Cardiac Resynchronization Therapy Reduces Subcellular Heterogeneity of Ryanodine Receptors, T-Tubules and Ca$^{2+}$ Sparks Produced by Dyssynchronous Heart Failure

Li et al: Subcellular Heterogeneity of Remodeling in HF

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Abstract

Background—Cardiac resynchronization therapy (CRT) is a major advance for treatment of patients with dyssynchronous heart failure (DHF). However, our understanding of DHF-associated remodeling of subcellular structure and function and their restoration after CRT remains incomplete.

Methods and Results—We investigated subcellular heterogeneity of remodeling of structures and proteins associated with excitation-contraction coupling in cardiomyocytes in DHF and after CRT. Three-dimensional confocal microscopy revealed subcellular heterogeneity of ryanodine receptor (RyR) density and the transverse tubular system (t-system) in a canine model of DHF. RyR density at the ends of lateral left ventricular cardiomyocytes was higher than in cell centers, while the t-system was depleted at cell ends. In anterior left ventricular cardiomyocytes, however, we found a similar degree of heterogeneous RyR remodeling despite preserved t-system. Synchronous heart failure (SHF) was associated with marginal heterogeneity of RyR density. We used rapid scanning confocal microscopy to investigate effects of heterogeneous structural remodeling on calcium signaling. In DHF, diastolic Ca\(^{2+}\) spark density was smaller at cell ends versus centers. After CRT, subcellular heterogeneity of structures and function was reduced.

Conclusions—RyR density exhibits remarkable subcellular heterogeneity in DHF. RyR remodeling occurred in lateral and anterior cardiomyocytes, but remodeling of t-system was confined to lateral myocytes. These findings indicate that different mechanisms underlie remodeling of RyRs and t-system. Furthermore, we suggest that ventricular dyssynchrony exacerbates subcellular remodeling in heart failure. CRT efficiently reduced subcellular heterogeneity. These results will help to explain remodeling of EC coupling in disease and restoration after CRT.

Key Words: remodeling heart failure, cardiac resynchronization therapy, myocyte, ryanodine receptors, excitation-contraction coupling, transverse tubular system
Cardiac resynchronization therapy (CRT) is an established clinical therapy for patients with moderate to severe heart failure (HF). CRT is based on biventricular pacing. It acutely improves left ventricular (LV) mechanical performance and reduces myocardial oxygen consumption in patients with HF and intraventricular conduction delays. Several clinical trials demonstrated that CRT leads to a reduction of HF-related rehospitalizations and overall mortality in the majority of patients with dyssynchronous HF (DHF). A clinical marker of DHF is prolonged QRS duration, which reflects interventricular delays of electrical activation. Consequently, mechanical contraction is regionally delayed, which aggravates weak cardiac performance in DHF patients.

While recent studies demonstrated remarkable remodeling of subcellular structures and function in DHF and partial restoration in response to CRT, we are only starting to understand pathological remodeling and effects of CRT at the microscopic scale. This study focuses on remodeling of excitation-contraction (EC) coupling in DHF and the ability of CRT to restore structure and function at subcellular level. EC coupling is the mechanism by which an action potential at the membrane of a muscle cell initiates a cascade of events that result in release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) and activation of contraction. Our current understanding of EC coupling in ventricular myocytes is organized around the concept of the couplon. A couplon comprises one or more voltage-gated L-type Ca\(^{2+}\) channels (LCCs) in the sarcolemma and a cluster of ryanodine receptors (RyRs) in the terminal cisternae of the SR. LCCs and RyRs are separated by a junction of ~12 nm. In ventricular myocytes, couplons are commonly associated with the transverse tubular system (t-system), which is a specialization of the sarcolemma penetrating into the interior of myocytes and in close proximity to the SR. The t-system facilitates rapid transmission of membrane voltage changes into the cell interior to enable synchronous activation of couplons. Our current knowledge of the mechanism by which...
coulons activate the Ca\(^{2+}\) transient is based on the classical work of Fabiato\(^{17}\) and insights into the subcellular arrangement of structures involved in EC coupling by microscopy.\(^{18}\) Couplons are activated when LCCs admit small quantities of Ca\(^{2+}\) into the junctional region and gate RyRs. Couplons are separated from one another by \(\sim 1\) \(\mu\)m.\(^{19}\) When Ca\(^{2+}\) is released by the RyRs, it produces a local Ca\(^{2+}\) release event, or Ca\(^{2+}\) spark.\(^{20,21}\) The spread of Ca\(^{2+}\) in the cytoplasm is governed by diffusion and buffering. Ca\(^{2+}\) transients are believed to result from the summation of the activation of many couplons.

Alterations of Ca\(^{2+}\) transients are a common feature of cardiac myocytes from HF hearts and thought to contribute to the progression of HF.\(^{22}\) Recent studies investigated the connection between alterations of Ca\(^{2+}\) transients and remodeling of the t-system. The studies suggested that remodeling of the t-system in ventricular myocytes is a consequence of tachycardia-induced HF,\(^{23-25}\) infarction\(^{26}\) and DHF.\(^{11}\) Our previous work on isolated myocytes revealed regional depletion of t-system in canine DHF models.\(^{11}\) In particular, lateral LV myocytes were affected by DHF, but not myocytes from the anterior LV wall. We demonstrated that t-system depletion in DHF was accompanied by increased occurrence of non-junctional RyR clusters. A remarkable recent finding is that t-system depletion is reversible. Partial restoration of t-system was found after CRT,\(^{11}\) SERCA2a gene therapy\(^{27}\) and mechanical unloading of the heart.\(^{28}\) In those studies t-system restoration was associated with restoration of Ca\(^{2+}\) transients, which emphasizes the crucial role of the t-system for efficient excitation-contraction coupling.

Beyond those studies remarkably little is known about the mechanisms and the degree of remodeling and restoration of the t-system. In particular, little is known about the subcellular
heterogeneity of remodeling of the t-system and associated proteins such as RyRs as well as the effects of this structural remodeling on cell function. Also, we have only sparse information on restoration of structures and function at the subcellular scale. Here, we studied an animal model of synchronous heart failure (SHF) based on right atrial (RA) pacing and a model of DHF based on right ventricular (RV) pacing to test the hypothesis that ventricular dyssynchrony accentuates the extent and heterogeneity of t-system and RyR remodeling in HF. While both models are based on rapid pacing and lead to congestive HF, the DHF model additionally reproduces clinical findings of interventricular delays of electrical activation and delayed mechanical contraction of the lateral LV wall. Furthermore, we used an animal model of CRT to test the hypothesis that CRT effectively reduces the extent and heterogeneity of remodeling after DHF.

We applied three-dimensional (3D) confocal microscopy and image analysis to provide insights into the spatial distribution of the t-system and RyRs in cardiac myocytes from animal models of SHF, DHF and CRT. Segmentation of myocytes in images of cardiac tissue allowed for 3D reconstructions of cells and quantitative analyses of subcellular distributions. We investigated for the first time the subcellular heterogeneity and degree of remodeling of t-system and RyRs in different models of HF and after CRT. Additionally, we measured Ca\(^{2+}\) spark density in isolated ventricular myocytes using dual-labeling with fluorescent dyes and rapid scanning confocal microscopy, which allowed acquisition of two-dimensional image sequences at high spatiotemporal resolution. We developed image-processing methods for analysis of these image sequences to investigate at the subcellular scale if structural heterogeneity is accompanied by functional heterogeneity.
Methods

Animal Models of HF and CRT

All procedures involving the handling of animals were approved by the Animal Care and Use Committees of the Johns Hopkins University and the University of Utah. Protocols complied with the published *Guide for the Use and Care of Laboratory Animals* published by the National Institutes of Health.

We generated canine models of SHF, DHF and CRT. These models have been described and characterized in detail previously.\(^1\), \(^2\), \(^29\)-\(^32\) In brief, adult mongrel canines (25-30 kg) were used as control (CTRL) and models of SHF, DHF and CRT. DHF was induced by either 6 weeks of RV pacing or left bundle branch ablation followed by 6 weeks of RA pacing. The CRT model was identical to the DHF model for the first 3 weeks, followed by 3 weeks of biventricular pacing. SHF animals underwent RA pacing for 6 weeks. The pacing rate for SHF, DHF and CRT animals was 170-200 bpm. Our methods for monitoring, tissue collection and cell isolation are described in the Supplemental Data.

Tissue Preparation, Fluorescent labeling and 3D Confocal Microscopy

Our approach for preparation and labeling of tissue is detailed in the Supplemental Data. We used wheat germ agglutinin (WGA) conjugated to a fluorophore for labeling of the sarcolemma, t-tubules and the interstitial space. RyRs were labeled with a monoclonal antibody (MA3-916, ThermoFisher Scientific, Waltham, MA, USA). After labeling, tissue sections were placed on a glass slide, embedded in Fluoromount-G (Electron Microscopy Science, Hatfield, PA, USA) and covered with a #1.5 coverslip. Three-dimensional image stacks were obtained from labeled tissue
sections using a Zeiss LSM 5 Live Duo confocal microscope (Carl Zeiss, Jena, Germany) with a 63x oil immersion objective (numerical aperture: 1.4). Alexa Fluor 488 and 555 were excited using a 488 nm and 543 nm laser, respectively. Emitted light was band-pass filtered at 505 to 530 nm and long-pass filtered at 560 nm, respectively. Typical stack dimensions were 1024×1024×240 voxels at a resolution of 0.1×0.1×0.1 μm.

**Analysis of T-System and RyR Clusters in Image Stacks**

A detailed description of the analyses is provided in Supplemental Data. In short, we applied methods for noise reduction, deconvolution and background removal to the image stacks. The WGA and RyR images were segmented using histogram-based thresholding. Segmented WGA images were used for semi-automatic segmentation of myocytes.33 Segmented cells then served as masks to analyze RyR clusters and sarcolemma including t-tubules. Cells were divided into two regions: 0-10 μm and 10-40 μm from a longitudinal cell end. Analysis of each RyR cluster yielded sum of intensity and intensity-weighted centroid position. Clusters with intensities higher than 90% of all clusters in the same cell (90th percentile) were defined as high-intensity clusters. To determine the distance of voxels to the nearest sarcolemma we calculated Euclidean distance maps from the segmented WGA images (Supplemental Figure 1). As a measure of t-system density we used the mean distance of intracellular voxels to the nearest sarcolemma (ΔSL). Accordingly, high and low distances indicate low and high t-system densities, respectively. Distances of RyR cluster centroids to the nearest sarcolemma (ΔRyR) were calculated. We analyzed RyR clusters in groups proximal (0-1 μm) and distal (>1μm) to the sarcolemma. RyR cluster density (ρRyR) was computed by dividing the number of clusters by the volume of interest. Additionally, we normalized the total intensity of RyR clusters in each segmented cell and in
subcellular regions to the corresponding volumes. Dividing intensity densities of subcellular regions by the intensity density of the whole cell yielded subcellular RyR intensity distribution (I_{RyR}).

**Rapid Scanning Confocal Microscopy and Analysis of Ca\textsuperscript{2+} Sparks**

Isolated LV lateral myocytes were incubated at room temperature in modified Tyrode’s solution containing 12.5 μM of the Ca\textsuperscript{2+} sensitive dye Fluo-4 AM (Invitrogen) for 20 min followed by incubation in 6.25 μM of the membrane staining dye Di-8-Anepps (Invitrogen) for 8 min. Cells were placed in a perfusion bath system and allowed to settle on the glass slide. The chamber was perfused with the modified Tyrode’s solution containing either 2 mM or 4 mM Ca\textsuperscript{2+} and held at 37°C. Imaging was performed using a Zeiss LSM 5 Live Duo confocal microscope equipped with a 63x oil immersion objective. Only brick-shaped cells with clear striations were imaged. A diode laser emitting a wavelength of 489 nm was used to excite both dyes simultaneously. Emitted light was filtered using a dichromatic mirror and two bandpass filters of 505-610 nm and 560-675 nm for capturing Fluo-4 and Di-8-Anepps signals, respectively. Cells were conditioned with a train of at least 10 stimuli at 0.5 Hz. Image acquisition was triggered 460 ms after the final stimulus to measure Ca\textsuperscript{2+} in the diastolic phase. Sequences of 100 two-dimensional images were acquired at a size of 256×1024 pixels and a frame rate of 9.3 ms/frame. Pixel sizes were 0.1×0.1 μm. Details of the detection algorithm are provided in the Supplemental Data. Briefly, cells were segmented using histogram-based thresholding in Di-8-Anepps images (Supplemental Figure 2). After preprocessing of the Fluo-4 images (Supplemental Figures 2 and 3) sparks were detected in the cell interior using mode plus five times standard deviation of the intensity values. We measured the total number of sparks, number of sparks within 10 μm of the cell end, and the
number of sparks between 10 and 40 μm from the cell end. Spark number was normalized by temporal duration and area used for detection, which yielded spark density (sparks/μm²/s).

**Statistical Analyses**

All results are presented as mean ± standard error. All statistical analyses were performed in Matlab R2012b (The Mathworks Inc., Natick, Ma, USA). We accounted for multiple measurements from an animal by averaging of data for each animal. We used one-way analysis of variance (ANOVA) followed by Tukey’s multiple-comparison for microstructural analyses. We applied the paired t-test for functional analyses. Significance of differences was defined as p<0.05.

**Results**

**Subcellular Heterogeneity of Remodeling of RyRs and T-System in DHF**

We first analyzed RyR clusters and t-tubules in myocytes in LV lateral tissue from control animals (Figure 1). All images were preprocessed, which improved signal-to-noise ratio and resolution (Figure 1A-D versus 1E-H). Control cells exhibited a dense t-system (Figure 1E) and homogenous intracellular RyR density (Figure 1F). This was also visible in 3D reconstructions of sarcolemma and RyR clusters in a segmented cell (Figure 2A-C). RyR clusters of high intensity were found evenly distributed in all cell regions. We measured the mean intracellular distance to the sarcolemma (Figure 2D), the mean distance of RyR clusters to the sarcolemma (Figure 2E), the mean density of RyR clusters (Figure 2F) and the normalized intensity of RyR signal (Figure 2G) in regions at a distance of 0-10, 10-20, 20-30, and 30-40 μm to a cell end.
These quantitative measures indicate homogeneity of t-system and RyRs within LV lateral cells in control animals.

In contrast to control, DHF tissue from lateral LV tissue showed a pronounced loss of t-tubules near longitudinal cell ends together with striking heterogeneity in RyR density (Figure 3). RyR intensities were considerably higher near cell ends as compared to cell centers. In this overview image, most cells were affected and exhibited this heterogeneity at least at one cell end. A high-resolution 3D image stack is shown in Figure 4A-C. Cell ends almost completely lacked t-tubules and exhibited increased RyR intensities. This was confirmed when inspecting 3D reconstructions of sections through a representative DHF cell (Figure 4D-F). T-tubules were virtually absent within 10 μm from the cell end, while RyR clusters of high intensity aggregated in this region. Quantitative data corresponded to these findings. The mean intracellular distance to the sarcolemma was increased within 10 μm from the cell end versus other regions (Figure 4G). The mean distance of RyR clusters to the sarcolemma decreased slightly from the cell end to center (Figure 4H). The increase of density and intensity of RyR clusters at the cell end was remarkable (Figure 4I and J, respectively). These findings suggest substantial subcellular heterogeneity of t-tubule and RyR cluster remodeling in LV lateral DHF myocytes.

**Partial Restoration in Response to CRT**

We next investigated whether CRT was able to reduce the DHF-associated subcellular heterogeneity of remodeling. Our model of CRT is based on application of the DHF model for three weeks followed by three weeks of resynchronization by rapid biventricular pacing. The RyR and t-system density in myocytes from LV lateral tissue of CRT animals appeared more
homogeneous than in DHF (Figure 5A-C), indicating partial structural restoration. Three-dimensional reconstructions of longitudinal and transverse sections through an example cell are presented in Figure 5D-F, showing low t-system densities in close proximity to the cell end. However, large regions devoid of t-tubules as found in DHF were not observed. Similarly, RyR clusters of high intensity still appeared in higher concentration near the cell end, but to a lesser degree than in DHF. Quantitative analyses confirmed partial restoration of subcellular homogeneity in this cell (Figure 5G-J).

**Statistical Analyses of RyR and T-System Remodeling**

We applied the same analyses used for the examples to a group of control, DHF and CRT cells. Extracted features binned by increasing distance from the cell end are presented in Supplemental Figure 4. Because t-system loss and increased RyR intensities in DHF occurred primarily near cell ends, we compared t-system and RyR properties in regions 0-10 μm with regions 10-40 μm from the cell end. The mean intracellular distance to the nearest sarcolemma indicated high t-system density in control, while DHF cells exhibited a pronounced loss of t-tubules at cell ends (Figure 6A). CRT was not able to completely restore this measure to levels of control, but the intracellular distance to the nearest sarcolemma was similar at cell ends and centers. In our analysis of the mean distance of RyR clusters to the sarcolemma (Figure 6B), we found increased values in DHF and CRT versus control near cell ends (0.67±0.4 and 0.66±0.09 vs 0.47±0.03 μm, respectively). The distance was similar in cell centers of DHF, CRT and control cells (0.52±0.03, 0.52±0.06 and...
0.51±0.04 μm, respectively). DHF cells exhibited significant subcellular heterogeneity of mean RyR-sarcolemma distance.

RyR cluster densities ranged from 0.75 to 0.91 μm$^{-3}$ and our analysis did not indicate significant subcellular heterogeneity in DHF and CRT (Figure 6C). In contrast, analyses of normalized RyR intensities (Figure 6D) revealed that DHF was associated with a substantial increase at cell ends (134±10% of cellular mean) and a decrease in centers (92±2%) versus control (110±6% and 98±1%, respectively). CRT cells presented RyR distributions similar to those of DHF.

Our data show that DHF is associated with pronounced heterogeneity of remodeling of t-tubules and RyR clusters at cell centers and ends. We next sought to determine whether similar changes occur when grouping RyR clusters by their distance to the sarcolemma (Supplemental Figure 5). We therefore analyzed cluster density and intensity within 0-1 μm and at a distance ≥1 μm to the sarcolemma. Cluster densities did not differ between groups or regions (Figure 6E). However, when analyzing RyR cluster intensities (Figure 6F), DHF cells exhibited decreased intensities 0-1 μm (91±2% of cell mean) and strikingly increased intensities ≥1 μm from the sarcolemma (144±12%). A similar, but less pronounced distribution was found in CRT cells (95±1% and 124±6%, respectively). RyR cluster intensity in control cells was almost equally distributed at distances 0-1 μm and ≥1 μm from the sarcolemma (101±2% and 99±8%, respectively). In DHF and less pronounced after CRT, we also found increased RyR intensities ≥1 μm from the sarcolemma in cell centers. RyR intensities in control, however, were homogeneous throughout the cell (Supplemental Figure 6A, B). In all experimental groups, the majority of RyR clusters was found within 1 μm of the sarcolemma (Supplemental Figure 6C).
In summary, control cells were more homogeneous at the subcellular scale than DHF and CRT cells. DHF led to a significant loss of t-tubules and increased RyR intensities at cell ends versus centers, causing pronounced subcellular heterogeneity. Increased RyR intensities were found particularly in regions ≥1 μm from the sarcolemma. CRT was able to partially reverse these structural changes.

**RyR Remodeling Despite Preserved T-System in Anterior Cells in DHF**

In previous work we found that subcellular remodeling of the t-system is regional, i.e. cells isolated from the lateral LV wall of DHF animals exhibited a higher degree of t-system depletion than cells from the anterior wall. To shed light on this regional heterogeneity of subcellular remodeling, we additionally studied cells from the anterior LV wall obtained from DHF animals. An example cell is shown in Supplemental Figure 7. The mean intracellular distance to the nearest sarcolemma did not differ from control proximal and distal to the cell end (Supplemental Figure 8A). In contrast to lateral cells, DHF in anterior cells was not associated with heterogeneous t-system remodeling. However, the distribution of RyR intensity in anterior DHF cells was heterogeneous as observed in lateral cells (Supplemental Figure 8D, F). This indicates that RyR remodeling is independent of t-system remodeling in anterior cells.

**Preserved T-System and Marginal RyR Remodeling in SHF**

To investigate the role of dyssynchrony in remodeling, we analyzed lateral cells from animals in SHF induced by RA pacing. Cells from this group did not show t-system remodeling or changes in the relationship between t-system and RyRs versus control (Supplemental Figures 9 and 10).
However, we found changes in RyR intensity as observed in DHF, although to a lesser degree. RyR intensity at cell ends was higher than at cell centers in SHF.

**Subcellular Heterogeneity of Ca\(^{2+}\) Sparks**

To investigate whether subcellular heterogeneity of structural remodeling was associated with heterogeneity of Ca\(^{2+}\) release events, we acquired Fluo-4 images from isolated myocytes using rapid scanning confocal microscopy. Processing of the Fluo-4 images (Figure 7A) enabled the detection of Ca\(^{2+}\) release events during the diastolic phase of ventricular myocytes. We applied filtering, attenuation correction and pixel-wise self-ratioing on the image data to improve signal quality. Results of this processing are presented for a single pixel (Figure 7B, C) and region with a spontaneous Ca\(^{2+}\) release event (Figure 7D, E). In Figure 7F-H, we present detected sparks localized in the Di-8-Anepps images. We detected the cell orientation and the most distal point of the cell. This enabled the creation of distance maps from the cell end. Subsequently, specific regions of interest at varying distances were compared. Figures 7F, 7G, and 7H show the investigated regions and centroids of detected sparks mapped onto the Di-8-Anepps images of an exemplary control, DHF and CRT cell, respectively. Example Fluo-4 and Di-8-Anepps images of these cells are presented in Supplemental Figures 2 and 3. Supplemental Videos 1, 2 and 3 illustrate the raw and processed Fluo-4 image sequences in control, DHF and CRT cells, respectively. While spark density was homogeneously distributed in the control and CRT cell, the DHF cell exhibited a reduced spark density at the end versus center.

Using this approach we performed a statistical analysis of sparks in control, DHF and CRT cells bathed in modified Tyrode’s solution containing either 2 or 4 mM Ca\(^{2+}\) (Figure 8 and
Supplemental Figure 11). We did not find significant changes in overall spark density for cells in either 2 or 4 mM Ca$^{2+}$ (Figure 8A, B). However, cells from DHF animals exhibited a significantly lower spark density in regions 0-10 μm versus 10-40 μm from cell ends. This heterogeneity was not visible in cells from either control or CRT animals (Figure 8D, E), indicating restoration of homogeneity of sparks after CRT.

**Discussion**

Our study revealed previously unknown subcellular heterogeneity of structural and functional remodeling in ventricular myocytes in HF. Three-dimensional analysis of confocal microscopic images of tissue sections from DHF myocytes indicated that t-system density and RyR distribution is heterogeneous at the subcellular scale. Both, DHF and SHF cells showed heterogeneous RyR distributions, but only lateral DHF myocytes exhibited t-system depletion at cell ends. The t-system was not affected in anterior DHF and lateral SHF myocytes. In subsequent studies using rapid scanning confocal microscopy we found that the subcellular heterogeneity of t-system and RyR distribution is associated with heterogeneous Ca$^{2+}$ spark densities. After CRT subcellular homogeneity of structures and function was, in part, restored.

Our finding of subcellular heterogeneity of the density of RyRs in HF is novel. Our studies revealed increased density and fluorescence intensity of RyR clusters at cell ends versus cell centers in DHF and SHF. In both HF models, the intensity of RyR clusters not associated with sarcolemma was higher than proximal to sarcolemma. This effect of HF was not restricted to cell ends (Supplemental Figure 6A), but also occurred in cell centers (Supplemental Figure 6B). In contrast, in control cells the intensity of RyR clusters was not affected by the distance to
sarclemma. This suggests that differences in optical properties or immunostaining do not underlie heterogeneous RyR intensities found in DHF cells. Instead, our study indicates that in HF cells the number of RyR channels is higher in non-junctional clusters than in couplons. Accordingly, RyR distribution along the longitudinal cell axis was especially heterogeneous in lateral DHF cells. Cell ends, where t-system density was low, presented a higher percentage of non-junctional RyR clusters. Since these clusters exhibited increased intensity, the overall RyR intensity at cell ends was increased. In agreement with this finding, SHF and anterior DHF cells exhibited an increase of RyR intensity ≥1 μm from the sarclemma although the t-system was normal (Supplemental Figures 8 and 10). This suggests that RyR remodeling is associated with rapid pacing underlying our HF models. However, subcellular heterogeneity of RyR signal intensity was higher in DHF than in SHF indicating that ventricular dyssynchrony exacerbates the remodeling.

Previously, significant reduction of RyR mRNA and protein expression has been reported in LV myocytes from DHF and CRT animals. The reduction was found in both anterior and lateral cells. Our analysis of RyR associated fluorescence revealed a ≈40% increase of RyR expression at ends versus centers of lateral DHF cells (134±10% vs 92±2%) (Figure 6D). Assuming an overall decrease of RyR protein expression in DHF lateral cells by ≈20%, this would translate into an increase of RyR expression to 107% at cell ends and a decrease to 74% at cell centers versus control. Similarly, RyR reduction in DHF cells can be explained by reduction of junctional RyRs (Figure 6F). Our data suggest that RyR protein expression is reduced to 73% within 0-1 μm and increased to 115% in regions ≥1 μm from the sarclemma. This reduced RyR expression within 0-1 μm from the sarclemma may reflect decreased junction size, which was
suggested to underlie delayed stochastic coulon activation and reduced amplitude of Ca^{2+} transients.34

It is well established that t-system depletion is a feature of HF. Our previous study on isolated ventricular myocytes suggested regional heterogeneity of t-system depletion. In particular this study indicated that t-system depletion is pronounced in the lateral LV region in DHF, but we did not investigate subcellular heterogeneity of t-system depletion. However, it has been observed in isolated cells that t-system depletion in tachypacing induced HF occurs preferentially at cell ends.23, 24 In agreement with those studies, remodeling of t-system in our DHF model appears to be regional and restricted to ends of myocytes. These findings indicate that ventricular dyssynchrony is a major contributor of this type of regional subcellular remodeling.

Previously, we found reduced amplitudes and slowed decays of Ca^{2+} transients in DHF myocytes and restoration after CRT to levels similar as in control.10, 11 In this study, we provide insights into structural subcellular heterogeneity in DHF, which may underlie these alterations. According to our recent model of Ca^{2+} release35 the detubulation and increase in non-junctional RyRs at cell ends would cause heterogeneous Ca^{2+} transients in DHF cells, in particular delayed onset times at ends versus centers of cells. This hypothesis is supported by a preliminary analysis of a DHF cell (Supplemental Data). The analysis also suggests reduced amplitude of the Ca^{2+} transients at cell ends as a further effect of heterogeneous microstructural remodeling in DHF.

Since diastolic Ca^{2+} sparks have been linked to arrhythmogenesis,36 we investigated whether diastolic sparks are affected by the structural heterogeneity in DHF cells. Spark densities in
control and CRT cells were homogeneous, but in DHF spark density was lower at cell ends than in cell centers. This agrees with a previous study in which regions void of t-tubules lacked sparks in normal and failing canine myocytes. Possible causes of subcellular heterogeneity of spark density include heterogeneous SR Ca\(^{2+}\) load and different spark probabilities of junctional and non-junctional RyR clusters. Interestingly, increased RyR density at cell ends was not associated with increased spark density. Instead spark density was increased in regions with intact t-system and, thus, high ratio of junctional to non-junctional RyRs. A potential explanation for increased spark probability in couplons is that spontaneous Ca\(^{2+}\) releases from junctional RyRs into the dyadic cleft have a higher probability of triggering neighboring RyRs than Ca\(^{2+}\) release from non-junctional RyRs into the cytosol. Overall spark density was modulated by bath Ca\(^{2+}\) concentration, which can be explained by a positive relationship between bath Ca\(^{2+}\) concentration, SR Ca\(^{2+}\) load and spontaneous spark probability. However, our study did not indicate significant differences of overall spark density in control, DHF and CRT cells. This suggests that increased spark density in centers of DHF cells is counterbalanced by reduced spark density at cell ends.

While our studies revealed striking subcellular remodeling and restoration, the underlying mechanisms remain unclear. A potential mechanism is related to heterogeneity of mechanical properties of cardiac myocytes and their strain profiles during a heartbeat. We previously proposed that changes in strain profiles directly affect t-system structure and maintenance. Here, we extend this hypothesis by suggesting that subcellular heterogeneity of strain profiles in myocytes lead to subcellular heterogeneous remodeling of t-system and RyRs. Based on principles of mechanics, for instance, Hooke’s law, we expect larger stretch in those regions of
strained myocytes, which have a smaller cross-sectional area. Indeed most cells taper towards their ends and thus have a smaller cross-sectional area at the end (e.g. Figure 2). In the cell population of our study, cross-sectional areas within 10 μm from the cell end were reduced to 62.8±1.2% of central cross-sectional areas (within 10-40 μm from the cell end). An alternative explanation of subcellular heterogeneity of strain profiles is subcellular heterogeneity of myocyte stiffness. Hooke’s law predicts larger stretch in those regions of strained myocytes, which have a smaller stiffness.

It is well established that DHF is associated with contraction in anterior and septal sites, but reciprocal stretch in lateral sites during early systole.10, 32 Opposite strains occur during late systole. Stretch during early systole in lateral sites is significantly larger than at end diastole. Based on our hypothesis we expect that lateral left ventricular myocytes are heterogeneously stretched during early systole, with pronounced stretch at the tapered cell ends that is larger than in the cell center. The pronounced stretch at the cell ends could directly destabilize t-system or interfere with its maintenance.

A similar argument based on mechanical principles can be made to suggest potential mechanisms for up-regulation of RyRs at cell ends in SHF and DHF in the context of a general RyR down-regulation in ventricular myocardium. Both, SHF and DHF are associated with increased left ventricular end diastolic blood pressure.11 Based on our new hypothesis we expect that ventricular myocytes in SHF and DHF are heterogeneously stretched during diastole, with larger stretch at the tapered cell ends than in the cell center. We speculate that the increased
stretch at cell ends provides a signal for local up-regulation of RyR expression. Alternatively, the locally increased stretch might interfere with protein degradation.

**Limitations**

Limitations of our animal models have been explained previously. Further limitations related to confocal microscopy, image analysis and tissue preparation are summarized in the Supplemental Data.

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**Disclosures**

None.
References


Figure Legends

Figure 1. Preprocessing of confocal microscopic images. XY sections at a depth of 27 μm from a 3D image stack (102.4x102.4x35μm) of control tissue. (A-D) Unprocessed data. (E-H) Preprocessed data. Green: RyRs, Blue: WGA. White squares: zoom-in regions shown in (C,D) and (G, H). Scale bars: 20 μm.

Figure 2. Visualization and analysis of control cell. (A-C) 3D visualizations of 4 μm thick sections through example control cell. (A) Longitudinal section, transverse section (B) 5 μm from cell end and (C) 40 μm from cell end. Red bars indicate section planes. Blue: sarcolemma. Green and red spheres: RyR clusters. Clusters of high intensity (90th percentile) are shown in red. Scale bars: 10 μm. Scale bar in (B) applies to (C). Analysis of (D) mean intracellular voxel distance to the nearest sarcolemma, (E) mean RyR cluster distance to nearest sarcolemma, (F) RyR cluster density and (G) RyR intensity per volume normalized to mean value of the whole cell. These parameters were analyzed at distances 0-40 μm from the left cell end in bins of 10 μm.

Figure 3. Confocal microscopic image of DHF tissue from lateral LV wall. (A) T-system density decreases at cell ends. (B) RyR clusters exhibit increased intensities near cell ends. Scale bar: 40 μm.

Figure 4. Visualization and analysis of DHF cell. (A-C) Confocal microscopic images of extracellular space (blue) and RyR clusters (green) in DHF tissue. (B) and (C) are zoom-ins from the region indicated by the white rectangle. Note the increase in RyR intensity and t-system
depletion near cell ends. (D-F) 3D visualizations of 4 μm thick sections through example DHF cell. (D) Longitudinal section, (E) transverse section 5 μm from cell end and (F) transverse section 40 μm from cell end. Red bars indicate section planes. Blue: sarcolemma. Green and red spheres: RyR clusters. Clusters of high intensity (90th percentile) are shown in red. All scale bars: 10 μm. Scale bar in (B) applies to (C). Scale bar in (E) applies to (F). Analysis of (G) mean intracellular voxel distance to the nearest sarcolemma, (H) mean RyR cluster distance to nearest sarcolemma, (I) RyR cluster density and (J) RyR intensity per volume normalized to mean value of the whole cell.

Figure 5. Visualization and analysis of CRT cell. (A-C) Confocal microscopic images of the extracellular space (blue) and RyR clusters (green) in CRT tissue. (B) and (C) are zoom-ins from the region indicated by the white rectangle. RyR and t-system remodeling are less pronounced than in DHF. (D-F) 3D visualizations of 4 μm thick sections through an example CRT cell. (D) Longitudinal section, (E) transverse section 5 μm from cell end and (F) transverse section 40 μm from cell end. Red bars indicate section planes. Blue: sarcolemma. Green and red spheres: RyR clusters. Clusters of high intensity (90th percentile) are shown in red. All scale bars: 10 μm. Scale bar in (B) applies to (C). Scale bar in (E) applies to (F). Analysis of (G) mean intracellular voxel distance to the nearest sarcolemma, (H) mean RyR cluster distance to nearest sarcolemma, (I) RyR cluster density and (J) RyR intensity per volume normalized to mean value of the whole cell.

Figure 6. Statistical analysis of myocytes from control (CTRL), DHF and CRT animals. Error bars indicate standard errors of the mean. Parameters were analyzed 0-10 μm and 10-40 μm from
the cell end. (A) Mean intracellular voxel distance to nearest sarcolemma. (B) Mean RyR cluster
distance to nearest sarcolemma, (C) RyR cluster density and (D) RyR intensity per volume
normalized to mean intensity of the whole cell. Parameters were analyzed 0-1 μm and ≥1 μm
from the sarcolemma. (E) RyR cluster density and (F) RyR intensity per volume normalized to
mean value of the whole cell. Brackets mark statistical significance between experimental
groups, asterisks between corresponding bins of groups.

Figure 7. Analysis of subcellular heterogeneity of diastolic Ca\(^{2+}\) sparks using rapid scanning
confocal microscopy. (A) Representative Fluo-4 images from control cell with a spark marked
by the red box. (B) Trace of Fluo-4 signal over time for pixel located at center of red box marked
in A. (C) Trace of processed Fluo-4 signal in B. (D) Image of Fluo-4 signal in region outlined by
red box in A. (E) Processed image of region in E. (F-H) Di-8-Anepps images overlaid with red
circles centered on all detected sparks. (F) Control cell with a spark density of 0.020
sparks/s/μm\(^2\) near the end and 0.0253 sparks/s/μm\(^2\) in the center of the cell. (G) DHF cell with a
spark density of 0.0227 sparks/s/μm\(^2\) near end and 0.0418 sparks/s/μm\(^2\) in the center of the cell.
(H) CRT cell with spark density 0.0166 sparks/s/μm\(^2\) near the end and 0.0167 sparks/s/μm\(^2\) in
the center of the cell.

Figure 8. Statistical analyses of diastolic spark density. Overall spark density in control (CTRL),
DHF and CRT cells in a bathing solution containing (A) 2 mM and (B) 4 mM Ca\(^{2+}\) was similar.
Analysis of spark density with regions less than 10 μm and between 10 and 40 μm from the cell
end in a bathing solution containing (C) 2 mM and (D) 4 mM Ca\(^{2+}\) revealed subcellular
heterogeneity in DHF cells. Asterisks mark statistical significance between corresponding bins of groups.
CTRL
Animals/cells: 8/13
DHF
Animals/cells: 7/9
CRT
Animals/cells: 3/7

CTRL
Animals/cells: 6/18
DHF
Animals/cells: 5/8
CRT
Animals/cells: 4/9
Cardiac Resynchronization Therapy Reduces Subcellular Heterogeneity of Ryanodine Receptors, T-Tubules and Ca\(^{2+}\) Sparks Produced by Dyssynchronous Heart Failure
Hui Li, Justin G. Lichter, Thomas Seidel, Gordon F. Tomaselli, John H. B. Bridge and Frank B. Sachse

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Supplemental Material

Supplemental Methods

Monitoring of Animal Models

After device implantation weekly ECGs were obtained to ensure correct lead placement and capture of the pacing. Only animals with increased QRS duration after DHF and decreased QRS duration after CRT were used for our studies (Supplemental Table I).

Tissue Collection and Cell Isolation

Animals were anesthetized by propofol injection and isoflurane inhalation. Hearts were excised quickly and perfused retrogradely through the aorta for 10 min with Ca\(^{2+}\) free modified Tyrode’s solution comprising (in mM) NaCl 92, KCl 4.4, MgCl\(_2\) 5, NaH\(_2\)PO\(_4\) 5, D-glucose 11, HEPES 24, NaOH 12.5, Taurine 20, Creatine 5, Na Pyruvate 1. Tissue samples were collected from the subepicardial anterior and lateral LV free wall. Tissue samples were flash-frozen in Tissue-Tek Cryo-Oct compound (ThermoFisher Scientific, Waltham, MA, USA). Cardiac cells were isolated by perfusion of Ca\(^{2+}\) free modified Tyrode’s solution containing Collagenase P (0.1-0.2 mg/ml, Roche Diagnostics, Indianapolis, IN, USA) and Protease Type XIV (0.06 mg/ml, Sigma-Aldrich, St. Louis, MO, USA). Once an acceptable degree of tissue dissociation was reached digestion was stopped by wash out with the modified Tyrode’s solution containing 50 µM Ca\(^{2+}\). Tissue from anterior and lateral LV were collected separately and manually chopped into small pieces. These samples were then gently shaken in a beaker at 37°C for 10 min. To remove undigested tissue chunks, cell solutions were filtered through a strainer. After 10-20 min the Ca\(^{2+}\) concentration was increased from 50 µM to 1 mM in 4 steps over 24 min.

Tissue Sectioning and Labeling

Tissue samples were sectioned into 80 µm slices using a cryostat (Leica Biosystems, CM1950, Buffalo Grove, IL, USA). The slices were fixed using 2% paraformaldehyde in phosphate buffered saline solution (PBS) for 30 min and then rinsed with PBS twice. Sarcolemma, t-tubules and the interstitial space were labeled by incubation of the tissue with wheat germ agglutinin (WGA) conjugated to Alexa Fluor 555 (Invitrogen, Carlsbad, CA, USA) at 30 µg/ml at room temperature overnight. The tissue sections were then rinsed 3 times with PBS and permeabilized with 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 2 h. Signal enhancer (Invitrogen) was applied to the tissue sections for 30 min. Tissue slices were incubated with blocking solution (0.05% Triton X-100, 4% goat serum in PBS) for 1 h to reduce unspecific labeling. RyRs were labeled with a monoclonal antibody (1:100, MA3-916, ThermoFisher Scientific, Waltham, MA, USA) in incubation solution (2% BSA, 2% goat serum, 0.05% TritonX-100 in PBS) overnight followed by incubation of a secondary antibody attached to Alexa Fluor 488 (1:100, A-11001, Invitrogen) in blocking solution overnight. Tissue sections were rinsed with PBS 3 times after each labeling procedure.

Isolated Cell Labeling

Ventricular myocytes were isolated, labeled with WGA and fixed as previously described.\(^1\) The cells were permeabilized by application of 0.3% Triton-X in PBS for 20 min. After washing with PBS, signal enhancer (Invitrogen) was applied for 30 min. After washing with PBS, blocking was performed by incubation for 1 h in PBS containing 10% goat serum. Cells were then incubated with a primary antibody against NCX (1:100, MA3-926, ThermoFisher Scientific) diluted in incubation solution (2% BSA, 2% goat serum, 0.05% TritonX-100 in PBS) overnight at 4°C.
Cells were washed with PBS and then incubated with a secondary antibody attached to Alexa Fluor 633 (1:100, A-21046, Invitrogen). Cells were washed with PBS and then imaged in the same manner as tissue sections. A washing step consisted of removal of supernatant, application of PBS and allowing cells to settle for 15 min. Washing steps were repeated at least 3 times.

**Analysis of T-System and RyR Clusters in 3D Image Stacks**

Processing of the WGA and RyR image stacks involved noise reduction, deconvolution and background adjustment. Noise was reduced in WGA and RyR image stacks by applying a Gaussian (5x5x5 voxels, $\sigma = \sigma_y = \sigma_z = 0.1$ µm) and mean filter (6 neighbors), respectively. Subsequently, images were deconvolved with the Richardson-Lucy algorithm using measured point spread functions as described previously.$^1,^2$ Finally, the mode in the histogram of image intensities was identified as background intensity and adjusted to 256.

After this processing, the interstitial space was segmented from the WGA image stacks by applying a threshold of mode plus one standard deviation. Single, non-connected voxels were considered as noise and removed. RyR clusters were segmented in the RyR image stacks using a threshold of mode plus four standard deviations. The segmented WGA images served as mask for semi-automatic segmentation of individual cells based on a recently published method.$^3$ In short, seeds for a morphological watershed were iteratively created on the distance map of the segmented WGA images followed by manual merging of intracellular segments.

Only cells with a long axis angle smaller than $20^\circ$ to the xy plane were selected for analyses. Cells without visible longitudinal end or a visible length of < 40 µm as well as cells showing signs of contracture were excluded. Segmented cells were used as masks to analyze RyR clusters and sarcolemma (including t-tubules). A bounding box was calculated for each cell to determine the distance from the longitudinal cell end of each voxel. Cells were divided into two regions: 0-10 µm and 10-40 µm from the cell end. Assuming a minimum myocyte length of ≈80 µm, regions further than 40 µm from the end were not included in the analyses because most cells were not captured completely due to limited image size (102.4 µm × 102.4 µm). To account for depth-dependent attenuation in confocal microscopy, analysis was restricted to the first 12 µm from the tissue surface.

Analysis of each segmented RyR cluster yielded sum of intensity and intensity-weighted centroid position. To determine the nearest sarcolemma distance we used distance maps (Supplemental Figure 1). RyR clusters with intensities higher than 90% of all clusters in the same cell (90th percentile) were defined as high-intensity clusters. RyR cluster density was calculated by dividing the number of clusters by the volume of interest, i.e. 0-10 µm and 10-40 µm from the cell end or 0-1 µm and >1 µm from the nearest sarcolemma. Total RyR intensity in these regions was calculated by summing up the intensity of segmented clusters. As a measure of t-system density, we calculated the mean distance of intracellular voxels to the nearest sarcolemma. Accordingly, high and low distances indicate low and high t-system densities, respectively.

For 3D visualizations of cells, we used ParaView (Kitware, Clifton Park, NY, USA). RyR clusters were displayed as spheres.

We performed analyses to investigate differences of the two different DHF model used in this study. Comparison of cells from animal models based on left bundle branch ablation or RV pacing did not reveal differences of t-system, the t-system-RyR relationship and the normalized
RyR intensity at cell ends/centers (Supplemental Figure XII). For subsequent structural analyses of DHF cells, we pooled data from the two DHF models.

**Analysis of Ca²⁺ Spark Density in 2D Image Sequences**

The cell membrane was detected using the Di-8-Anepps images (Supplemental Figure IIA). The images were filtered using a Gaussian mask (10 x 10 pixels, \(\sigma = 0.2 \mu m\)). The resulting image was segmented using a threshold of mode plus twice the standard deviation of the image intensities. A morphological closing operator was used to fill holes in the image and obtain a mask of the entire cell (Supplemental Figure IIC). These cell masks were then visually inspected for each cell and adjusted manually for images with poor quality. For subsequent processing, mode and standard deviation were calculated only for the regions contained by this cell mask.

We created distance maps to the cell end by manually selecting the most distal point of the cell. Cell orientation was automatically detected and adjusted when necessary. A line through this point and perpendicular to cell orientation was used as the zero distance marker. The distance of each pixel to this line was then calculated. The distance mask was used for the subsequently described analysis of subcellular heterogeneity of spark density.

Sparks were detected after preprocessing of the Fluo-4 images (Supplemental Figure IIB). Preprocessing of these images included noise removal and bleaching correction. Low-frequency noise was removed by filtering in the frequency domain. High-frequency noise in the images was reduced by filtering with a discretized Gaussian function (25 x 25 pixels, \(\sigma = 0.5 \mu m\)). Bleaching correction was performed by fitting a mono-exponential function to the mode of each frame of the image sequences. The decay was inverted and used for correction of each frame (Supplemental Figure IID). Image sequences were then self-ratioed using a moving window minimum in which each pixel was divided by its minimum value from the 5 previous to the 5 following frames. Potential sparks were segmented in normalized images using a threshold of mode plus five times the standard deviation of the image. These potential regions were then filtered through a set of criteria including: spanning less than 5 frames (42 ms), having circularity greater than 0.5 but less than 2, a minimum area of 79 pixels (~0.5 \(\mu m\) radius), and a maximum intensity greater than the mean intensity of that frame in the non-normalized image. Circularity was defined as:

\[
\text{Circularity} = \frac{p^2}{4\pi A}
\]

with the perimeter \(P\) and the area \(A\). These criteria were applied to the segmentation of the potential spark found in the frame where the maximum intensity of that potential spark occurred. Additionally, all images were inspected for motion artifacts, abnormal cell morphology or cell debris. If necessary, regions and frames of interest were manually selected in the image sequences. Spark detection for these cells was restricted to designated regions of interest.

**An Example of the Analysis of Ca²⁺ Transients in 2D Image Sequences**

To shed light on potential effects of subcellular micro-structural heterogeneity on EC coupling we performed an exemplary analysis on Ca²⁺ transients in two regions, end and center, of a left lateral myocyte from a DHF animal (Supplemental Figure XIII A). Our approach is based on methods previously introduced by us⁴ and detailed in section Methods. Image acquisition at a frame rate of 4.6 ms/frame was triggered at 35.4 ms before electrical stimulation of the cell. Two-dimensional Ca²⁺ transients at different time points are presented in Supplemental Figure XIII B. After stimulation the cell exhibited a heterogeneous distribution of signal intensities with
higher intensities in the central region. The measurement of averaged raw signals in the end and central region revealed a delayed upstroke and reduced amplitude of the Ca\textsuperscript{2+} signal at the cell end versus center (Supplemental Figure XIII C). We applied filtering and attenuation correction to improve image quality. We averaged and self-ratioed signals from the end and central region (Supplemental Figure XIII D), which confirmed our findings in the raw signals.

**Limitations**

We discussed limitations of confocal microscopy for studies of RyR cluster and t-system previously.\cite{1, 2} A limitation of our imaging approach is that RyR cluster sizes are below the resolution limit of a conventional confocal microscope.\cite{5-7} Segmented RyR clusters therefore appear elongated and larger than their actual size. Thus, an increase in RyR cluster density, i.e. decreased distances between clusters, may have caused detection of fewer, but larger clusters. However, the presented sum of RyR intensity is not affected by this limitation. Our results show that high RyR intensity correlated with high RyR cluster density suggesting only a negligible effect of apparent cluster merging.

We used mounted tissue sections for 3D imaging and image analyses. While this preparation might avoid issues with detubulation that can occur during cell isolation, there are several limitations. The mounting medium to embed tissue sections is based on glycerol, polyvinyl alcohol and water. Evaporation of water increases the refractive index leading to improved image quality. The volume loss may lead to shrinkage of the tissue and compression of tissue between the glass slides. However, average distances of RyRs to the nearest sarcolemma were similar as in our previous study with isolated cells embedded in aqueous solutions.\cite{1} Our imaging and analysis approach did not account for nuclei in cardiac myocytes. In most cells the nucleus membrane was weakly labeled by WGA and visible, for instance, in Figure II A. In cell centers, distances to the sarcolemma might be increased due to the presence of nuclei, which are devoid of t-tubules. The presence of nuclei together with the absence of transverse cell membrane might explain the marginal increase in sarcolemmal distance in the center of control cells (Figure VI). Nevertheless, all experimental groups were similarly affected by this issue and we do not expect that it impairs the ability of the developed approach to detect differences of subcellular remodeling between the experimental groups.

A potential limitation is related to our approach for labeling of the sarcolemma. While WGA conjugated fluorescent signals exhibited high colocalization with extracellular fluorescent signals in the t-system of control rabbit myocyte,\cite{2} a previous study suggested deficiencies of WGA labeling for some components of the t-system in human myocytes explained by heterogeneous glycosylation within the t-system.\cite{3} We used a dual labeling approach to investigate heterogeneity of glycosylation in our preparation and, in particular, if this heterogeneity explains the subcellular heterogeneity of t-system in our DHF model (Supplemental Figure XIV). Our investigation revealed a high degree of colocalization of WGA and NCX. We were not able to identify significant components of the t-system that are labeled by NCX only. Thus, in our preparations, WGA appears to be a reliable marker of t-system.
<table>
<thead>
<tr>
<th></th>
<th>QRS Duration (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>44.67 ± 2.32</td>
</tr>
<tr>
<td>SHF</td>
<td>36.67 ± 1.23</td>
</tr>
<tr>
<td>DHF</td>
<td>81.78 ± 0.97 *</td>
</tr>
<tr>
<td>CRT-3 weeks</td>
<td>83.50 ± 2.97 *</td>
</tr>
<tr>
<td>CRT-6 weeks</td>
<td>44.00 ± 3.21</td>
</tr>
</tbody>
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**Supplemental Table I.** QRS durations measured in control, SHF, DHF and CRT models. Measurements for the CRT animals were performed after 3 weeks of RV pacing (CRT-3 weeks) and after additional 3 weeks of resynchronization (CRT-6 weeks). Asterisks marks significance versus control.
Supplemental Figure I. Processing of 3D image stacks from ventricular myocardium labeled with WGA and for RyRs. Image stacks were segmented using thresholds of mode+1 standard deviation and mode+4 standard deviation, respectively. Filtering removed the t-system from the WGA image allowing watershed-based segmentation of cells in the tissue. Segmented cells were extracted and used as masks for RyR analyses. Distance maps from the WGA images and connected component analysis of RyRs yielded their distances to cell end and sarcolemma as well as sum of RyR intensity.
Supplemental Figure II.
Processing of images from example control cell. (A) Raw Di-8-Anepps signal. (B) Raw Fluo-4 signal. (C) Automatically segmented cell. (D) Attenuation correction of Fluo-4 signals in each image. After application of a Gaussian filter (blue), a mono-exponential function was fitted to the mode of fluorescence intensity over time (black). The fit was used to correct for time-dependent attenuation (red).
Supplemental Figure III. Example (A, C) Di-8-Anepps and (B, D) Fluo-4 images from a (A, B) DHF and (C, D) CRT cell.
Supplemental Figure IV. Distribution along the cell long axis of (A) intracellular voxel distance to nearest sarcolemma, (B) RyR cluster distance to nearest sarcolemma, and (C) RyR intensity per volume normalized to mean value of the whole cell. Parameters were grouped in bins of 2.5 µm and with increasing distance from the cell end. Means and standard errors were calculated from all analyzed cells.
Supplemental Figure V. Distribution of (A) RyR cluster density, (B) RyR intensity, and (C) RyR intensity per volume normalized to mean value of the whole cell. Parameters were grouped with increasing distance to the sarcolemma. Means and standard errors were calculated from all analyzed cells.
Supplemental Figure VI. Statistical analysis of intensity and occurrence of RyR clusters in lateral LV cardiomyocytes from CTRL, DHF and CRT animals. Fluorescence intensity of RyRs 0-1 and ≥1 μm from the sarcolemma, normalized to volume and mean cell intensity within (A) 0-10 μm and (B) 10-40 μm from cell end. (C) Percentage of RyR clusters detected >1 μm from the sarcolemma, which was significantly lower than within 1 μm from the sarcolemma in all animal models. The percentage did not differ between our animal models, which suggests together with the data presented in (A) and (B) that in DHF cells non-junctional RyR clusters were larger than junctional clusters. Brackets indicate statistical significance between groups, asterisks between bins of one group.
Supplemental Figure VII. Visualization and analysis of DHF cell from the anterior LV wall. (A-C) Confocal microscopic images from DHF anterior tissue showing the extracellular space (blue) and RyR clusters (green). (B) and (C) are zoom-ins from the region indicated by the white rectangle in (A). T-system is intact, but RyR intensity increases at cell ends. (D-F) 3D visualizations of 4 µm thick sections through an example cell. (D) Longitudinal section. (E) Transverse section 5 µm from cell end. (F) Transverse section 40 µm from cell end. Red bars in (D) indicate section planes. RyR clusters of high intensity (90th percentile) are shown in red, others in green. All scale bars: 10 µm. Scale bar in (B) applies to (C), scale bar in (E) applies to (F). Analysis of (G) mean intracellular voxel distance to the nearest sarcolemma, (H) mean RyR cluster distance to nearest sarcolemma, (I) RyR cluster density, and (J) RyR intensity per volume normalized to mean value of the whole cell. These parameters were analyzed within 0-40 µm from the left cell end and grouped in bins of 10 µm.
Supplemental Figure VIII. Statistical analysis of myocytes from LV lateral tissue of CTRL animals as well as LV lateral tissue (DHF-LAT) and LV anterior tissue (DHF-ANT) of DHF animals. Parameters were analyzed 0-10 µm and 10-40 µm from the cell end. (A) Intracellular voxel distance to nearest sarcolemma. (B) RyR cluster distance to nearest sarcolemma, (C) RyR cluster density, (D) RyR intensity per volume normalized to mean value of the whole cell. Parameters were analyzed 0-1 µm and ≥1 µm from the sarcolemma. (E) RyR cluster density, (F) RyR intensity per volume normalized to mean value of the whole cell. Brackets indicate statistical significance (p<0.05) between groups, asterisks between bins of one group.
Supplemental Figure IX. Visualization and analysis of SHF cell. (A-C) Confocal microscopic images of SHF tissue showing the extracellular space (blue) and RyR clusters (green). (B) and (C) are zoom-ins from the region indicated by the white rectangle in (A). T-system is intact and RyRs are equally distributed. (D-F) 3D visualizations of 4 µm thick sections through an example cell. (D) Longitudinal section. (E) Transverse section 5 µm from cell end. (F) Transverse section 40 µm from cell end. Red bars indicate section planes. RyR clusters of high intensity (90th percentile) are shown in red, others in green. All scale bars: 10 µm. Scale bar in (B) applies to (C), scale bar in (E) applies to (F). Analysis of (G) mean intracellular voxel distance to the nearest sarcolemma, (H) mean RyR cluster distance to nearest sarcolemma, (I) RyR cluster density, and (J) RyR intensity per volume normalized to mean value of the whole cell. These parameters were analyzed within 0-40 µm from the left cell end and grouped in bins of 10 µm.
Supplemental Figure X. Statistical analysis of cardiomyocytes from CTRL and SHF animals. Parameters were analyzed 0-10 µm and 10-40 µm from the cell end. (A) Intracellular voxel distance to nearest sarcolemma. (B) RyR cluster distance to nearest sarcolemma, (C) RyR cluster density, (D) RyR intensity per volume normalized to mean value of the whole cell. Parameters were analyzed 0-1 µm and >1 µm from the sarcolemma. (E) RyR cluster density, (F) RyR intensity per volume normalized to mean value of the whole cell. Brackets indicate statistical significance between groups, asterisks between bins of one group.
Supplemental Figure XI. Statistical analyses of diastolic spark density. Overall spark density in control (CTRL), DHF and CRT cells in a bathing solution containing (A) 2 mM and (B) 4 mM Ca$^{2+}$ was similar. Analysis of spark density with regions less than 10 µm and between 10 and 40 µm from the cell end in a bathing solution containing (C) 2 mM and (D) 4 mM Ca$^{2+}$ revealed subcellular heterogeneity in DHF cells. Asterisks mark statistical significance (p<0.05) between corresponding bins of groups. Box bars present the median, 25% percentile and 75% percentile of samples. Whiskers mark the minimum and maximum of samples.
Supplemental Figure XII. Statistical analysis of cardiomyocytes from animals in DHF induced either by right-ventricular pacing (RVP) or left bundle branch ablation (LBBA). Parameters were analyzed at 0-10 µm and 10-40 µm from cell end. (A) Mean intracellular voxel distance to nearest sarcolemma. (B) RyR cluster distance to nearest sarcolemma, (C) RyR cluster density, (D) RyR intensity per volume normalized to mean value of the whole cell. Parameters were analyzed 0-1 µm and ≥1 µm from the sarcolemma. (E) RyR cluster density, (F) RyR intensity per volume normalized to mean value of the whole cell. Brackets indicate statistical significance between groups, asterisks between bins of one group.
Supplemental Figure XIII. Imaging and analysis of dual-labeled isolated lateral DHF myocyte. (A) Di-8-ANEPPS labeling reveals the sarcolemma and t-system. (B) Labeling with fluo-4 produced low intensity signals in cells at rest and heterogeneously increased intensity after stimulation at 35.4 ms. (C) Fluo-4 signals at the sites marked with box in (A). The box adjacent to the cell end and in the cell center has a width of 10 and 30 µm, respectively. (D) The signals after filtering and self-ratioing with the marked region. Scale bar in (A) has a length of 10 µm and also applies to (B).
Supplemental Figure XIV. 2D confocal image of isolated lateral DHF myocyte. (A) Stained with WGA. (B) Stained for NCX. (C) Overlay. (D, E) Zoomed in region marked in (C). Scale bar in (A) has a length of 10 µm and also applies to (B) and (C). Scale bar in (D) has a length of 5 µm and also applies to (E).
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4. Torres NS, Sachse FB, Izu LT, Goldhaber JI, Spitzer KW, Bridge JH. A modified local control model for ca2+ transients in cardiomyocytes: Junctional flux is accompanied by release from adjacent non-junctional ryrs. *J Mol Cell Cardiol*. 2014;68:1-11


Legends for Video Files

Video 1. Raw and processed image sequences from confocal microscopy of CNTRL ventricular myocyte.

Video 2. Raw and processed image sequences from confocal microscopy of DHF ventricular myocyte.

Video 3. Raw and processed image sequences from confocal microscopy of CRT ventricular myocyte.