major limitations.

**Objective:** Thyroid and glucocorticoid hormones are critical for heart maturation. We hypothesized that their addition to standard protocols would promote t-tubule development and mature EC coupling of hiPSC-CM when cultured on extracellular matrix with physiological stiffness (Matrigel mattress).

**Methods and Results:** HiPSC-CM were generated using a standard chemical differentiation method supplemented with triiodo-L-thyronine (T3) and/or dexamethasone (Dex) during days 16-30 followed by single-cell culture for 5 days on Matrigel mattress. HiPSC-CM treated with T3+Dex, but not with either T3 or Dex alone, developed an extensive t-tubule network. Notably, Matrigel mattress was necessary for t-tubule formation. Compared to adult human ventricular CM, t-tubules in T3+Dex-treated hiPSC-CM were less organized and had more longitudinal elements. Confocal line scans demonstrated spatially and temporally uniform Ca release that is characteristic of EC coupling in the heart ventricle. T3+Dex enhanced elementary Ca release measured by Ca sparks as well as promoted ryanodine receptor (RyR2) structural organization. Simultaneous measurements of L-type Ca current and intracellular Ca release confirmed enhanced functional coupling between L-type Ca channels and RyR2 in T3+Dex cells.

**Conclusions:** Our results suggest a permissive role of combined thyroid and glucocorticoid hormones during the cardiac differentiation process which, when coupled with further maturation on Matrigel mattress, is sufficient for t-tubule development, enhanced CICR, and more ventricular-like EC coupling. This new hormone maturation method could advance the utility of hiPSC-CM for disease modeling and cell-based therapy.

**Keywords:** Induced pluripotent derived stem cell, t-tubules, calcium induced calcium release, Matrigel mattress, T3, Dexamethasone, electrophysiology, excitation-contraction coupling

**Nonstandard Abbreviations and Acronyms:**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>hiPSC-CM</td>
<td>human induced pluripotent stem cell-derived cardiomyocytes</td>
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<tr>
<td>EC</td>
<td>excitation-contraction</td>
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<tr>
<td>CICR</td>
<td>calcium induced calcium release</td>
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<tr>
<td>T3</td>
<td>tri-iodo-l-thyronine</td>
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<tr>
<td>Dex</td>
<td>dexamethasone</td>
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<tr>
<td>RyR2</td>
<td>ryanodine Receptor 2</td>
</tr>
<tr>
<td>τ</td>
<td>Tau (decay constant)</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>LTCC</td>
<td>L-type calcium channel</td>
</tr>
<tr>
<td>PSC</td>
<td>iPSC or embryonic stem cell derived pluripotent stem cell</td>
</tr>
<tr>
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<td>caveolin-3</td>
</tr>
<tr>
<td>NCX</td>
<td>Na/Ca Exchanger</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarco/endooplasmic reticulum Ca ATPase</td>
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INTRODUCTION

Human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CM) are increasingly being used in biomedical research for cardiac disease modeling, cardiotoxicity screening, and regeneration of injured myocardium. Although considerable advances have been made in the maturation of hiPSC-CM, the lack of t-tubule formation and respective functional consequences remain a major limitation. T-tubules are organized invaginations in the cardiomyocyte sarcolemma and are critical for the synchronous Ca induced Ca release (CICR) that underlies efficient excitation-contraction (EC) coupling in human ventricular myocardium. Reduced t-tubule formation contributes to abnormal EC coupling in heart failure and may be responsible for the ventricular arrhythmias observed after cardiac transplantation of hiPSC-CM. Early t-tubule development in PSC-CM may be possible using complex techniques such as nano-patternning or tissue engineering, however, the extent of t-tubule formation and its functional contribution to EC coupling has not been demonstrated.

Thyroid and glucocorticoid hormones are critical for cardiac maturation during development. While standard hiPSC-CM induction media contains both factors, supplementing the culture media with higher concentrations of thyroid and/or glucocorticoid was found to enhance CM maturation. Independently, we and others have shown improved contractility and electrophysiological maturation when culturing hiPSC-CM on extracellular matrix substrates of physiological stiffness. We hypothesized that a combination of the thyroid hormone tri-iodo-l-thyronine (T3) and the glucocorticoid dexamethasone (Dex) with the flexible Matrigel substrate method would enhance the structural and functional maturation of hiPSC-CM. As such, at days 16-30 of the standard chemical differentiation protocol, the maintenance medium was supplemented with a combination T3 and Dex. Cells were dissociated after day 30 followed by single-cell culture on Matrigel mattress for an additional 5 days before experimental analyses.

METHODS

T3+Dex treated cells were generated as depicted in Figure 1A. In brief, media was supplemented with 100 nmol/L T3 and 1000 nmol/L Dex in 3 mL of media/well during days 16-30 of hiPSC-CM maintenance. Detailed materials and methods are included in the Online Data Supplement.

RESULTS

T3+Dex promotes t-tubule formation and synchronizes intracellular Ca release.

HiPSC-CM were stained using a lipophilic membrane dye and imaged using a confocal microscope. Images were deconvolved, and thresholded to the mean fluorescence intensity of the entire cell, and t-tubule density calculated by normalizing the supra-threshold signal within the cell interior to cross-sectional cell area. Consistent with previous reports, vehicle treated cells essentially lacked t-tubules (Figure 1B). Treatment with either T3 or Dex alone did not significantly impact t-tubule density, whereas the combination of T3+Dex promoted t-tubule formation (Figure 1B and Online Video I and II). Notably, maturation on Matrigel mattress was required for t-tubule formation, as T3+Dex treated cells plated without thick Matrigel mattress were not different from control cells (Figure 1B). Cellular staining for bridging-integrator 1 (BIN1), junctophilin-2 (JP2), and caveolin-3 (Cav3), three regulators of t-tubule genesis, demonstrated striking changes in their subcellular localization. Whereas these t-tubule markers were located mostly perinuclear in vehicle-treated cells, after T3+Dex treatment staining was much more prominent.
throughout the whole cell (Online Figure IA). Importantly, co-immunostaining of sarcomeric α-actinin with JP2, which is required for anchoring and maturation of developing t-tubules\textsuperscript{17}, demonstrated increased accumulation of JP2 along Z-lines (Online Figure IB,C).

We next compared t-tubule density of hiPSC-CM to that of adult human myocardium (Figure 1B and Online Video III). Ventricular tissue was acquired from organ donors deemed unsuitable for transplantation due to surgical reasons, and right atrial tissue from patients undergoing open heart surgery. T-tubule density was calculated from confocal images of cell-membrane stained cryosections (Online Figure IIA and Online Methods). T-tubule density of T3+Dex hiPSC-CM significantly exceeded that of healthy human atrial CM (p<0.05), but remained sparser and less organized than the t-tubule network of human ventricular CM (p<0.001) (Figure 1B and Online Video IV). Adult human ventricular CM exhibited a greater fraction of transversely-oriented tubules in comparison to longitudinal elements, while T3+Dex cells exhibited similar proportions of t-tubules in these two orientations (Online Figure IIA, B, C). This less organized appearance of t-tubules is analogous to that previously reported for rat CM during the postnatal period of development\textsuperscript{17}.

In addition to promoting t-tubule development, T3+Dex also significantly increased the size of iPSC-CM, primarily cell width and cell volume (Online Figure IIIA, B). Results were confirmed in a second, independently-generated iPSC line (Online Figure IIIC). However, T3+Dex treated hiPSC-CM were still significantly smaller than adult human atrial or ventricular CM (Online Figure IIID).

Are the newly formed t-tubules functional and do they contribute to EC coupling? The juxtaposition of the L-type Ca channel (LTCC) located in the t-tubule membrane with the ryanodine receptor (RyR2) located in the junctional sarcoplasmic reticulum (SR) would be expected to synchronize SR Ca release throughout the cell. Consistent with their lack of t-tubules\textsuperscript{4}, transverse confocal line-scans of Fluo-4 loaded vehicle treated hiPSC-CM demonstrated a U-shaped Ca release, with significantly faster Ca release at the cell periphery compared to the cell center (Figure 1C). In contrast, T3+Dex treated cells exhibited a uniform Ca release across the width of the cell, with a comparable amplitude and rate of Ca rise at both center and periphery of the cell (Figure 1C). Together, our findings indicate functional t-tubule development in hiPSC-CM as evidenced by increased t-tubule staining, synchronization of Ca release, and improved subcellular distribution of t-tubule related proteins.

\textit{T3+Dex treated hiPSC-CM demonstrate greater dependence on SR Ca release for EC coupling.}

We next examined the effect of the hormone treatment on cellular Ca handling using the ratiometric Ca indicator Fura-2 AM in electrically stimulated hiPSC-CMs (Figure 2A)\textsuperscript{14}. Although diastolic and peak Fura-2 fluorescence were not significantly different between the groups, both time to peak and Ca decay rates were significantly accelerated by hormone treatment (Figure 2B, C, D and Online Table I). To determine what was responsible for the accelerated Ca removal, we performed a Ca flux balance analysis\textsuperscript{18} and calculated transport rate constants for SERCA, NCX, and non-NCX pathways (Online Table I). T3+Dex treated cells displayed faster Ca efflux via SERCA2 and NCX, although no differences in SR content were found (Online Table I). Consistent with the improved Ca handling, cell shortening and contractile kinetics measured by edge detection\textsuperscript{14} were also significantly enhanced by T3+Dex (Online Table II).

To examine the contribution of SR Ca release to Ca handling, we repeated whole-cell Ca fluorescence recordings after blocking SR Ca release with thapsigargin and ryanodine (Figure 2E). T3+Dex treated cells displayed a greater reduction in Ca transient amplitude following SR blockade (Figure 2F). Both groups demonstrated a similar delay in time to peak following SR blockade (42% and 54% longer) (Figure 2G). Furthermore, Ca decay was significantly slower (i.e. increased τ) in the T3+Dex treated
compared to vehicle-treated cells (Figure 2H). Taken together, T3+Dex produced cells with a greater contribution of SR Ca release to CICR and an overall improvement in EC coupling.

**T3+Dex increases cell capacitance and EC-coupling gain.**

We next quantified the efficiency of coupling between LTCC current and SR-mediated Ca release by measuring the EC coupling gain (ECC gain) in voltage-clamped hiPSC-CM. ECC gain was calculated as the ratio of the amplitude of the cytoplasmic Ca release to the corresponding amplitude of the LTCC current at each membrane voltage. T3+Dex treated cells exhibited a greater rise in intracellular Ca for the respective membrane Ca current at each membrane potential (Figure 3A). On average, T3+Dex treatment significantly increased Ca transient amplitude despite reduced LTCC current density (Figure 3B). As a result, ECC gain was increased at most membrane potentials (Figure 3C). Equivalent SR Ca content was confirmed by rapid caffeine application following each voltage protocol (Figure 3D). Cell capacitance, a measure of cell surface membranes that includes t-tubules, was significantly increased in the T3+Dex group (Figure 3E). The increase in cell capacitance (93%) was greater than the increase in cell surface area measured by 3D reconstruction, which excludes any membrane invaginations (54%, Online Figure IIIB). This result provides further evidence that the t-tubules formed after T3+Dex treatment were electrically connected to the sarcolemma.

A characteristic consequence of greater spatial proximity of LTCC, located primarily in t-tubules, and RyR2, located in the junctional SR, is faster LTCC inactivation. To assess this, a longer depolarization step was utilized to measure both voltage-dependent inactivation ($\tau_1$) and Ca-dependent inactivation ($\tau_2$) (Online Figure IVA). The significant acceleration of $\tau_2$ but not $\tau_1$ (Online Figure IVB, C) also supports closer spatial proximity of RyR2 and LTCC that occurs when t-tubules are present. Taken together, these results establish that T3+Dex enhances the efficiency of CICR in hiPSC-CM.

**T3+Dex enhances elementary Ca release (Ca sparks) and RyR2 organization.**

In adult ventricular myocardium, t-tubules form dyads where sarcolemmal LTCCs are closely juxtaposed to clusters of SR RyR2 Ca-release channels. As such, dyad formation promotes RyR2 clustering and hence RyR2-mediated Ca release. The activity of RyR2 clusters but not that of single isolated RyR2 can be assessed by measuring Ca sparks. To date, only a few reports exist on the presence of Ca sparks in hiPSC-CM. Representative longitudinal line scans of Ca sparks are shown in Figure 4A. T3+Dex treatment increased spark frequency, spark amplitude, and spark mass, with no significant differences in SR Ca content (Figure 4B, C, D, E and Online Table III). As RyR2 function was improved, we next assessed the structural organization of RyR2. Consistent with literature reports, vehicle treated cells showed diffuse RyR2 staining with predominant perinuclear localization. In contrast, T3+Dex treated cells displayed reduced perinuclear RyR2 staining with striated organization and RyR2 clustering (Figure 4F). Taken together, these results provide evidence for enhanced organization and function of RyR2 in T3+Dex treated cells.
DISCUSSION

Here we report that a simple culture method – addition of T3+Dex during days 16-30 of cardiac induction followed by a 5 day culture on a Matrigel mattress – was sufficient to generate extensive t-tubule development in hiPSC-CM. Although still less organized than in adult human myocardium, the new t-tubules were electrically coupled to the cell surface, and drastically enhanced CICR and ECC gain. T3+Dex promoted a more ventricular-like RyR2 organization, which likely contributed to the robust appearance of Ca$^{2+}$ sparks and the greater participation of SR Ca handling to the EC coupling process. This finding is also supported by our data showing a significant reduction (Figure 3) in LTCC current in T3+Dex treated cells, further evidence for increased coupling between the RyR2 and LTCC that allows for more efficient negative feedback of SR Ca release on LTCC (i.e., increased Ca-dependent inactivation of LTCC, Online Figure IV). The results reported here represent a significant advancement, because previous approaches that accelerate hiPSC-CM maturation (i.e., addition of growth factors, length of cultivation, culturing on various substrate/patterning, in vivo maturation) have not enhanced t-tubule formation or provided evidence of ventricular-like EC coupling to the extent shown here$^2$. 

The Matrigel mattress method (and others) has facilitated maturation of electrophysiological and Ca handling properties of hiPSC-CM, thereby demonstrating the importance of extracellular matrix for maturation$^2, 8, 14$. Thyroid and glucocorticoid hormones are essential for optimal fetal and neonatal heart development and can also enhance hiPSC-CM maturation$^{13}$. However, our results suggest that standard differentiation medium contains suboptimal concentrations of both hormones. Our findings highlight the importance of increasing fetal maturation factors during hiPSC-CM differentiation as well as the substrate dependence for promoting optimal CM development. Although mechanisms underlying this process are not yet elucidated, our data suggest that addition of T3+Dex primes the CM for functional and structural maturation and, when receiving proper substrate cues, is able to express these improvements.

In summary, our results suggest a permissive role of combined thyroid and glucocorticoid hormones during the cardiac differentiation process which, when coupled with further maturation on Matrigel mattress, is sufficient for robust t-tubule development, enhanced CICR, and more ventricular-like EC coupling in single hiPSC-CM. This provides proof of principle that functional t-tubule development can be achieved in single cell culture of hiPSC-CM. The hiPSC-CM structural and functional maturation appear to be mediated by two features: 1. Activation of pathways downstream of thyroid and glucocorticoid receptors and 2. Interaction with an extracellular matrix substrate of physiological stiffness. However, as our work does not provide an exhaustive list of molecular signatures of cardiomyocyte maturation, further assessment of the effects of T3+Dex mattress method on cellular metabolism and gene expression changes specific to EC coupling are warranted. Furthermore, t-tubule development in T3+Dex treated hiPSC-CM after 5 days is less organized than in adult human myocardium, and more reminiscent to that found in day 15-20 postnatal rat hearts$^{17}$. Hence, future studies are required to establish optimal culture conditions to achieve further t-tubule maturation (e.g., longer culture time, tuning substrate tension, and optimizing hormone concentrations). Indeed, our discovery encourages investigation of gene expression pathways downstream of thyroid and glucocorticoid receptors and we anticipate this will yield novel targets for further maturation of hiPSC-CM. Nevertheless, the new maturation technique reported here overcomes a major barrier in the stem cell field, which will help improve the utility of hiPSC-CM for disease modeling and enable future research into the molecular pathways underlying t-tubule development.
SOURCES OF FUNDING
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DISCLOSURES
None.

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FIGURE LEGENDS

Figure 1: T3+Dex promotes t-tubule formation and synchronizes intracellular Ca release. A) Hormone-based cardiac differentiation protocol (D=day, see online methods for details). B) Representative examples of t-tubule staining in hiPSC-CM (upper panels) and human adult myocardium (lower panels) with quantitation of t-tubule index (% cell area) (n=19-59 cells/group, *p<0.05 and ***p<0.001 vs vehicle). Images are planar projections of 3D reconstructions. C) Representative transverse line scans of Fluo-4AM loaded hiPSC-CM electrically-stimulated at 0.2 Hz (left panels) and summary data (right panel) comparing Ca transient time to peak between periphery (P) and center (C) of cell. Scan line was positioned across the middle of each cell. n=18-21 cells/group; *p<0.05, **p<0.01, ***p<0.001, vs vehicle, ns – non significant. All data are reported as mean±SEM.

Figure 2: T3+Dex treated hiPSC-CM demonstrate greater dependence on SR Ca release for EC coupling. A) Representative examples of intracellular Ca transients recorded from Fura-2AM loaded hiPSC-CM with an overlay of the Ca transient from a T3+Dex treated cell (red trace) and a vehicle-treated cell (black trace). Cells were paced at 0.2 Hz followed by rapid caffeine (Caff) application to measure SR Ca content. Note the significantly faster rate of Ca rise and rate of Ca decay in the T3+Dex treated cells. B-D) Summary data for Ca transient amplitude (B), time to peak (C), and transient decay tau (D); (n=68-73 cells/group). E) Representative paced Ca transients before (black) and after (grey) block of SR Ca release with thapsigargin and ryanodine. Note the larger contribution of SR Ca release to the Ca transient in T3+Dex treated cells. F-H) Summary data following SR blockade for Ca transient amplitude (F), time to peak (G), and decay tau (H). Data are expressed as percent change from baseline before SR block. Data reported as mean±SEM (n=16-21 cells/group); *p<0.05, **p<0.01, and ***p<0.001 vs vehicle.

Figure 3: T3+Dex increases cell capacitance and EC-coupling gain. A) Representative examples of simultaneously-recorded intracellular Ca fluorescence traces (top) and L-type Ca currents (bottom) from vehicle-treated (black) and T3+Dex treated (red) hiPSC-CM. Records were obtained from voltage-clamped hiPSC-CM loaded with the fluorescent Ca indicator Fluo-4 and measured using the indicated voltage protocol. B) Average current-voltage relationship with associated intracellular Ca fluorescence at indicated membrane potentials in T3+Dex versus vehicle treated hiPSC-CM. C) EC coupling gain calculated as a ratio of normalized Ca fluorescence to normalized Ca current. D) SR Ca content. E) Cell capacitance. All data reported as mean±SEM (n=6-7); *p<0.05, **p<0.01, ***p<0.001, vs vehicle, ns – non significant.

Figure 4: T3+Dex enhances elementary Ca release (Ca sparks) and RyR2 organization. A) Representative line-scans of Ca sparks recorded from saponin permeabilized hiPSC-CM. B) Overlay of representative Ca spark. C-E) Summary data for spark parameters for the different treatment groups (n=15-37 cells/group). F) Representative cell images and fast Fourier transform of RyR2 immunostaining in T3+Dex and vehicle treated cells. Summary data of peak power of RyR2 immunostaining (n=8-10 cells/group). All data reported as mean±SEM; *p<0.05 and ***p<0.001 vs vehicle.
NOVELTY AND SIGNIFICANCE

What Is Known?

- Human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CM) are utilized for modeling heart disease and under development for regenerating the injured myocardium.

- Inefficient excitation-contraction (EC) coupling, fetal cardiomyocyte-like Ca handling and electrophysiological properties remain a major limitation of hiPSC-CM.

- Central to efficient EC coupling in the human ventricle are functional t-tubules and ryanodine receptor 2 (RyR2) clustering, both of which are largely absent in hiPSC-CMs.

What New Information Does this Article Contribute?

- The addition of thyroid and glucocorticoid hormones to standard protocols promotes robust t-tubule development and RyR2 clustering of hiPSC-CM when cultured on extracellular matrix with physiological stiffness (Matrigel mattress).

- The new t-tubules are functional, resulting in spatially and temporally uniform Ca-induced Ca-release and efficient EC coupling that is characteristic of healthy adult ventricular myocardium.

The absence of t-tubules has remained a major concern for the utilization of hiPSC-CM. Although a number of methods have been reported that improve hiPSC-CM maturation (e.g. culture on soft, patterned substrates, increased length of culture time, hormone maturation), none of them produce functional t-tubules. We similarly observed that Matrigel mattress alone or treatment with hormone alone is not sufficient for development of t-tubules. Our work provides compelling evidence that combined activation of pathways downstream the thyroid and glucocorticoid receptors is sufficient for functional t-tubule development if hiPSC-CM are allowed to mature on a substrate of extracellular matrix with physiological stiffness. Furthermore, T3+Dex treated cells demonstrated more widespread cellular distribution of key t-tubule related proteins and displayed improved RyR2 organization. As such, our discovery not only helps to overcome a major barrier in the field, but also provides novel mechanistic insight into the genesis of t-tubules.
**Figure 1**

A. Schematic diagram showing the culture timeline of hiPSC-CM on Matrigel. D0 to D10: Chir and IWR treatment, D10 to D16: metabolic selection, D16 to D30: T3 + Dex treatment.

B. Images showing hiPSC-CM under vehicle and T3+Dex conditions. Comparing the T-tubule density and % cell area in human atrial and ventricular myocytes.

C. Graphs demonstrating time to peak in the center and periphery of Vehicle and T3+Dex treatments.
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SUPPLEMENTAL MATERIAL

Thyroid and Glucocorticoid Hormones Promote Functional T-tubule Development in Human Induced Pluripotent Stem Cell Derived Cardiomyocytes

Short Title: T3+Dex generates functional t-tubules in hiPSC-CM

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Detailed Methods
HiPSC Culture and Maintenance

Human induced pluripotent stem cells (hiPSC) were generated using fibroblasts isolated from dermal punch biopsies. These cells were acquired from two consenting healthy individuals (one male, one female) and reprogrammed to hiPSC using a non-integrating episomal based reprogramming approach. In brief, Epi5TM episomal oriP/EBNA1 vectors (Thermo) containing 5 reprogramming factors (Oct4, Sox2, Lin28, Klf4, and L-Myc) were electroporated into fibroblasts using the Thermo NEON® Transfection System as per manufacturer’s guidelines. Single colonies were manually picked and clonally expanded. Clones were subjected to a battery of analyses (data not shown) for verification of stemness and genomic stability as per including karyotyping, embryoid body differentiation, and immunostaining (Tra1-60, SSEA4, SSEA5, and Oct4). HiPSCs were maintained on growth factor reduced Matrigel (Corning) coated plates in mTeSR1 medium (Stem Cell Technologies). Plates were coated with 1:200 Matrigel diluted in DMEM/F12 for at least 1 hour at 37 °C prior to use. HiPSC were passaged every four days. Cells were washed with 1xPBS without CaCl2 or MgCl2 and dissociated with 0.5 mmol/L EDTA in PBS without calcium or magnesium for 7 minutes at room temperature (RT) followed by resuspension in mTeSR1 with 10 µmol/L RHO kinase inhibitor Y-27632 (Calbiochem) and immediately seeded on Matrigel coated plates. Cells received fresh mTeSR1 daily and were maintained at 37 °C with 5% CO2 and 5% O2. The male iPSC line was used as the primary source of cardiomyocytes (CM) for imaging and functional studies. The female iPSC line was used to confirm the concordant effect of the hormone treatment on CM morphology.

Differentiation and Maturation of iPSC to CM
HiPSC-cardiomyocytes (hiPSC-CM) were generated using a small molecule based cardiac differentiation protocol1, 2. HiPSCs were cultured until they reached 65-85% confluence, at which point the chemical differentiation protocol was initiated (D0) using the media listed in Table 1.

Table 1: Differentiation Media Components

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<td>B27 minus insulin (A1895601, Life Technologies)</td>
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<tr>
<td>M2</td>
<td>RPMI 1640 no glucose (11879 Life Technologies)</td>
<td>B27 minus insulin (A1895601, Life Technologies)</td>
</tr>
<tr>
<td>M3</td>
<td>RPMI 1640 with glucose (11875 Life Technologies)</td>
<td>B27 (17504044, Life Technologies) With 1% Pen-Strep (Life Technologies)</td>
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</table>

At D0 the hiPSC transition from mTeSR1 to M1 was supplemented with 6 µmol/L GSK3 inhibitor CHIR99021 (Selleck Chemicals). On day 2, the media was changed to fresh M1. On day 3, the media was changed to M1 with 5 µmol/L IWR-1 (Sigma). On days 5 to 9 the media was changed every other day with M1. Metabolic selection was started with M2 media on day 10 and cells received fresh M2 media on days 12 and 14. On day 16, cells were transitioned to 3 mL M3 media. For generation of hormone treated cells, M3 was supplemented with the combination of hormones or their vehicles as in Table 2. All solutions were generated, stored at -20 °C, and used within 3 months of constitution. Media supplemented with the final concentrations of hormones was prepared fresh daily.
Table 2: Hormone Treatments

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Supplement</th>
<th>Concentration of Stock</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>DMSO and NaOH</td>
<td>-</td>
<td>0.01% DMSO + 0.065 mmol/L NaOH</td>
</tr>
<tr>
<td>T3</td>
<td>Triiodo-L-thyronine hormone (Sigma T2877)</td>
<td>0.031 mmol/L in 20.0 mmol/L NaOH</td>
<td>0.0001 mmol/L in M3</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>Dexamethasone (Cayman 11015)</td>
<td>63.70 mmol/L in DMSO</td>
<td>0.001 mmol/L in M3</td>
</tr>
<tr>
<td>T3 + Dexamethasone</td>
<td>Both</td>
<td>-</td>
<td>0.0001 mmol/L T3 and 0.001 mmol/L Dex in M3</td>
</tr>
</tbody>
</table>

hiPSC-CM Dissociation and Plating on Matrigel Mattress
At D30 of the chemical differentiation process, hiPSC-CM were dissociated and plated as previously described. Primarily, freshly differentiated cells were utilized for experiments. Freshly differentiated hiPSC-CM were washed with 1x DPBS without calcium and magnesium and then incubated in 1 mL of TrypLE Express (Life Technologies) for 10 minutes at 37°C. A 10 mL serological pipet was then used to add equal volumes of M3 to neutralize TrypLE and for resuspension. Cells were centrifuged at 200 x g for 5 minutes, re-suspended in 1-3 mL of M3, and filtered through a 40 µM filter (Falcon). The filtered cells were counted and seeded on Matrigel mattress as previously described. Cells were allowed to attach for at least 10 minutes at RT before adding an additional 1 mL of normal M3 media and returned to the incubator for experimental use at days 4-6. M3 media was refreshed on a daily basis until experimentation.

Cell Selection for Imaging and Functional Measurements
The Matrigel mattress method generates single, elongated CM located in a glass bottom culture dish that can be used for imaging, Ca fluorescence, and patch-clamp studies without the need for cell transfer. Since not all iPSC-CM achieve a rod shape on mattress, iPSC-CM were selected for experimental studies using the same morphological and functional selection criteria that are widely used in studies of CM acutely-isolated from adult hearts: (1) rod-shape, (2) no contact with other cells, and (3) robust contractile response to field stimulation (for Ca and patch-clamp studies). In all cases, the experimenter that selected cells for studies was blinded to the treatment group.

T-Tubule Staining and Analysis
HiPSC-CM were seeded at a density of 10-15,000 cells on Delta TPG Culture Dish (Fisher) using the Matrigel-mattress. Cells were loaded (as per manufacture’s recommendations) with CellMask Orange for 20 minutes followed by three 5 minute washes at RT in Tyrodes solution consisting of (mmol/L): 140 NaCl, 5 KCl, 1 MgCl₂, 1.8 CaCl₂, 10 glucose, and 10 HEPES (pH = 7.4). Images were collected as Z-stacks using a Zeiss LSM510 inverted laser scanning confocal microscope. Images were first deconvolved, and thresholded to the mean fluorescence intensity of the entire cell, and t-tubule density calculated by normalizing the supra-threshold signal within the cell interior to cross-sectional cell area. Cross sectional area was determined using ImageJ. Presented t-tubule densities correspond to images taken within the central plane of the cell. 3D reconstruction of the t-tubular network was performed as described previously. For rendering of the surface sarcolemma, the membrane was identified manually across entire Z-stacks using ImageJ, and smoothed by dilation and re-erosion. Sarcolemma Z-stacks were then merged with the t-tubule stacks and rendered in 3D.

T-tubule development in hiPSC-CMs was compared with t-tubule density and organization in the left ventricles and right atria of healthy individuals. This tissue was obtained using protocols approved by Norwegian South-Eastern Regional Committee for Medical Research Ethics (Permit numbers s-07482a and
in agreement with guidelines outlined in the Declaration of Helsinki. Tissue from the left ventricular free wall was obtained from non-diseased hearts considered for transplantation, but deemed unsuitable due to surgical reasons (n=4). These hearts were kept in a cardioplegic solution at 4 °C for 1-4 hours prior to tissue collection and freezing. Cause of death in these patients was cerebrovascular accident, and none had a history of heart disease. Approval for study inclusion was obtained from next of kin.

Tissue from the right atrial appendage was obtained during open-heart surgery in patients undergoing coronary artery bypass surgery. Atrial tissue was rapidly excised and frozen in liquid nitrogen until further use. All patients (n=3) provided informed written consent.

Using a cryostat, human tissue was sliced into 10 μm thick sections at -20 °C, collected on Poly-prep Slides (Sigma-Aldrich, St. Louis, MO), and fixed in 4% paraformaldehyde. T-tubules were stained with wheat germ agglutinin (WGA) conjugated to Alexa Fluor 480 prepared in PBS. An LSM 710 (Zeiss, GmbH, Jena, Germany) confocal scanning system with a 40× objective was used to visualize t-tubules and cellular membranes. T-tubule density was analyzed as described above for hiPSC-CMs. Organization of t-tubules was compared by calculating the amount of transverse and longitudinal elements in the representative 3D cells.

Paced Calcium Transients
HiPSC-CM were incubated for 30 min at RT in 5 µM Fluo-4 AM (Invitrogen) with 0.02% pluronic (Invitrogen) followed by three 5-minute washes in Tyrodes solution consisting of (mmol/L): 140 NaCl, 5 KCl, 1 MgCl2, 1.8 CaCl2, 10 glucose, and 10 HEPES (pH = 7.4). Single-cell Ca2+ transients were recorded at RT using a Zeiss LSM 510 inverted laser scanning confocal microscope in line scan mode (521 lines per second) with a 40X / 1.30 NA planar oil immersion objective. The pinhole settings corresponded to a measured optical slice of 0.85 μm. HiPSC-CM were stimulated by an electric field of 40 mV with a 5 ms bipolar pulse duration at 0.2 Hz using a MyoPacer (IonOptix). The focal plane was positioned in the middle of the Z-dimension from the top and bottom edges of the cell and transverse lines were drawn across the widest part of the cell, avoiding nuclei. Fluo-4 was excited at 488 nm with an argon laser and the emission was passed through a 505 nm long pass filter to photomultiplier tube detectors. Images were normalized to the intrinsic background fluorescence and ImageJ was used to measure the calcium transient amplitude and rate based parameters at the peripheral and central regions of the cell. Time-zero for time to peak measurements were defined as the point of calcium rise.

Immunostaining
HiPSC-CM were cultured on Matrigel mattress-coated cell culture chambers (Falcon 354104) and fixed with 4% paraformaldehyde (Thermo #28906) for 10 minutes at RT. Cells were washed three times with PBS for 10 minutes at RT and permeabilized with 0.4% Triton X-100 in PBS for 1 hour, after which they were blocked for 1 hour (5% Goat serum, 0.4% Triton X-100 in PBS). Cells were incubated with primary antibody overnight at 4 °C as per Table 3. Then cells were washed 3 x 5 minutes with 0.4% Triton X-100 in PBS and incubated with secondary antibody for 1 hour. After washing cells with 0.4% Triton X-100 in PBS 3 x 5 minutes followed by PBS another 3 x 5 minutes each, the cells were counterstained with ProLong Diamond Antifade with DAPI-containing Mountant (Invitrogen #P36962) and mounted on a cover slide for imaging. Imaging of t-tubule markers was completed using the Zeiss LSM 510 followed by calculation of percent stained area using ImageJ. For co-labeling of t-tubule markers and α-actinin, imaging was performed using the LSM 880 inverted laser scanning confocal microscope with a 63X / 1.40 Plan-APOCHROMAT oil immersion objective. Each channel was sequentially scanned using photomultiplier tube detectors. Alexa Fluor 488 was excited using a 488 nm Argon laser and the emitted light was collected from 499 – 570 nm; Alexa Fluor 568 was excited using a diode-pumped solid state 561-10 laser and collected from 570 – 712 nm; DAPI was excited using a 405-30 diode laser and collected from 409 – 497 nm. Using ImageJ, immunostaining images were background corrected by subtracting the mean value of background next to the cell and the contrast was linearly adjusted. Each image from a particular
immunostain (e.g. BIN-1) was adjusted equally and all images were cropped and scaled equally. Regularity of RyR2, Cav3, BIN1, α-actinin and JP2 localization along z-lines was assessed using fast Fourier transforms (FFT), computed by the FFT tool in Matlab (MathWorks). The power spectrum was plotted as a function of 1 / axial spatial frequency, and the amplitude of the first peak measured. Co-localization analysis was then completed using the Manders split coefficients in ImageJ.

Table 3: Antibodies for Immunostaining

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Host</th>
<th>Catalogue number</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bin1</td>
<td>Abcam</td>
<td>Rabbit</td>
<td>Ab185950</td>
<td>1:100</td>
</tr>
<tr>
<td>Cav3</td>
<td>Abcam</td>
<td>Rabbit</td>
<td>Ab2912</td>
<td>1:100</td>
</tr>
<tr>
<td>Sarcomeric α-actinin</td>
<td>Sigma</td>
<td>Mouse</td>
<td>A7811</td>
<td>1:100</td>
</tr>
<tr>
<td>Sarcomeric α-actinin</td>
<td>Abcam</td>
<td>Rabbit</td>
<td>Ab137346</td>
<td>1:100</td>
</tr>
<tr>
<td>Junctophilin2</td>
<td>Abcam</td>
<td>Mouse</td>
<td>Ab180362</td>
<td>1:100</td>
</tr>
<tr>
<td>RyR2</td>
<td>Thermo</td>
<td>Mouse</td>
<td>MA3-916</td>
<td>1:50</td>
</tr>
<tr>
<td>anti-mouse 488</td>
<td>Invitrogen</td>
<td>Goat</td>
<td>A11029</td>
<td>1:250</td>
</tr>
<tr>
<td>anti-rabbit 568</td>
<td>Invitrogen</td>
<td>Donkey</td>
<td>A10042</td>
<td>1:250</td>
</tr>
</tbody>
</table>

Sparks Measurements in Permeabilized Myocytes
Isolated hiPSC-CM were permeabilized with saponin (20 µg/mL) for 30 seconds and then bathed for 5 minutes in a freshly-made internal solution (pH = 7.2) containing (mmol/L): K-aspartate (120), KCl (15), K₂HPO₄ (5), MgCl₂ (5.6), HEPES (10), dextran (4% w/v), MgATP (5), Phosphocreatine-Na₂ (10), creatine phosphokinas (10 U/mL), reduced L-glutathione (10), EGTA (0.5), CaCl₂ (0.12), and Fluo-4 pentapotassium salt (0.03). Free [Ca] was 100 nmol/L (calculated with MaxChelator). Confocal imaging was performed on a Zeiss LSM 510 inverted laser scanning confocal microscope equipped with a 40X / 1.30 NA planar oil immersion objective. Fluo-4 was excited by an argon laser line at 488 nm and the fluorescence emission was passed through a 505 nm long pass filter to PMT detectors. Cell sparks were imaged in line-scan mode in isolated cells that had no contact with other cells. Lines were positioned longitudinally near the center of the cell. SR Ca load was measured as the Ca transient amplitude induced by application of 10 mmol/L caffeine. Image analysis was performed in ImageJ with the SparkMaster plugin using a background setting of 5 and criteria of 3.8. Spark mass was calculated from the equation: spark mass = 1.206 * Amplitude * FWHM^3.

Measurement of Intracellular Calcium and Contractility
HiPSC-CM were seeded 10-15,000 cells on Matrigel mattress on Delta TPG Culture Dish (Fisher). After 5 days of acclimation on mattress with daily M3 media changes, cells were loaded with Fura-2 AM (Molecular Probes Inc, Eugene, OR) as previously described. Fura-2 AM was reconstituted in DMSO at 2 mmol/L in a light-protected vessel. HiPSC-CM were incubated at a final concentration of 2 µmol/L Fura-2 AM in M3 medium for 8 minutes at RT and then washed twice with 1.2 mmol/L calcium containing Tyrode’s solution with 250 µmol/L probenecid. Measurements were made with a 40x LUCPlanFLN Ph2 (NA 0.6, 0-2/FN22, WD 2.7-4 mm) objective using a Nikon Eclipse T5100 fitted with an IonOptix video microscopy system (Ionoptix, Milton, MA). Tyrode’s solution contained in mmol/L: CaCl₂ as indicated below, NaCl 134, KCl 5.4, MgCl₂ 1, glucose 10, and HEPES 10, with the pH adjusted to 7.4 with NaOH.

A. Calcium Kinetics: Calcium transients were recorded from cells which elicited a response during 0.2 Hz electrical field stimulation in Tyrode’s solution with 2 mmol/L Ca (2Cal) for 20 seconds at RT. Stimulation was then stopped and immediate puffer based application of 10 mmol/L caffeine in 2Cal for 5 seconds was initiated followed by 5 seconds of recording with 2Cal alone to assess for sarcoplasmic
reticulum (SR) calcium content and SERCA-independent calcium extrusion. The recording was then paused for 1 minute while cells were continuously perfused with 2Cal solution. Cells were then exposed to 0 mmol/L Na 0 mmol/L calcium solution (0Na0Ca) for 10 seconds followed by buffer-based application of 10 mmol/L caffeine containing 0Na0Ca solution for 30 seconds in addition to 10 seconds of recording in 0Na0Ca solution to estimate non-NCX mediated calcium extrusion. For examination of SR calcium contribution to the calcium flux, hiPSC-CM were pretreated for 5 minutes with 10 µmol/L thapsigargin (Sigma) and 50 µmol/L ryanodine (Sigma) in 2Cal after the last wash. For each cell, the following parameters were determined: baseline and peak amplitude of transient, time to peak and baseline, and tau (τ) of A) field stimulated calcium transients, B) 2Cal Caffeine transient, and C) 0Na0Ca Caffeine transient. Analyses of calcium transients and calculation of calcium flux balance was completed as previously2. Time zero for time to peak measurements were defined as the time of electrical stimulation.

B. Contractility: Video based edge detection was used to simultaneously assess cellular shortening of the hiPSC-CM during the first 20 seconds of field stimulation. In brief, during acquisition of calcium transients on single HiPSC-CM, edge detectors were placed to define the ends of the cells using IonWizzard™ v6.5 acquisition software (Ionoptix, Milton, MA). Analysis of traces was completed using the instrument packaged IonWizard™ v6.5 data analysis software. Cell selection was limited to cells which were amenable to 0.2 Hz field stimulation and captured by edge detection.

Morphometric Measurements
HiPSC-CM were seeded on Matrigel mattress. Cellular volume measurements were taken as previously described1, 2. Briefly, Z-stacks of single D35 hiPSC-CM were obtained using a Zeiss LSM 510 inverted laser scanning confocal (40x oil immersion 1.30 Plan-NEOFLUAR lens). HiPSC-CM were loaded with 5 µmol/L calcein AM (Molecular Probes, Eugene, OR) in Tyrode’s solution for 30 minutes at RT followed by three five-minute washes at RT to remove any residual extracellular dye. Calcein AM was exited at 488 nm with an argon laser. Z-stack images were collected in 0.44 µm intervals and reconstructed using IMARIS (Bitplane, South Windsor, CT) to allow for the measurement of volume and surface area. Manual measurements for length and width were completed using ImageJ measurement tools. Image acquisition and analysis was completed with the user blinded to the treatment groups.

Measurement of Excitation Contraction Coupling (ECC) Gain and L-type Ca current inactivation
To measure ECC gain, hiPSC-CM from both groups (30 days after cardiac induction, 3-5 days after plating) were studied using patch clamp technique in whole-cell mode. External solution contained (in mmol/L): NaCl 134, CsCl 5, MgCl2 1, CaCl2 2, glucose 10, HEPES 10, adjusted to pH 7.4 with NaOH. The pipette solution contained (in mmol/L): CsCl 110, MgCl2 1, MgATP 5, cAMP 0.2; EGTA 1; Hesper 20; adjusted to pH = 7.25 with CsOH. For Ca fluorescence measurements, 100 µmol/L Fluo-4 pentapotassium salt was added to the pipette solution. Currents were elicited by 50 ms depolarizing steps ranging from -40 mV to +40 mV (in 10 mV increments), from the holding potential of -70 mV. A 10 ms conditioning pulse to -45 mV was applied prior to the test pulse in order to inactivate Na currents. To assess inactivation kinetics of ICa, a single 500 ms depolarizing step to 0 mV (from the holding potential of -70 mV, with 10 ms conditioning pulse to -45 mV) was applied. Inactivation of ICa was fit with a double exponential curve. All experiments were carried out at RT.

Statistics
GraphPad Prism v7 was utilized for testing statistical differences with Student’s t-tests or, when applicable, ANOVA followed by post hoc analysis. P-values of <0.05 were considered statistically significant. All data are presented as mean±SEM. Image acquisition and analyses were conducted in a blinded fashion.
References


Online Videos
## Online Table I: Calcium Handling

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>T3+Dex</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diastolic Ca (F_{Ratio})</td>
<td>1.3 ± 0.029</td>
<td>1.27 ± 0.027</td>
<td>0.22</td>
</tr>
<tr>
<td>Ca transient amplitude (F_{Ratio})</td>
<td>0.94 ± 0.044</td>
<td>0.88 ± 0.034</td>
<td>0.23</td>
</tr>
<tr>
<td>Time to peak (s)</td>
<td>0.59 ± 0.025</td>
<td>0.49 ± 0.022</td>
<td>0.0036</td>
</tr>
<tr>
<td>Time to peak 50% (s)</td>
<td>0.21 ± 0.026</td>
<td>0.17 ± 0.023</td>
<td>0.036</td>
</tr>
<tr>
<td>Time to baseline 50% (s)</td>
<td>0.82 ± 0.03</td>
<td>0.60 ± 0.017</td>
<td>1.10E-09</td>
</tr>
<tr>
<td>SR Ca content (F_{Ratio})</td>
<td>1.1 ± 0.054</td>
<td>0.97 ± 0.045</td>
<td>0.093</td>
</tr>
<tr>
<td>Ca transient decay (t_{twitch}, s)</td>
<td>1.21 ± 0.053</td>
<td>0.84 ± 0.033</td>
<td>1.33E-08</td>
</tr>
<tr>
<td>Caff decay (t_{caff}, s)</td>
<td>2.46 ± 0.086</td>
<td>2.12 ± 0.098</td>
<td>0.011</td>
</tr>
<tr>
<td>0Na0Ca Caff decay (t_{Caaff0Na}, s)</td>
<td>12.82 ± 0.77</td>
<td>10.67 ± 0.85</td>
<td>0.065</td>
</tr>
<tr>
<td>K_{SERCA}</td>
<td>0.43 ± 0.036</td>
<td>0.65 ± 0.057</td>
<td>0.02</td>
</tr>
<tr>
<td>K_{NCX}</td>
<td>0.36 ± 0.021</td>
<td>0.44 ± 0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>K_{non-NCX}</td>
<td>0.089 ± 0.0054</td>
<td>0.10 ± 0.0062</td>
<td>0.089</td>
</tr>
</tbody>
</table>

**Online Table I:** Ca kinetic measurements of T3+Dex treated vs vehicle treated hiPSC-CM paced at 0.2 Hz. Data presented are mean±SEM (n=68 for control and 79 for T3+Dex). Unpaired, two tailed Student’s t-test, p-value as listed.
### Online Table II: Contractile Kinetics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>T3+Dex</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell shortening (% of resting length)</td>
<td>5.55 ± 0.50</td>
<td>7.53 ± 0.70</td>
<td>0.02</td>
</tr>
<tr>
<td>Time to peak (s)</td>
<td>1.01 ± 0.04</td>
<td>0.71 ± 0.02</td>
<td>1.88E-07</td>
</tr>
<tr>
<td>Time to peak 50%</td>
<td>0.48 ± 0.03</td>
<td>0.37 ± 0.02</td>
<td>0.0028</td>
</tr>
<tr>
<td>Time to peak 90%</td>
<td>0.78 ± 0.03</td>
<td>0.56 ± 0.02</td>
<td>7.03E-06</td>
</tr>
<tr>
<td>Time to baseline 50%</td>
<td>0.58 ± 0.04</td>
<td>0.41 ± 0.09</td>
<td>2.07E-07</td>
</tr>
<tr>
<td>Time to baseline 90%</td>
<td>1.87 ± 0.02</td>
<td>1.20 ± 0.09</td>
<td>4.60E-06</td>
</tr>
</tbody>
</table>

**Online Table II:** Contractile kinetic measurements of T3+Dex treated vs vehicle treated hiPSC-CM paced at 0.2 Hz. Data presented are mean±SEM (n=68 for control and 79 for T3+Dex). Unpaired, two tailed Student’s t-test, p-value as listed.
### Online Table III: Calcium Spark Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle (Mattress)</th>
<th>T3 (Mattress)</th>
<th>Dex (Mattress)</th>
<th>T3+Dex (Mattress)</th>
<th>T3+Dex (Non-mattress)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spark frequency (sparks/100µm/s)</td>
<td>4.02 ± 0.51</td>
<td>5.82 ± 1.06</td>
<td>4.09 ± 0.78</td>
<td>8.31 ± 0.67***</td>
<td>4.95 ± 0.94</td>
</tr>
<tr>
<td>Spark amplitude (ΔF/F₀)</td>
<td>0.29 ± 0.02</td>
<td>0.26 ± 0.01</td>
<td>0.25 ± 0.01</td>
<td>0.46 ± 0.03**</td>
<td>0.31 ± 0.03</td>
</tr>
<tr>
<td>Spark mass (ΔF/F₀*µm³)</td>
<td>6.62 ± 0.99</td>
<td>7.36 ± 1.64</td>
<td>7.53 ± 1.61</td>
<td>33.36 ± 8.37***</td>
<td>12.76 ± 4.0</td>
</tr>
<tr>
<td>FWHM (µm)</td>
<td>2.52 ± 0.06</td>
<td>2.59 ± 0.07</td>
<td>2.56 ± 0.07</td>
<td>2.84 ± 0.05***</td>
<td>2.91 ± 0.07**</td>
</tr>
<tr>
<td>FDHM (ms)</td>
<td>27.84 ± 0.86</td>
<td>25.3 ± 0.63</td>
<td>25.42 ± 0.88</td>
<td>36.49 ± 0.86***</td>
<td>27.9 ± 1.21</td>
</tr>
<tr>
<td>Time to peak (ms)</td>
<td>16.24 ± 0.61</td>
<td>15.0 ± 0.61</td>
<td>16.73 ± 0.98</td>
<td>21.69 ± 0.78***</td>
<td>16.03 ± 0.82</td>
</tr>
<tr>
<td>Caffeine transient amplitude (ΔF/F₀)</td>
<td>2.79 ± 0.15</td>
<td>2.54 ± 0.13</td>
<td>2.46 ± 0.2</td>
<td>2.92 ± 0.27</td>
<td>2.72 ± 0.13</td>
</tr>
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</table>

**Online Table III:** Full width half max (FWHM), Full duration half max (FDHM). Data are reported as mean±SEM. One-way ANOVA with Tukey's post hoc test; *p < 0.05, **p < 0.01, and ***p < 0.001 vs vehicle.
Online Figure I: T3+Dex treated cells exhibit increased subcellular distribution of t-tubule related proteins.

A) Representative cell images after co-labeling for α-actinin and the indicated t-tubule related proteins Cav3, BIN1, or JP2 in vehicle and T3+Dex treated cells (scale bar = 10 µm for all cells). Summary data show percent stained area of indicated protein of total cell area. Data reported as mean±SEM (n=17-23 cells).

B) Fast Fourier transform for JP2 and α-actinin in vehicle and T3+Dex treated cells. 

C) Representative merge of α-actinin and JP2 with percent co-localization of JP2 with α-actinin (n=5 cells/group). Unpaired, two tailed Student’s t-test: ***p<0.001.
Online Figure II: T-tubule organization: comparison of longitudinal vs transverse t-tubules
A) Representative examples of planar projection after 3D reconstruction of t-tubules in T3+Dex vs human ventricular myocardium demonstrate intracellular location of t-tubule membrane structures. B) Comparison of longitudinal vs transverse t-tubules in 3D reconstructed T3+Dex CM vs human ventricular CM, (white represents transverse t-tubules, red represents longitudinal t-tubules) C) Summary data showing the fraction of transverse vs longitudinal t-tubule of total t-tubule density. Data are mean±SEM (n=18-19 cells). Unpaired, two tailed Student’s t-test: *p<0.05 vs transverse.
Online Figure III: T3+Dex treated cells exhibit increased cell size

A) Representative examples T3+Dex vs control treated hiPSC-CM. Images are planar projections of 3D reconstructions using Calcein-AM. B) and C) Summary data: morphometric measurements obtained from hiPSC-CM derived from two independently-generated iPSC lines (male and female). Data reported as mean±SEM (n=51-108 for male line from 4 independent differentiations and 24-35 for female line). D) Summary data for cross sectional cell area used for calculating t-tubule density for indicated groups. Note atrial and ventricular are of human origin (n=19-59). One-way ANOVA with Tukey's post hoc test: *p<0.05, **p<0.01, and ***p<0.001 vs vehicle.
Online Figure IV: Ca-dependent inactivation of L-type Ca current is accelerated in the T3 +Dex treated cells.

A) Representative traces of $I_{Ca}$ from a conditioning step of -45mV to 0mV in T3+Dex versus vehicle treated cells. Double exponential fit yields estimates of voltage dependent inactivation (tau 1) and Ca dependent inactivation (tau 2). B-C) Summary data for tau 1 and tau 2. Data reported as mean±SEM (n=9-12). Unpaired, two tailed Student’s t-test: *p<0.05 vs vehicle, ns – non significant.