

Mechanical load regulates transverse-tubule structure and function in left ventricular cardiomyocytes

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Abstract

Chronic mechanical load variation triggers a wide range of responses in the heart, a part of which includes cellular remodelling. Over the past 15 years, evidence has amassed that a part of this remodelling process involves changes to a sophisticated structure in the cell membrane, called the transverse (t)-tubule system. The t-tubules are a series of regular membrane invaginations, which contain a high density of ion channels responsible for local Ca^{2+} induced Ca^{2+} release (CICR).

This thesis addresses the question of whether the t-tubule system can be said to be specifically load sensitive, the nature of that load sensitivity and its molecular regulators. Using surgical models, the influence of mechanical load variation of different durations, degrees and settings are studied. Local CICR and t-tubule structure are investigated.

First, it was found that prolonged mechanical unloading induces subtle changes to the t-tubule system, which functionally uncouples the Ryanodine receptors (RyR) and L-type Ca^{2+} channels (LTCC) and induces a loss of whole cell Ca^{2+} release synchrony.

Second, heart failure was found to be associated with loss of t-tubule structure and Ca^{2+} handling abnormalities. Following mechanical unloading, the t-tubule system recovered with enhanced LTCC-RyR uncoupling, resulting in improved Ca^{2+} handling.

Third, the t-tubules were found to be unchanged initially during graded mechanical load variation. Prolonged myocardial unloading or overloading impaired t-tubule structure, with loss of normal CICR.

Telethonin (Tcap), a member of the cardiomyocyte stretch sensing complex, is a candidate regulator of the t-tubules. In a Tcap knock-out (KO), cardiomyocytes show a primary t-tubule defect, which becomes more pronounced following mechanical overload.

These results support the notion that the t-tubule system is dynamically regulated by mechanical overload and unloading, via a molecular pathway including Tcap.

Declaration: This thesis represents my own work. Where I have used material from other investigators, this is clearly stated. This thesis has not been submitted for a degree to any other academic institution.

Michael Ibrahim, Harefield, May 2012.

The work in this thesis has resulted in a number of publications.

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*To Angela, Ashraf, my family, the distinguished surgeons who continue to mentor me
and, above all, the great human heart.*

“CASUALLY CONSIDERED, the heart is merely a pump.

In fact, it is a sophisticated organ. Comprised of four valves, two holding chambers, two pumping chambers, a spiderweb of vessels to nourish it, and an intricate network of nerves to control it, the heart must harmoniously propel blood through approximately sixty thousand miles of arteries, capillaries, and veins – two thousand gallons of blood through the average-size person each and every day.

It does not pump autonomously. The heart must properly respond to sometimes severe, sometimes subtle changes in temperature, exertion, emotion, and stress. It should last a normal lifetime: some two and a half billion beats over the course of seventy-five years. Evolution has confirmed the heart’s importance by protecting it with bone. The brain and bone marrow, foundation of the immune system, are among the few other parts of the body so honoured.

Congenital disease can warp the heart with great variety. Valves can be sealed tight, missing parts – or absent altogether. Major vessels can be misplaced, narrowed, or blocked completely. A chamber can be too small or missing, a wall too thick or thin. The heart’s electrical system – its nerves – may go haywire. The muscle can be weak. Holes may occur almost anywhere, in almost any size. Studying heart pathology, one is reminded that the genetic symphony that produces a normal baby is indeed a wondrous and delicate one”

-King of Hearts

The True Story of the Maverick Who Pioneered Open Heart Surgery

[Dr Walt Lillehei]

GW Miller

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Lastly, I humbly recognise the sacrifice of many beautiful creatures in the pursuit of a better understanding of the mechanisms of cardiac failure, for the advancement of human and animal health.

List Of Abbreviations

$[Ca^{2+}]_i$	Intracellular Concentration Of Ca^{2+}
$[Ca^{2+}]_o$	Extracellular Concentration Of Ca^{2+}
$[Ca^{2+}]_{SR}$	Concentration Of Ca^{2+} In The Sarcoplasmic Reticulum
$[Na^+]_i$	Intracellular Concentration of Na^+
AP	Action Potential
AV	Aortic Valve
CICR	Ca^{2+} -Induced Ca^{2+} Release
CO	Cardiac Output
DHPR	Dihydropyridine Receptor
EDV	End Diastolic Volume
EF	Ejection Fraction
EM	Electron Microscopy
F/Fo	Peak Fluorescence/Baseline Fluorescence
FDHM	Ca^{2+} Spark Full Duration At Half-Maximal
FKBP12.6	FK506 Binding Protein 12.6
FS	Fractional Shortening
FWHM	Ca^{2+} Spark Full Width At Half-Maximal
HAHLT	Heterotopic Abdominal Heart-Lung Transplantation
HAHT	Heterotopic Abdominal Heart Transplantation (S-UN)
ICa,L	L-Type Ca^{2+} Current
IVC	Inferior Vena Cava

KO	Knock-out
LA	Left Atrium
LTCC	L-Type Ca^{2+} Channel
LV	Left Ventricle
LVAD	Left Ventricular Assist Device
LVd	Left Ventricular Cavity Diameter in Diastole
LVEDV	Left Ventricular End Diastolic Volume
LVESV	Left Ventricular End Systolic Volume
LVPWd	Left Ventricular Posterior Wall Thickness In Diastole
LVPWs	Left Ventricular Posterior Wall Thickness In Systole
LVs	Left Ventricular Cavity Diameter in Systole
NCX	$\text{Na}^+/\text{Ca}^{2+}$ Exchanger
NT	Normal Tyrode
PA	Pulmonary Artery
PKA	Protein Kinase A
PV	Pulmonary Veins
RA	Right Atrium
RV	Right Ventricle
RyR	Ryanodine Receptor
S-UN	Severe Unloading (HAHT)
SERCA2a	Sarcoplasmic Reticulum ATPase 2a
SICM	Scanning Ion Conductance Microscopy
SR	Sarcoplasmic Reticulum
SUMO-1	Small Ubiquitin-Related Modifier-1
SVC	Superior Vena Cava

T-Tubule	Transverse Tubule
T50	Time To 50% Decline Of The Ca^{2+} Transient
T90	Time To 90% Decline Of The Ca^{2+} Transient
TAC	Thoracic Aortic Constriction
Tcap	Telethonin
TTP	Time To Peak Of The Ca^{2+} Transient
UN	Moderate Unloading (Used In Chapter 6)
VTTP	Variance Of The Time To Peak Of The Ca^{2+} Transient
WT	Wild-type

Chapter 1

General Introduction

1.1 Myocardial Load Variation

The heart is characterised by its ability to adapt its structure and function to external stressors (Catalucci *et al.*, 2008). Acute mechanical load variation has a major impact on cardiac contractility, with changes in cardiac preload resulting in altered cardiac contractility, termed Starling's Law of the Heart (Patterson and Starling, 1914) (Figure I-1). These early experiments proved a number of important concepts, which can be observed in the relationship between load (in this case, changes to cardiac preload in the form of altered end-diastolic volume (EDV)) and contractility (in this case, cardiac output, (CO)) (Figure I-1). First, they show that increased stretch causes increased contractility i.e. that the heart is able to adapt acutely to changes in load. Second, such adaptations are normally reversible, in that as soon as the stimulus is reduced (reduced EDV), contractility will fall. Third, that beyond a certain stage, further stretch is no longer accompanied by increased contractility, so-called failure. Further work showed that the augmentation of cardiac contractility under increased loading conditions is mediated by a number of mechanisms including increased myofilament sensitivity to Ca^{2+} , decreased myofilament lattice spacing and others (reviewed in (Shiels and White, 2008)). This demonstrates the important role of Ca^{2+} in regulating the myocardial response to altered mechanical load.

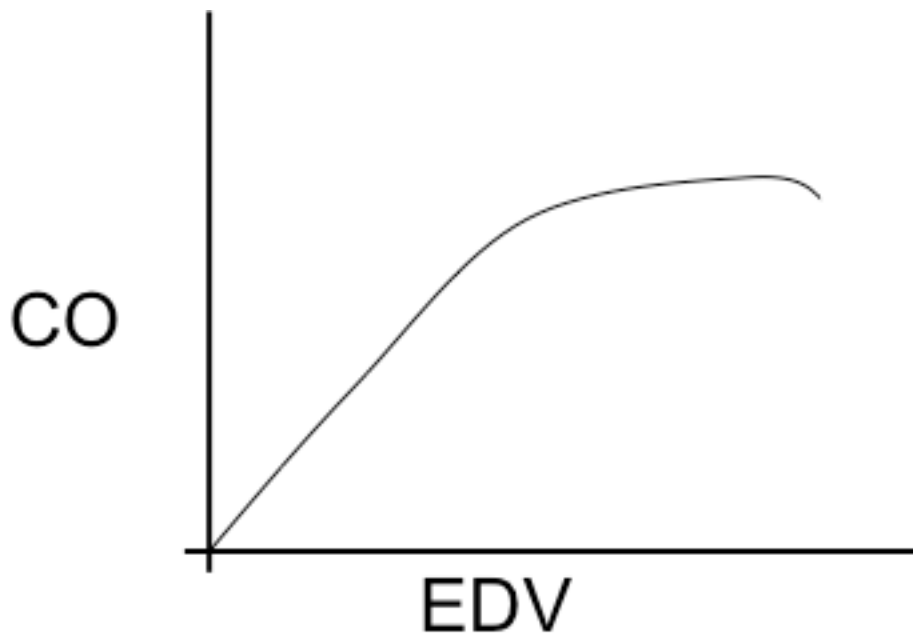


FIGURE I-1: STARLING'S LAW OF THE HEART

Acutely, CO is enhanced by increased EDV within a physiological range.

Chronic mechanical overload and unloading trigger adaptive responses, which cause the heart to undergo physiological remodelling as a part of a wider response to meet increased or reduced demand (Ellison *et al.*, 2012). Causes of mechanical load variation are listed in Table I-1.

Mechanical Overloading	Mechanical Unloading
<u>Increased Afterload</u>	<u>Pressure unloading</u>
-Aortic Stenosis	-Ventricular Assist Devices
-Hypertension	-Septal Defects*
-Atherosclerosis	-Mitral Valve Regurgitation*
-Coarctation of the aorta	-Bed Rest
-Renal Artery Stenosis	-Possibly space flight
	-Aortic Valve Bypass
<u>Increased Preload</u>	<u>Volume Unloading</u>
-Mitral Valve Regurgitation*	-Ventricular Assist Devices
-Septal Defects*	-Diuresis
-Renal Failure	-Mitral Valve Regurgitation
-Ischaemic Heart Disease	-Heterotopic, “piggy-back”
-Increased circulating volume (e.g. pregnancy)	Transplantation
-Arrhythmia	-Fontan Circulation*
	-Any cause of prolonged venodilatation e.g. sepsis.

TABLE I-1 CAUSES OF MYOCARDIAL MECHANICAL LOAD VARIATION

*These may cause overloading or unloading depending on the prevailing conditions of the circulation.

Chronically elevated demand causes depletion of the adaptive reserve and leads to heart failure (HF) (Heineke and Molkentin, 2006). A central concept is the notion of physiological versus pathological change. Physiological changes allow the myocardium to meet the increased demand placed by altered loading conditions. These include cardiomyocyte hypertrophy, augmentation of the Ca^{2+} transient and altered contractility amongst many other factors (Ellison *et al.*, 2012). These

physiological responses are so-called because they are reversible upon removal of the stimulus that triggered them. Pathological changes are thought to begin as physiological, adaptive changes. This pathophysiological pathway is termed remodelling and has been defined as (Table I-2):

“The molecular, cellular, interstitial and genomic changes which are manifested clinically as changes in size, shape and function of the heart following cardiac injury.”
-(Pfeffer and Braunwald, 1990)

Systems

- Neuroendocrine changes that coordinate skeletal, cardiac and smooth muscle structure, function and metabolic activity

Organ

- Ventricular wall hypertrophy or atrophy and changes in cavity size and shape
- Changes in heart rate which alter preload

Tissue

- Changes to electrophysiological properties of conduction
- Changes to extracellular matrix

Cellular

- Cardiomyocyte:
 - Hypertrophy/atrophy*
 - Changes to activity and expression of ion channels*
 - Changes to the membrane structure*
 - Changes to sarcomere size and biochemistry*
- Fibroblasts
 - Changes in number*
 - Changes in secretory function*
 - Possible changes in myocyte:fibroblast coupling*
- Immune and other cellular changes

TABLE I-2: THE SPECTRUM OF REMODELLING

The defining difference between physiological and pathological remodelling is thought to be its reversibility. This is shown in an elegant study of chronic aortic banding in rats for a period of time followed by removal of the band (Derumeaux *et al.*, 2002) (Figure I-2). If the band remains in place for two months, removal of the band leads to recovery of cardiac contractility (measured as the systolic mean velocity gradient (sMVG), recorded using echocardiography). However, if the band remains in place for nine months, removal of the band does not induce reversal of the phenotype, with chronically blunted contractility. This shows that one essential determinant of the transition between physiological and pathological remodelling is the duration of mechanical overload.

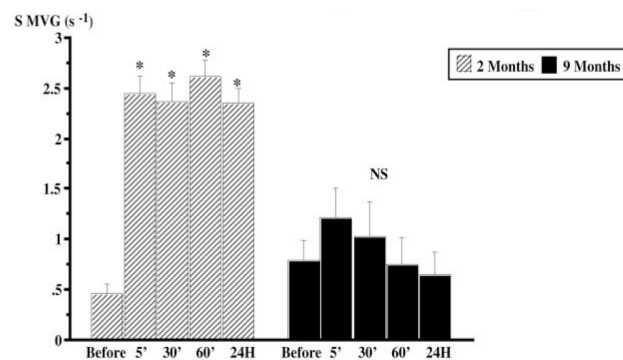


FIGURE I-2: PHYSIOLOGICAL REMODELLING VERSUS PATHOLOGICAL REMODELLING

sMVG is slowed after chronic mechanical overloading (induced by aortic banding), presumably to reduce the energetic cost of increased contractile force. If debanding is performed after two months, sMVG recovers, indicating physiological, reversible remodelling. If debanding is performed at nine months, such remodelling is irreversible. Figure from (Derumeaux *et al.*, 2002). Y axis shows myocardial velocity gradient before, 5, 30, 60 minutes and 24 hours following debanding.

1.2 Clinical Entity Of Heart Failure

HF is defined as the inability of the heart to meet the demands of the metabolising tissues (Jessup and Brozena, 2003). HF may involve the right, left or both ventricles (the role of the atria in HF is complex, understudied and is emerging (Corradi *et al.*, 2011)). When right-sided, symptoms and signs include peripheral oedema, hepatomegaly, with the attendant symptoms of bruising (coagulopathy) and lethargy among others. When left-sided, pulmonary oedema occurs, resulting in chronic breathlessness and marked anxiety. The end organs are under-perfused resulting in neuroendocrine derangement, skeletal muscle cachexia and severe lethargy. Two symptoms may be said to be pathognomonic of HF – paroxysmal nocturnal dyspnoea (a distressing attack of night-time breathlessness that wakes the patient) and orthopnoea (breathlessness on lying flat). These two symptoms describe a state where the ventricles are unable to drive sufficient flow to prevent pulmonary oedema in the supine position. A failing left or right ventricle can cause failure of both ventricles, congestive cardiac failure.

HF is progressive and its impact on function can be described according to the New York Heart Association (NYHA) class system¹. Class I describes those patients who have no functional restriction and whose symptoms do not impact on their ability to perform any activity. Class II describes those patients in whom normal levels of daily physical activity are possible, but induce symptoms of HF including shortness of breath. Class III describes those patients who experience significant functional limitations but are generally comfortable at rest. These patients show a specific feature which is that they can start but not finish normal daily activity. Class IV

¹ The Criteria Committee of the New York Heart Association. Nomenclature and Criteria for Diagnosis of Diseases of the Heart and Blood Vessels. Boston: Little Brown, 1964.

describes those patients who are not able to conduct any physical activity without discomfort and who are symptomatic at rest.

HF is associated with more morbidity and a lower quality of life than other multi-system diseases (Hobbs *et al.*, 2002). In terms of prognosis, up to 40% of patients die within one year of diagnosis (Rector and Cohn, 1994). The cause of death is either pump failure or arrhythmia, which each account for approximately 50% of deaths due to HF.

The scale of the HF problem is substantial². In the United Kingdom, there are 63000 new diagnoses of HF every year, with a total patient population of over 800000. One million inpatient bed days (2%) are occupied by HF, and this is projected to double over the next 25-years. HF has an annual cost to the NHS of approximately £625 million. Therefore, HF, which is the end-result of chronic mechanical overload, is a disease that is common, debilitating and often rapidly lethal.

1.3 Current Therapy For Heart Failure

The therapeutic rationale for HF has changed substantially in the last few decades (Eichhorn and Bristow, 1996). In the pre-clinical trial era of the 1950s, treatment was aimed at improving symptoms using digitalis (to enhance cardiac contractility, and improve symptoms by reducing breathlessness), diuretics (to reduce the circulating volume and reduce peripheral and pulmonary oedema) and prolonged bed rest (to reduce cardiac work). In the 1960-1980s, focus shifted to thinking of HF as a cardiovascular disease, based on pathological preload, afterload and contractility.

² British Heart Foundation. Heart Failure Supplement. <http://www.bhf.org.uk/publications/view-publication.aspx?ps=1001402>

Treatment was still aimed at relieving symptoms, using vasodilators (to reduce pre- and afterload) and inotropes. In the 1980s-90s, clinical trials showed that these strategies for improving symptoms had no benefit on survival (Cohn *et al.*, 1986, Packer *et al.*, 1991). From the 1980s, clinical trials showed that angiotensin-converting enzyme inhibitors (ACEi) and β -adrenergic blockers, which do not enhance short-term cardiac performance, improve mortality (Currie *et al.*, 1984, Ikram and Fitzpatrick, 1981). These agents have become the mainstay of treatment for prognostic benefit. Thus in fifty years, treatment has changed from positive inotropes to negative inotropes, from rest to exercise training (Belardinelli *et al.*, 1995) and from vasodilators to ACEi. Today the mainstay of therapy is ACEi, diuretics and β -adrenergic blockers.

1.4 Left Ventricular Assist Devices

Left Ventricular assist devices (LVADs) provide relief from HF by supporting the circulation

(Terracciano *et al.*, 2010). The inflow cannula removes blood from the left ventricle (LV), via a tract made in the apex of the LV and pumps this blood into the ascending aorta. This induces pressure and volume mechanical unloading of the LV.

The first ventricular assist devices were essentially extensions of the cardiopulmonary bypass machine and were entirely extracorporeal (Terracciano *et al.*, 2010). Later pulsatile ‘paracorporeal’ ventricular assist devices allowed some mobility. A major breakthrough was the development of totally implantable devices, powered by batteries carried in a holster by the patient. After internalisation, the next major step was achieved by the HeartMate pump (Thoratec Laboratories Corp., Pleasanton, CA),

which abolished the need for warfarin (which is a major source of complications) due to the development of anticoagulant surfaces. Second generation, axial, continuous flow pumps then emerged, which use grooves to drive rotational blood flow (Figure I-3). They are smaller, safer, and more durable and can provide up to six litres of flow. The third, and current, generation devices are small and centrifugal pumps which lack ball bearings and in which the rotor is magnetically (or mechanically) suspended. Some of these devices can be implanted totally within the pericardial space. Newer devices are emerging which provide “partial” mechanical support and can be implanted without median sternotomy (Meyns *et al.*, 2011).



FIGURE I-3: VARIOUS TYPES OF VENTRICULAR ASSIST DEVICE

(a, b) Examples of first-generation pulsatile VAD (HeartMate® I, Thoratec Corp., left) and second-generation continuous-flow (HeartMate II®, Thoratec Corp., right) (from <http://www.thoratec.com>). (c, d) A second-generation LVAD (Jarvik 2000, Jarvik Heart®) with intraventricular implantation (<http://www.jarvikheart.com>). (e, f) A third-generation centrifugal VAD (HVAD®, HeartWare, <http://www.heartware.com.au>), with small dimension and hydrodynamically suspended impeller. Figure from (Terracciano *et al.*, 2010).

Apart from their role as permanent circulatory support (“destination therapy”) and as bridges to transplantation, their most interesting use is to induce substantial reverse remodelling of myocardial structure and function (Yacoub, 2001) termed “Bridge to Recovery” (BTR). BTR is clinically defined as explantation of the device without needing cardiac transplantation.

The seminal discovery of BTR was accidental when devices had to be removed due to complications (Frazier *et al.*, 1996). The rate of recovery initially observed appeared to be very small (1-10%) (Mann and Willerson, 1998, Mancini *et al.*, 1998, Maybaum *et al.*, 2007). In some studies, the rate of recovery could be substantially increased by combining LVAD treatment with pharmacological therapy (up to 70%) (Birks *et al.*, 2011, Birks *et al.*, 2006). There are many unanswered questions with respect to BTR including which is the optimal device, in what mode of operation and, especially, for which patients. The indications for BTR are still evolving. To date, BTR has been offered mainly to patients with severe HF due to non-ischemic dilated cardiomyopathy (DCM), whether idiopathic, post-viral, familial, postpartum or post-chemotherapy, regardless of age, sex or duration of HF (Yacoub and Miller, 2008). Their use in ischaemic and other cardiomyopathies is expanding.

One important issue in the clinical use of LVADs is the decision about the timing of application, with some advocating increased use of LVADs in less sick patients (Miller, 2011). Timing of LVAD intervention can have an important influence on outcome, in both the short and long term. Virtually all patients recruited for BTR are from the larger pool of bridge-to-transplantation patients, and therefore by definition have an extremely advanced grade of HF (Kirklin *et al.*, 2008). This can have an adverse effect on early mortality, which is usually caused by multi-organ damage.

Rapid progress in the field has resulted in the development of several types of implantable LVAD (Figure I-3) with different performance characteristics, which can influence both the mode and degree of interaction with the native heart (Terracciano

et al., 2010). Although the pulsatile devices such as Heart Mate I are thought to provide better unloading of the LV, which could enhance recovery, their use has declined due to their much bigger size and higher incidence of complications. Recent series utilising the continuous flow Heart Mate II reported results similar to those obtained by Heart Mate I (Birks *et al.*, 2011). Further definition of the haemodynamic interaction between the device and the native heart should help in optimising the choice of device.

Although the process of reverse remodelling starts within a very short time after the onset of unloading, it continues for many weeks or months and varies in different types of animal models and from patterns observed in humans (Maybaum *et al.*, 2007, Oriyanhan *et al.*, 2007) with relatively wide variations between individuals. Tests for cardiac recovery in patients are problematic and clear protocols are not established. The tests to monitor recovery are usually started four weeks after leaving intensive care, when the patient is fully ambulant, with no evidence of infection or any other complication. Measuring LV size and ejection fraction (EF) during LVAD support provides a rough estimate of the degree of recovery (Dalby *et al.*, 2003). However, accurate evaluation of cardiac function requires discontinuing the contribution to cardiac output by the device. This can be achieved by switching the device off in pulsatile pumps or by reducing the pump speed in continuous flow pumps. This should be performed after full heparinisation, with close monitoring of haemodynamic and echocardiographic parameters. If the patient tolerates this period, a six-minute walk test followed by repeating echocardiography to test for inotropic reserve is performed. These tests are repeated at two-weekly intervals, and continued for up to 18 months so long as there is any degree of improvement in the parameters

measured. Additional tests in the form of measuring exercise capacity with gas exchange and cardiac catheterisation are performed at the appropriate stage to confirm recovery (Birks *et al.*, 2006).

There are currently no specific biological markers of recovery and assessment is based on clinical parameters alone. The development of such markers is predicated on our understanding of the molecular mechanisms of cardiac recovery.

1.5 Biological Remodelling in Heart Failure And Its Regression Following LVAD Therapy

Since the phenomenon of cardiac recovery was discovered at the clinical level, efforts in the laboratory have attempted to define its mechanisms. Patients treated with LVADs offer the unprecedented opportunity to study myocardial structure and biology at two time points in the same patient: before treatment (tissue taken during device implantation) and after treatment (tissue taken at explantation or transplantation). Given the limited amount of tissue available at explantation in BTR patients, most of the studies have been performed on bridge to transplantation samples, and it is therefore difficult to correlate the relationship between the changes observed and clinical consequences. In addition, a large body of information has amassed from studies using animal models of mechanical unloading. The array of LVAD-induced reverse remodelling has been reviewed extensively (Hall *et al.*, 2007, Terracciano *et al.*, 2010, Baba *et al.*, 2000, Wohlschlaeger *et al.*, 2010), and in this section, a brief summary of the most interesting and relevant changes is given. The physiology and pathophysiology of cellular electrophysiology is discussed in detail in the following sections.

1.5.1 System And Organ Effects Of LVADs

There are a number of effects of LVAD therapy. First, they induce major volume and pressure unloading of the LV, reducing myocardial wall tension and normalising CO. The enhanced CO improves brain (with improved neuroendocrine function (Klotz *et al.*, 2009)), kidney (with diminished anaemia, and adequate circulatory volume management), liver (with enhanced immune and metabolic function), gut (with enhanced metabolic and immune function) and other end-organ perfusion. Importantly, enhanced coronary blood flow is a major effect of the enhanced cardiac output. This can improve cardiac function independently. Cardiac improvements are listed in Table I-3. Within the heart, the main players are the cardiomyocytes, the extracellular matrix, immune and other cells.

Changes after LVAD therapy	Study
Decreased neurohormones	(Klotz <i>et al.</i> , 2005)
Decreased Cytokines	(Thompson <i>et al.</i> , 2005)
Decreased Apoptosis	(Hattori, 2003)
Decreased whole heart dimensions	(Levin <i>et al.</i> , 1995)
Improves LV geometry and CO	(Mancini <i>et al.</i> , 1998)
Reduces Cell size	(Terracciano <i>et al.</i> , 2004)
Normalisizes beta-adrenergic pathways	(Klotz <i>et al.</i> , 2005)
Alters collagen pattern [or fibrosis, varied reports]	(Bruggink <i>et al.</i> , 2006)
Normalisation of the cytoskeleton	(Birks <i>et al.</i> , 2005)
Improved cellular Ca ²⁺ handling	Figure I-4

TABLE I-3: CHANGES AFTER LVAD THERAPY

1.5.2 Metabolic Impact Of LVAD Therapy

The metabolic derangements of the failing heart, partly mediated by dysfunctional mitochondrial performance, has led to the failing heart being characterised as an engine out of fuel (Neubauer, 2007). LVADs, apart from reducing cardiac workload, also induce improvement of metabolic and mitochondrial function in HF. This is mediated in part by restored respiratory capacity of mitochondria, and by enhanced endogenous nitric oxide regulation of mitochondrial function (Mital *et al.*, 2000).

1.5.3 Impact Of LVAD Therapy On Fibrosis

LVAD therapy increases fibrosis in some studies (Klotz *et al.*, 2005), and decreases fibrosis in other studies (Bruckner *et al.*, 2004). Klotz *et al* showed that collagen cross-linking increased after LVAD therapy with consequent increase in myocardial stiffness, which could impact on ventricular filling properties (Klotz *et al.*, 2008). Bruggink *et al* demonstrated a biphasic response with an initial increase in fibrosis followed by a subsequent regression with prolonged LVAD therapy (Bruggink *et al.*, 2006).

1.5.4 Effect Of LVAD Therapy On Tissue And Cell Electrophysiology

Possibly related to altered extracellular matrix conditions, reverse electrophysiological remodelling occurs at the myocardial level during LVAD therapy, demonstrated by reduction of the QT interval (Harding *et al.*, 2001, Drakos *et al.*, 2011). Cellular electrophysiology is discussed in detail in section 1.6. Briefly, the depolarisation of the cell membrane in the form of the action potential (AP) opens the voltage-gated L-type Ca^{2+} channels (LTCC). These allow a small amount of Ca^{2+} to enter the cell, which triggers the opening of the Ryanodine receptors (RyR) which

allow the release of the intracellular Ca^{2+} stores from the sarcoplasmic reticulum (SR), resulting in contraction. The whole cell Ca^{2+} transient is then terminated by extrusion of Ca^{2+} via the $\text{Na}^+/\text{Ca}^{2+}$ Exchanger (NCX) and sarcoplasmic reticulum ATPase 2a (SERCA2a) (Bers, 2001).

Multiple elements of this system are affected by LVAD therapy (Figure I-4). LVAD therapy induces a regression of the pathologically prolonged AP duration occurs with possible improvements to depressed L-type Ca^{2+} current (ICa_L) (Terracciano *et al.*, 2004). SR Ca^{2+} release function is possibly improved after LVAD therapy (Marx *et al.*, 2000), with an increase in SR Ca^{2+} content (Terracciano *et al.*, 2004). These mechanisms could partly explain the observed increase in the Ca^{2+} transient amplitude (Dipla *et al.*, 1998) and therefore also the enhanced cellular contractility. Removal of diastolic Ca^{2+} is also improved by LVAD therapy, through increased SR Ca^{2+} uptake (Frazier *et al.*, 1996), presumably because of an increase in the amount of SERCA2a protein (Heerdt *et al.*, 2006). Therefore, the whole of local CICR appears to undergo some degree of local CICR reverse remodelling (Figure I-4). These mechanisms play some role in the enhanced whole heart function observed after LVAD use, which leads to clinical recovery.

1.5.5 Cardiomyocyte Contractility

Both basal cellular contractility and the contractile response to β -adrenergic stimulation (Dipla *et al.*, 1998) are improved after LVAD therapy. There is also evidence that LVAD therapy enhances EF in a number of clinical studies (Maybaum *et al.*, 2007), suggesting improved whole heart function.

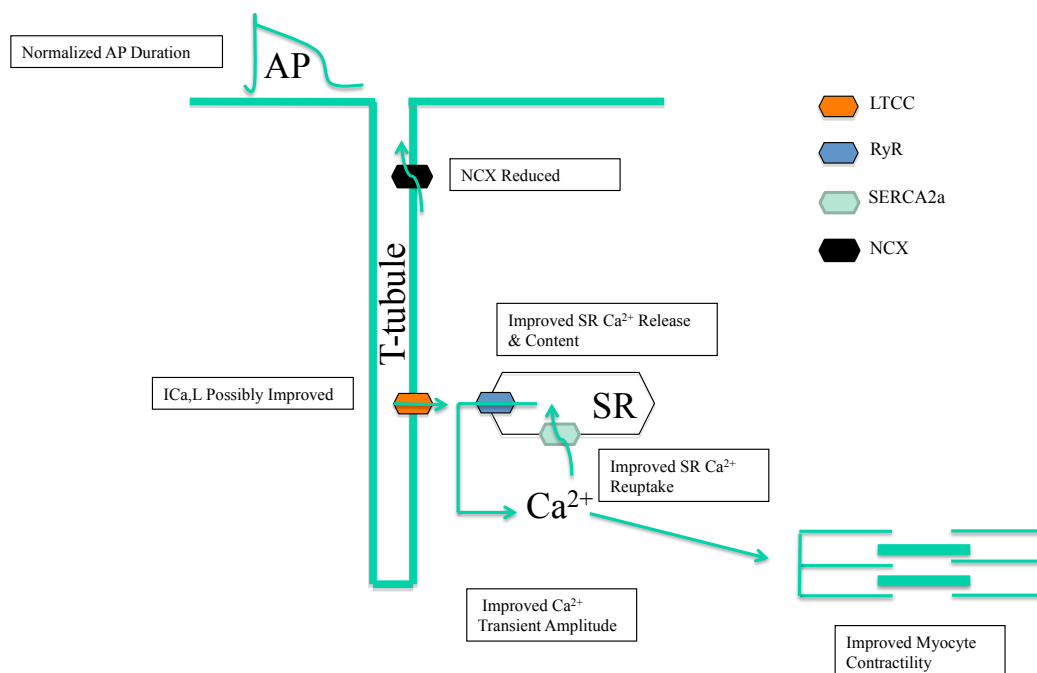


FIGURE I-4: REVERSE REMODELLING OF LOCAL CICR AFTER LVAD THERAPY

Multiple elements of the excitation-contraction coupling system undergo reverse remodeling after LVAD therapy.

1.5.6 Regression Of Cellular Hypertrophy

The cardiomyocytes form approximately 35% of myocardial cells by number, but almost 75% of the ventricular volume (Nag, 1980). These cells are linked to one another through intercalated disks, which provide a low resistance route for excitation. As such, the cardiomyocytes form an electric and mechanical syncytium. The cardiomyocytes are the sole force generators in the heart and the mechanisms that regulate their contractility will be discussed below. The hypertrophic cellular response to mechanical overload and HF has been described by a number of investigators (Del Monte *et al.*, 2002). Regression of cardiomyocyte hypertrophy has been documented after LVAD therapy (Zafeiridis *et al.*, 1998). It is widely assumed that regression of

hypertrophy improves clinical prognosis (and possibly cellular function), partly because of LVAD studies that show both regression of cellular hypertrophy and improvements in functional features such as cellular contractility (Dipla *et al.*, 1998). In a study comparing cardiomyocyte Ca^{2+} handling and cell size in patients who recovered clinically and those who did not, regression of hypertrophy was found to be common to both patient groups. Clinical recovery occurred in patients whose SR Ca^{2+} content recovered (Terracciano *et al.*, 2004).

1.5.7 LVADs Alter Cardiomyocyte Cell Cycle Regulation

HF is associated with increased apoptosis, which reduces cell number further, leading to increased stretch and dysfunction in the remaining cells. There is some evidence that the enhanced cell death of HF is reduced by LVAD support; LVAD therapy increases the expression of anti-apoptotic proteins (Bartling *et al.*, 1999) and decreases markers of cellular apoptosis (Baba *et al.*, 2000).

Cardiomyocyte division may occur in response to cardiac injury, but is clearly limited given the overt failure of neomyogenesis in the failing heart. The favourable environment during LVAD support, which improves the neuroendocrine environment by reducing plasma adrenaline, noradrenaline (NA), renin, aldosterone and vasopressin (Klotz *et al.*, 2009), as well as atrial and brain-type natriuretic peptide plasma levels (Thompson *et al.*, 2005), could promote conditions for cardiac regeneration. Wohlschlaeger *et al* recently showed a dramatic halving of mean cardiomyocyte DNA and a doubling in number of diploid cardiomyocytes, together with a significant decrease in the number of polyploid cardiomyocytes (Wohlschlaeger *et al.*, 2010). These results may imply that LVAD therapy promotes cardiomyocyte cell division.

1.5.8 Cardiomyocyte Cytoskeletal Structure

LVAD therapy alters the expression of multiple sarcomere, cytoskeletal and linker proteins within the cardiomyocyte, suggesting some role for internal cardiomyocyte reorganization of the cell (Margulies *et al.*, 2005, Vatta *et al.*, 2002, Latif *et al.*, 2007). De Jonge *et al* demonstrated that the disrupted cytoskeletal architecture observed in HF is improved after LVAD therapy (involving a number of proteins including actin, tropomyosin, troponin T and troponin C) (de Jonge *et al.*, 2002). Among cytoskeletal proteins, the integrin family of proteins span the membrane and link the extracellular matrix with the cytoskeleton. As such, they play an important role in mechanosensation and cell signalling. LVADs could induce recovery at multiple levels in the integrin pathway, which is dysfunctional in HF (Hall *et al.*, 2007). Whether this molecular recovery restores mechanosensation and the diverse actions of integrins is not yet known.

1.5.9 β -Adrenergic Subcellular Signalling

One of the major regulators of cellular and myocardial contractility is the β -adrenergic system, which is altered in HF. The domains of different β -adrenergic signalling pathways within the single cell, which are normally spatially segregated, have recently been shown to be more globalised in failing cells compared to healthy cells in an animal model of post-ischaemic HF (Nikolaev *et al.*, 2010). In addition, LVADs enhance the β -adrenergic response of the myocardium, both by increased receptor density (Ogletree-Hughes *et al.*, 2001) and by improved localization of β -receptors (Bick *et al.*, 2005).

1.5.10 Nature Of LVAD- Induced Gene Expression Changes

LVAD therapy alters the expression of a large number of proteins and their post-transcriptional regulation. MicroRNAs are small RNA sequences, which do not code for protein directly, but can alter patterns of translation by binding with coding RNA sequences and by influencing the pattern of protein expression. Patterns of microRNA expression appear to be normalized by LVAD therapy and could be a more sensitive marker for reverse remodelling than changes in gene expression (Matkovich *et al.*, 2009).

1.6 Physiological And Pathological Excitation-Contraction Coupling And The Transverse-Tubule System

The ventricular ability to contract resides in the function of the single cardiomyocytes. This contractility is triggered and regulated by a series of messengers (Bers, 2002), which were introduced briefly above. The cell membrane messenger in this case is electrical depolarisation of the cell membrane in the form of the morphologically complex AP. Depolarisation of the cell membrane opens voltage-gated LTCC (also termed dihydropyridine receptors, DHPR), which allows some entry of Ca^{2+} into the cytoplasm, in the form of the ICa_L . Through a positive feedback mechanism, this Ca^{2+} triggers the opening of the RyRs, which control the release of Ca^{2+} from the SR. This Ca^{2+} then bathes the myofilaments, triggering contraction. Ca^{2+} is then rapidly removed and re-uptaken into the SR (by SERCA2a) and out of the cell (via NCX) terminating contraction. These coordinated events can be observed in the whole cell Ca^{2+} transient, which precedes cellular contraction. Each of these components will be discussed below.

1.6.1 The Action Potential

The AP was first described by Hodgkin and Huxley in 1952 using giant squid axon (Hodgkin and Huxley, 1952). The AP describes a voltage waveform (as the consequence of the activity of different ion channels (DiFrancesco and Noble, 1985)), which is generated along the membrane of excitable cells, altering the membrane potential (E_m). APs are all-or-nothing (Noble, 1962), meaning that (i) their size is not affected by the original stimulus size because when triggered the amplitude is constant at any further point; this is because of the positive feedback mechanism which regulates the voltage-gated Na^+ channel; (ii) they are not triggered if the stimulus is below a certain threshold voltage. This threshold is set by the interaction between the negative feedback of voltage-gated K^+ channels (which tend to repolarise the cell and are dominant near the resting potential) and the positive feedback of voltage-gated Na^+ channels (which tend to depolarize the cell and as more Na^+ enters the cell, the greater the depolarization, and so on) (Noble and Hall, 1963). There is a voltage at which the positive feedback overcomes the negative feedback, a threshold. So, for a stimulus sufficient to tip the balance toward the positive feedback of Na^+ , the AP will be generated and, if there are no conduction defects, will regeneratively pass through the electrical syncytium.

The AP is different in morphology in different cardiac tissues (pacemaker, atrium and ventricle) due to the presence of different types of ion channels, which open in a different manner when depolarised (Nerbonne, 2000). The cardiac ventricular AP has a plateau phase due to the balance achieved by ICa,L and outward currents such as the delayed rectifier current (Charpentier *et al.*, 2010). In conjunction with the electrochemical gradient, this plateau allows inward Ca^{2+} flow through open LTCC.

Therefore changes in AP duration can have a major impact on the inward flow of Ca^{2+} .

1.6.2 The Long Lasting Type Ca^{2+} Current

In 1967, Reuter described the Long-lasting type Ca^{2+} current (Reuter, 1967). LTCCs are a type of voltage gated Ca^{2+} channel which persist in an open state, in contrast to the transient (T)-type Ca^{2+} channels which have a far smaller role in cardiac ventricular excitation-contraction coupling. Dihydropyridines stabilize the closed state of LTCC, and due to their ability to inhibit Ca^{2+} influx, have a wide therapeutic use for the management of angina and hypertension through their effects on smooth and cardiac muscle (Elliott and Ram, 2011).

The LTCC opens in response to membrane depolarisation at a voltage threshold that depends on physical parameters like temperature and $[\text{Ca}^{2+}]$, but under physiological conditions, it is approximately -40mV. Between -40mV and +10 mV, $\text{I}_{\text{Ca,L}}$ reaches a peak in 2-3 ms (Rosenberg *et al.*, 1986).

The inactivation of $\text{I}_{\text{Ca,L}}$ depends on $[\text{Ca}^{2+}]_i$, membrane potential, and time (Lee *et al.*, 1985) and generally occurs over 60-200 ms. The Ca^{2+} -dependent inactivation of $\text{I}_{\text{Ca,L}}$ is demonstrated by the fact that use of Ca^{2+} free extracellular solutions (using Ba^{2+} for example) lead to a prolongation of $\text{I}_{\text{Ca,L}}$ (Hofer *et al.*, 1997, Grandi *et al.*, 2010). Such Ca^{2+} -dependent inactivation provides a mechanism for limiting the amount of Ca^{2+} entering the cell during the AP. Moreover, this effect is observed even when the extracellular Ca^{2+} is highly buffered with the use of extracellular Ca^{2+} chelators, suggesting that Ca^{2+} local to LTCC can have an effect on $\text{I}_{\text{Ca,L}}$ inactivation

(Hofer *et al.*, 1997). Sipido *et al.* showed that SR Ca^{2+} release could also inhibit the ICa_L , providing one method for regulating Ca^{2+} influx (Sipido *et al.*, 1998).

The LTCC are present throughout the membrane, but are concentrated in the t-tubules (Gu *et al.*, 2002). Their close interaction with RyR has a number of implications for their function, which is discussed later.

1.6.3 Ca^{2+} - Induced Ca^{2+} Release

Ca^{2+} entering through the LTCC is insufficient to activate the myofilaments, both because of the amount of Ca^{2+} and also because of Ca^{2+} buffering (Fabiato, 1983). Approximately 60-70 $\mu\text{mol/L}$ cytosolic Ca^{2+} is required to activate a normal ventricular twitch (40% maximal force at 25-30 °C). This means that the diastolic $[\text{Ca}^{2+}]$ of ~150nM needs to rise to 600nM (Puglisi *et al.*, 1999). The amount of Ca^{2+} entering through the LTCC is approximately 14 μmol per litre of cytosol (under voltage-clamp conditions using an AP waveform) (Yuan and Bers, 1994), which is not sufficient alone to activate ventricular contraction.

In skinned canine Purkinje fibres (which lack t-tubules), Fabiato (Fabiato, 1985a, Fabiato, 1985b, Fabiato, 1985c) showed that 1ms exposure of Ca^{2+} (simulating the LTCC) could lead to SR Ca^{2+} release, in a process termed Ca^{2+} -induced Ca^{2+} release (CICR). In other words, the Ca^{2+} signal was amplified. In ventricular cardiomyocytes, portions of expanded SR, termed junctional SR, reside close to the t-tubule's zone of concentrated LTCC, and would be exposed to ICa_L very quickly. This unit is a dyad, which has been defined as the “*structural complex and functional unit of muscle excitation-contraction coupling. Anatomic elements include the LTCC or voltage*

sensors, the Ca^{2+} release units (RyR), and the subspace between them” (Cheng and Lederer, 2008). This provides a structural basis for that amplification, through a mechanism of positive feedback. This amplification must be regulated such that the amount of SR Ca^{2+} release is graded to the ICa_L . Fabiato showed that the amount of SR Ca^{2+} release depends on the trigger $[\text{Ca}^{2+}]$ and the time taken to reach that $[\text{Ca}^{2+}]$. Interestingly, SR Ca^{2+} release shows a refractory period where further addition of Ca^{2+} is unable to trigger SR release. SR Ca^{2+} depletion cannot explain this because caffeine addition does result in further SR Ca^{2+} release. Fabiato described a model involving SR activation sites with a high on-rate but low affinity and other inactivating sites with a higher affinity, but a slower activation constant (Fabiato, 1983). When rapid increases in $[\text{Ca}^{2+}]$ occur, SR Ca^{2+} release occurs, and the inactivation site binds Ca^{2+} more slowly to terminate Ca^{2+} release. This theory has been replaced with others (stochastic attrition, luminal SR Ca^{2+} release) described later.

Initially, it was uncertain whether Ca^{2+} or voltage were the essential steps of Ca^{2+} release. The evidence presented so far is consistent with either voltage-dependent or Ca^{2+} -dependent Ca^{2+} release. Nabauer *et al* (Nabauer *et al.*, 1989) presented evidence in rat ventricular cardiomyocytes showing that during a stimulus wave, a typical ICa_L trace is observed as well as a Ca^{2+} transient. In the presence of ethylene glycol tetraacetic acid (a Ca^{2+} chelating agent), the extracellular $[\text{Ca}^{2+}]$ is reduced to submicromolar levels and the current flow consists mainly of Na^+ . Under these conditions, no Ca^{2+} transient is observed. This is despite the fact that caffeine is able to produce a large Ca^{2+} release. Therefore, SR Ca^{2+} release appears to be dependent upon Ca^{2+} , indicating that Ca^{2+} dependent not voltage-dependent SR Ca^{2+} release is occurring. Niggli and Lederer (Niggli and Lederer, 1990) showed that the application

of Ca^{2+} (using caged photo-dependent release) is able to produce SR Ca^{2+} release under voltage clamp. In addition, CICR was the same irrespective of the Em.

Although these experiments provide a great deal of information related to the general regulation of CICR at the level of the whole cell, it has become apparent that SR Ca^{2+} release occurs in localized regions, visualized first by Cheng *et al* as Ca^{2+} sparks (Cheng *et al.*, 1993). Ca^{2+} sparks initiate along z-lines, raise local $[\text{Ca}^{2+}]$ by $\sim 200\text{nM}$, rise in 10ms, fall with time constant of $\sim 25\text{ms}$ and a spatial spread of approximately $2\mu\text{m}$ (Bers, 2001). Importantly, the scale of optical measures of Ca^{2+} sparks is 100 times the depth of the junctional complex, so they do not resolve the true structure of the dyadic Ca^{2+} handling. During resting states in cardiomyocytes, the frequency of Ca^{2+} sparks is typically very low, approximately 100/s. Activation of ICa_L by the AP leads to simultaneous activation of thousands of sparks to give rise to the whole cell Ca^{2+} transient (Cheng and Lederer, 2008).

In the local control theory of CICR initially proposed by Stern (Stern, 1992), SR Ca^{2+} release is effectively all-or-none within a cluster of RyRs. However, the physical separation between clusters, and the high local $[\text{Ca}^{2+}]$ required to activate a cluster means that activation cannot spread between clusters. Parker *et al* (Parker *et al.*, 1996) showed that occasionally one Ca^{2+} spark could activate another in the lateral direction along the z-line ($\sim 0.76\mu\text{m}$ away), but could never extend to a second z-line ($\sim 1.8\mu\text{m}$ away). This implies that in order to obtain gradation of CICR, recruitment of a variable number of RyR clusters is required.

In rat cardiomyocytes, approximately 55% of SR Ca^{2+} is released during a normal twitch for SR Ca^{2+} contents of approximately 100 μmol per litre of cytosol (Delbridge *et al.*, 1997). The SR Ca^{2+} released varies with the ICa_L amplitude (Bassani *et al.*, 1995a, Bassani *et al.*, 1995b). When SR Ca^{2+} load is halved, fractional release is zero, indicating that ICa_L cannot trigger such low amounts of SR Ca^{2+} load. This could be due to an altered RyR sensitivity by changes to SR luminal $[\text{Ca}^{2+}]$. Ca^{2+} spark frequency increases as SR Ca^{2+} content rises, possibly because sensitisation of RyR means that even diastolic $[\text{Ca}^{2+}]$ is able to trigger Ca^{2+} sparks.

The intrinsic positive feedback which drives the amplification of SR Ca^{2+} release requires a termination mechanism to regulate it. Three mechanisms have been suggested including local luminal depletion of SR Ca^{2+} , RyR inactivation and stochastic attrition. Stochastic attrition requires that all LTCC and RyR involved in a cluster close at the same time, therefore terminating SR Ca^{2+} release. If the dyad involved a single LTCC and single RyR, then stochastic release would be a feasible mechanism for Ca^{2+} release termination, but as there are multiple channels this mechanism appears unlikely. SR Ca^{2+} depletion is also unlikely given that the application of caffeine can produce Ca^{2+} sparks which are much longer than those normally observed (>200 ms) (Cheng and Lederer, 2008). Importantly during these extremely long releases, Ca^{2+} spark amplitude does not fall, indicating that SR Ca^{2+} content does not fall. However, Satoh *et al* showed that the Ca^{2+} sparks may be regulated in some way by SR Ca^{2+} content, because as described above, ICa_L cannot trigger Ca^{2+} release when SR Ca^{2+} content is severely reduced (Satoh *et al.*, 1997). This is likely to be mediated by reduced RyR sensitivity. Therefore reduced RyR sensitivity may play some role in Ca^{2+} spark termination under normal conditions.

Sham *et al* used Ca^{2+} spike measurements to understand the mechanisms mediating Ca^{2+} spark termination (Sham *et al.*, 1998). Early LTCC opening produced Ca^{2+} sparks, but prolonged opening did not. This indicates that some mechanism within the SR leads to the Ca^{2+} spark termination, likely including an element of intrinsic RyR inactivation. The mechanisms mediating this are not understood.

Ca^{2+} sparks offer the most practical direct assessment of the function of the RyR clusters and SR Ca^{2+} release. However, although Ca^{2+} spark imaging can reveal many details of SR Ca^{2+} release function, it does not provide the real biophysical characteristics of Ca^{2+} sparks. Imaged Ca^{2+} spark amplitude is a representation of true Ca^{2+} spark amplitude, which depends on the optical slice and line scan selected. Imaged, representative Ca^{2+} spark amplitude underestimates true spark amplitude (Cheng and Lederer, 2008). Importantly, the F/Fo value (fluorescence peak over fluorescence of the baseline background) depends heavily on the $[\text{Ca}^{2+}]$ at Fo , which likely varies between cells. Ca^{2+} sparks, even from the same RyR clusters, show morphologic heterogeneity (Lipp and Niggli, 1993).

Ca^{2+} spark width (reported as the full width at half-maximal, FWHM) depends on Ca^{2+} diffusion properties and reaction. Ca^{2+} sparks approximately measure $2.0\mu\text{m}$ FWHM when measured at the time of the peak signal, which appears to be more than twice the value obtained by mathematical models of Ca^{2+} spark formation (Cheng and Lederer, 2008). This Ca^{2+} spark width paradox could be related to non-Fickian diffusion properties of Ca^{2+} sparks, the biological mechanisms of which are uncertain. The total duration of a Ca^{2+} spark is approximately 30ms. This extremely brief

duration underpins the tight spatial localisation of Ca^{2+} spark signalling. Luminal transfer of Ca^{2+} within the SR, and regulated Ca^{2+} release linked to $[\text{Ca}^{2+}]_{\text{SR}}$ occasionally allow for very long sparks (Bers, 2002).

Despite the fact that Ca^{2+} sparks from the same RyR cluster can be morphologically different, Ca^{2+} sparks are autonomous. That is, Ca^{2+} sparks caused by ICa_L or spontaneous sparks are essentially similar in morphology. Despite large changes in ICa_L the individual Ca^{2+} sparks remain constant. Once activated, Ca^{2+} spark amplitude and duration are autonomous of ICa_L . This is not to say that ICa_L does not modulate Ca^{2+} sparks at all. Indeed, more sparks are evoked at negative rather than positive voltages for a given amount of ICa_L supporting the idea that the microscopic properties of ICa_L are also important determinants of SR Ca^{2+} release function (Santana *et al.*, 1996). Understanding Ca^{2+} spark function is a crucial element of dissecting the changes underlying the Ca^{2+} transient. Underpinning the relationship between ICa_L and Ca^{2+} release is the tight spatial proximity between LTCC and RyRs. These two channels are brought into tight spatial contact by the t-tubules.

1.6.4 Termination Of The Ca^{2+} Transient Via NCX And SERCA2a

To terminate the whole cell Ca^{2+} transient, the raised $[\text{Ca}^{2+}]_i$ must be lowered via Ca^{2+} extrusion. This occurs via two routes, NCX and SERCA2a. NCX, which is concentrated in the t-tubules (Despa *et al.*, 2003), is the major Ca^{2+} extrusion pathway from the cell under normal conditions (Noble *et al.*, 1991). NCX can also modulate both local Ca^{2+} release (Sher *et al.*, 2008) and the AP (Armoundas *et al.*, 2003). Under the conditions of AP prolongation and raised $[\text{Na}^+]_i$ in HF, NCX may contribute a significant amount of Ca^{2+} , augmenting the weakened Ca^{2+} transient (Despa *et al.*,

2002). Enhanced NCX activity in HF may have multiple effects on the AP depending on the intracellular $[Na^+]_i$. When $[Na^+]_i$ is high, NCX may contribute to AP repolarisation but when $[Na^+]_i$ is low, NCX may prolong the AP (Armoundas *et al.*, 2003).

SERCA2a is responsible for the uptake of Ca^{2+} into the SR; SERCA2a protein expression is depressed in HF, which partially explains the loss of contractile performance and SR Ca^{2+} reuptake (Hasenfuss *et al.*, 1994). Replenishment of SERCA2a, using gene therapy, results in restoration of cardiac performance, cellular contractility and augments SR Ca^{2+} content (Lipskaia *et al.*, 2010, Lyon *et al.*, 2011). Recent evidence shows that SERCA2a is powerfully regulated by post-translational modification in health and disease; restoration of normal levels of small ubiquitin-related modifier-1 (SUMO-1) restores SERCA2a protein levels in a mouse HF model and rescues SR Ca^{2+} content and Ca^{2+} transient dynamics (Kho *et al.*, 2011). Although the relative contribution of NCX and SERCA2a in lowering $[Ca^{2+}]_i$ differs by species, SERCA2a typically accounts for 70-80% of Ca^{2+} removal from the cytoplasm under normal conditions in adult mammalian cardiomyocytes (Bassani *et al.*, 1994, Bassani and Bassani, 2002, Bassani *et al.*, 1992, Fowler *et al.*, 2005, Bers and Bridge, 1989, Bers *et al.*, 1989, Hryshko *et al.*, 1989). NCX accounts for the removal of the vast majority of the remaining systolic Ca^{2+} , with the rest (approximately 1%) extruded by *slow* pathways involving the plasma membrane Ca^{2+} -ATPase and mitochondrial Ca^{2+} uniporter.

1.7 The Transverse Tubules

The t-tubules are invaginations of the external membrane of skeletal and cardiac muscle cells (Figure I-4 and I-5), which are rich in ion channels important for excitation-contraction coupling (Brette and Orchard, 2003) (Figure I-6). The transverse t-tubule network of mammalian ventricular cardiomyocytes was first discovered by Linder, using electron microscopy (EM) in 1956 (Lindner, 1957). The first report of axial t-tubules was in 1971 (Sperelakis and Rubio, 1971).

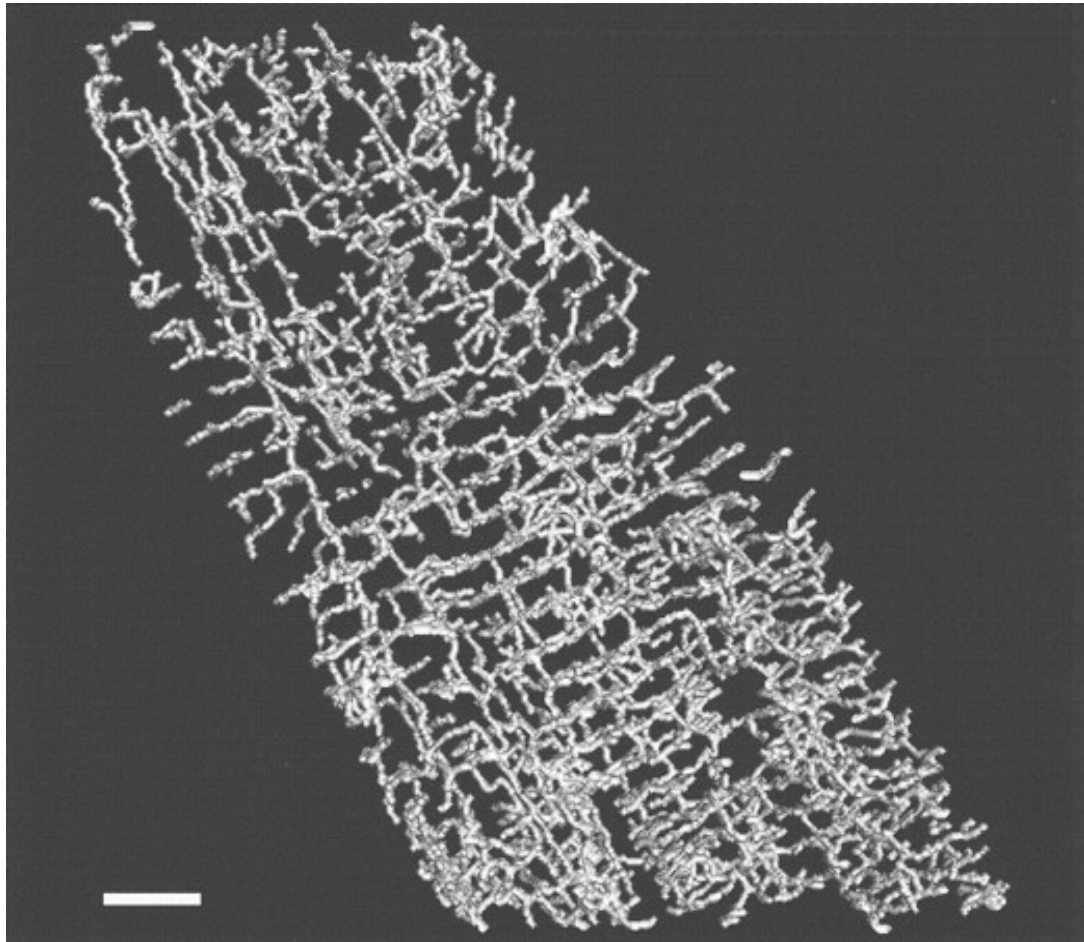


FIGURE I-5: TWO-PHOTON IMAGING OF THE T-TUBULE NETWORK IN RAT VENTRICULAR CARDIOMYOCYTES

The t-tubule network runs deep into the cell and extends in multiple directions, with a diameter varying from 20 to 450 nm. Figure from (Soeller and Cannell, 1999).

EM studies (Franzini-Armstrong and Porter, 1964, Porter and Palade, 1957) confirmed that the t-tubules were invaginations of the sarcolemma, and described their transverse and axial radiations, which paralleled findings in skeletal muscle cells. These studies described in ventricular cells a less regular but much larger t-tubule system. The finding that the external membrane regularly penetrated the cell centre was used to explain the earlier finding that it took less than 40ms for excitation to travel from the external membrane to the centre of the cell, a distance of

approximately 50 μ m (Hill, 1949). This also explained the observation that cells with well demarcated invaginations (t-tubules) contracted more rapidly than those without such structures (Peachey and Huxley, 1962).

Soeller and Cannell's two-photon live-imaging study elaborated the complexity of the t-tubule system (Figure I-5), which runs deep into cardiomyocytes and varies in its diameter (Soeller and Cannell, 1999). Recent work has reconstructed a three-dimensional model of the network and provided a new level of detail using computational approaches (Savio-Galimberti *et al.*, 2008). It confirms that the cardiac t-tubules are not transverse only, but have protrusions in many directions and a diameter that varies from 20 to 450nm. Skeletal muscle t-tubules are much smaller, with a diameter between 20 and 40nm (Franzini-Armstrong *et al.*, 1975).

The scheme of CICR described earlier relies heavily on the juxtaposition of sarcolemmal LTCCs and RyR to form a dyad (Figure I-4) (Franzini-Armstrong *et al.*, 1999). This close association (approximately 12nm) is critically dependent on normal t-tubule structure and guarantees that the Ca²⁺ trigger results in adequate Ca²⁺ release from the intracellular stores. This process of CICR is most effective in ventricular cells where the t-tubules ensure that Ca²⁺ is released in close proximity to all sarcomeres, regardless of how deep they lie within the cell (Brette and Orchard, 2003). The t-tubule network is responsible for just one third of the capacitance of the membrane, but most of the influx of Ca²⁺ which triggers the release of intracellular SR Ca²⁺ enters across the t-tubular membrane fraction (Kawai *et al.*, 1999). This is because there is a different complement of ion channels in the surface and t-tubular

fractions, with a three to nine fold higher number of LTCCs in the t-tubule fraction than in the surface sarcolemma (Scriven *et al.*, 2000, Brette and Orchard, 2003) (Figure I-6).

The myofilaments represent 45-60% of the cardiomyocyte volume. These contractile complexes are organised into sarcomeres with only a minority of these units close to the surface sarcolemma. In cells without t-tubules, the wave of Ca^{2+} propagates from the periphery of the cell into the centre (Huser *et al.*, 1996) either by simple diffusion or by a wave of propagated CICR (Figure I-7). Such a system would first activate the peripheral sarcomeres, and then the deeper sarcomeres, resulting in sub-maximal force production. A system where current is simultaneously relayed to the core of the cell would mean a larger instantaneous force is produced which is more equally shared between sarcomeres. The t-tubules make this possible by triggering SR Ca^{2+} release near to all sarcomeres simultaneously.

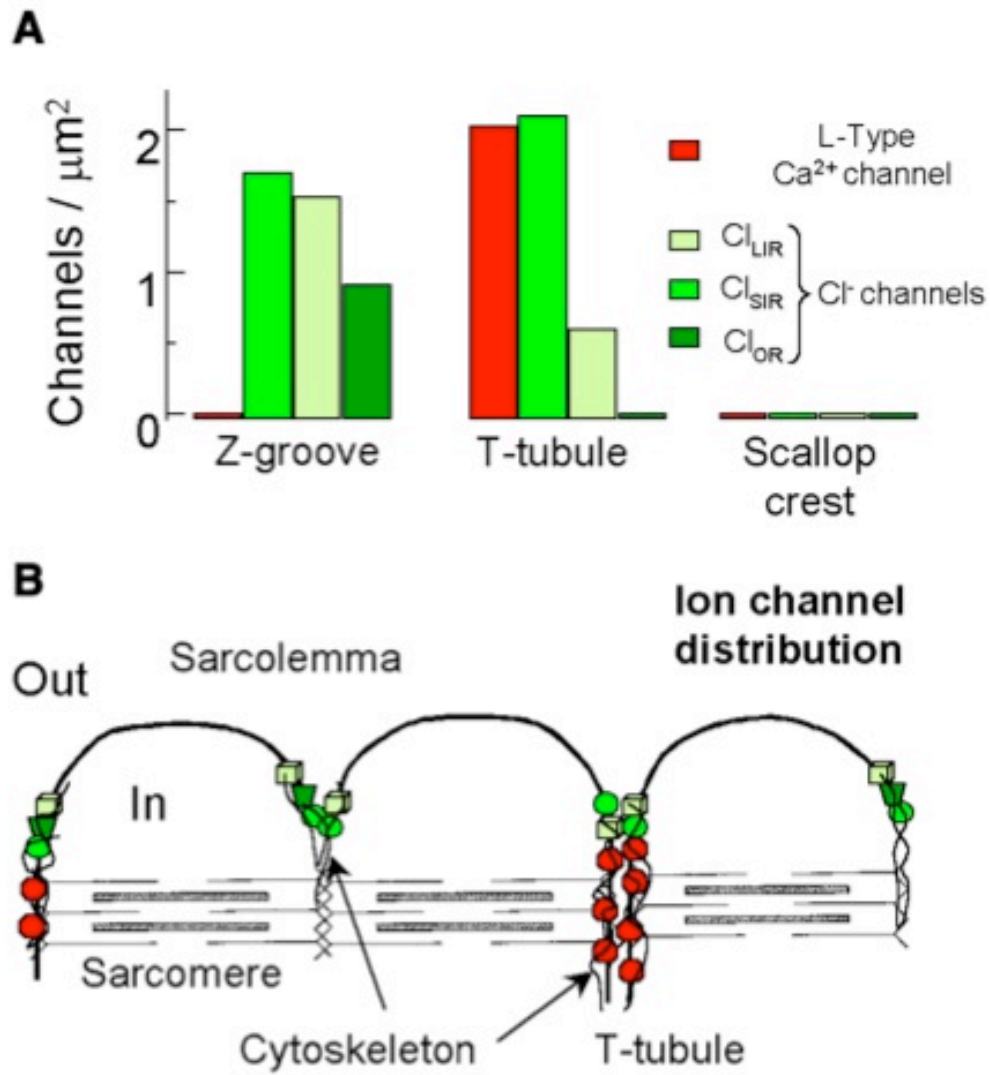


FIGURE I-6: Ca^{2+} CHANNELS ARE CONCENTRATED AT THE T-TUBULE

(A) Variation of the density of chloride and LTCCs at different positions on the cell surface (B) Functional schematic of the sarcomeric unit showing clustering and colocalisation of chloride and Ca^{2+} channels on the cell surface. From (Gu *et al.*, 2002).

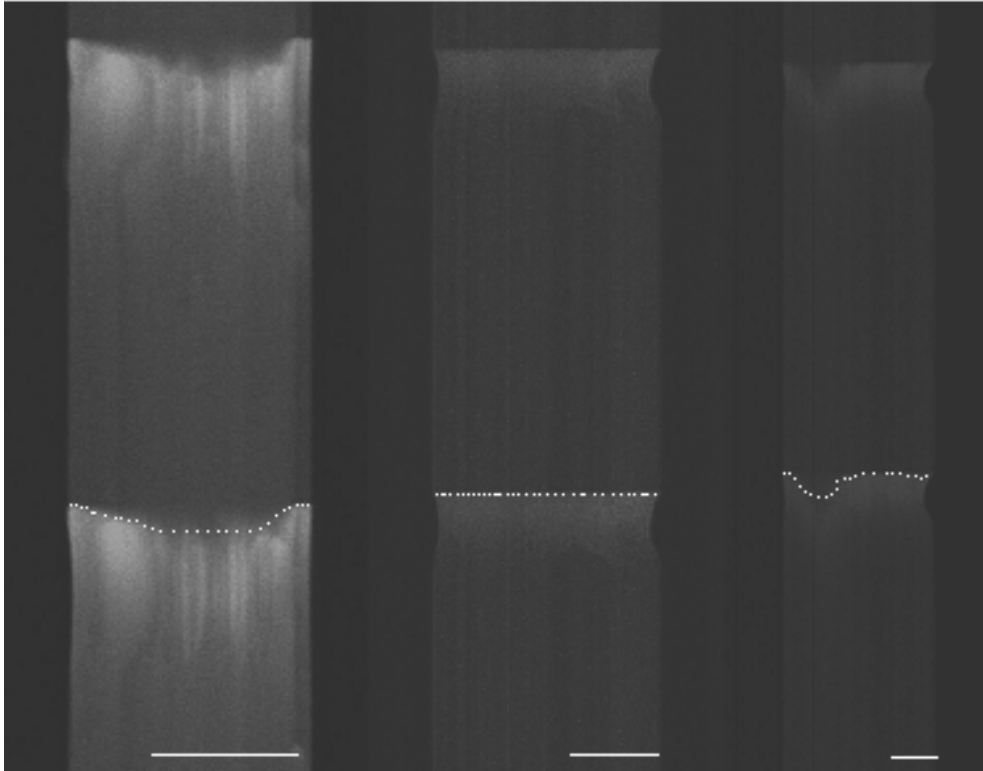


FIGURE I-7: EFFECT OF T-TUBULE NETWORK ON SR Ca^{2+} RELEASE EFFICACY

Confocal line scans of isolated cardiomyocytes from (left to right), human atrial, normal rat ventricular and failing rat ventricular tissue labelled with the Ca^{2+} sensitive dye, Fluo-4-AM. In atrial cells, the t-tubular density is reportedly very low, and this results in non-synchronous release of Ca^{2+} , with the cell exterior (edges of trace) activated before the centre of the cell (middle of trace). Normal rat ventricular cardiomyocytes show normal dense t-tubules and this allows synchronous activation of Ca^{2+} release throughout the cell. However, in failing ventricular cells the t-tubular membrane shows defects and this results in a loss of Ca^{2+} release synchronicity. The traces show two stimulated activations, the lower of which has been outlined by the dotted line for clarity. Each trace is shown at a different magnification for clarity, and the white scale bar represents $50\mu\text{m}$. (Original figure obtained using isolated cells labelled with Fluo-4-AM, and imaged using a confocal microscope).

This role for t-tubules in cardiac muscle is, to a certain extent, supported by comparative biology studies. Although there is little evidence that differences in t-tubule density are related to cell size, there appears to be a correlation with species

(which reveals a possible association with heart rate). For example, the mouse, which has a heart rate of approximately 600-800 beats per minute (bpm) at rest, has a denser t-tubule network than the pig, whose heart rate is less than 100 bpm (Heinzel *et al.*, 2002). This trend suggests the t-tubules are more important in hearts where the rapid cycling of Ca^{2+} is necessary in order to cope with high heart rates.

The t-tubules restrict diffusion of the extracellular fluid, creating a microdomain of ions of a concentration that is relatively stable in comparison to the wider extracellular space. This may be a mechanism to prevent rapid changes in the extracellular fluid from adversely affecting CICR. However, investigators have argued that it is theoretically possible that as more Ca^{2+} is drawn out of the t-tubule cleft, it may become Ca^{2+} depleted (Orchard *et al.*, 2009). Savio-Galimberti *et al.* have visualised single t-tubules at very high resolution and their analysis suggests that as the cardiomyocyte contracts, the t-tubules are squeezed and this ‘pumps’ the microdomain contents into the extracellular space (Savio-Galimberti *et al.*, 2008). It is not yet clear whether such a mechanism is quantitatively important in replenishing the contents of the t-tubular microdomain, and more studies are needed. However, recent computational studies which have modelled the t-tubular microdomain as containing ion concentrations either fixed at those of the extracellular space or altering by diffusion, suggest that the accumulation and depletion of ions in the t-tubule may have a significant effect on membrane currents (Pasek *et al.*, 2008a, Pasek *et al.*, 2008b). Whether this scenario is prevented by t-tubule pumping or other mechanisms (e.g. Ca^{2+} extrusion at the t-tubules by NCX (Despa *et al.*, 2003)) is a matter to be established by experimental approaches.

The density of LTCCs is higher in t-tubules than in the surface sarcolemma (Gu *et al.*, 2002) (Figure I-7). Brette *et al* estimate that approximately 75-80% of ICa,L flows across the t-tubules, indicating they are the main source of the Ca²⁺ trigger (Brette *et al.*, 2006). The Ca²⁺ current of the t-tubules is more readily inactivated by Ca²⁺ flow than the same current at the surface, indicating that Ca²⁺ released from the SR may have a greater autoregulatory role on the Ca²⁺ current in the t-tubules, further supporting their special role in CICR (Brette *et al.*, 2004b).

The t-tubules are not only a site for Ca²⁺ influx, but also for Ca²⁺ extrusion. NCX also appears more highly concentrated in the t-tubule (Despa *et al.*, 2003). T-tubular NCX appears to have privileged access to the Ca²⁺ released from the SR (Trafford *et al.*, 1995) and may occur in a specialised zone in the t-tubule. SERCA2a is preferentially expressed close to the t-tubules (Greene *et al.*, 2000), indicating that the major Ca²⁺ extrusion pathways are near to the influx pathways at the t-tubule. Recent evidence suggests that Ca²⁺ efflux through SERCA2a occurs only in the t-tubule, and Ca²⁺ efflux through NCX occurs predominantly through the t-tubule. Just as the major influx pathways in the t-tubules allow rapid contraction throughout the cell (Chase and Orchard, 2011), so the extrusion pathways allow rapid relaxation throughout the cell and further implicate the t-tubule in Ca²⁺ homeostasis. These data suggest the hypothesis that the t-tubules may have a specialised role in the extrusion of intracellular Ca²⁺ but this requires further experimental studies.

1.7.1 Signalling Functions

The β -adrenergic system is known to modulate the function of a number of key Ca²⁺ channels and contractile proteins. Indeed, a number of key proteins in this signalling

pathway (e.g. stimulatory G-protein, G_s) are localised at the t-tubule (Laflamme and Becker, 1999). Reports suggest that the β_2 -adrenoceptor (AR) system is more tightly coupled to the modulation of the ICa,L at the t-tubule than at the surface sarcolemma (Jurevicius and Fischmeister, 1996), as β -adrenergic stimulation causes a greater increase in the Ca^{2+} current in normal cells compared to detubulated cells (Brette *et al.*, 2004a). This may have a structural basis, as a subpopulation of LTCCs resides in caveolae associated with the t-tubule network, and may play signalling functions (Davare *et al.*, 2001, Balijepalli *et al.*, 2006). These LTCCs colocalise with a β_2 -AR/G-protein macromolecular signalling complex, raising the possibility that they process small Ca^{2+} signals which could be important in inducing hypertrophy and controlling contractility. There are conflicting reports as to the effect of caveolae disruption on β_2 -AR stimulation of the ICa,L , with some reports that caveolae are necessary (Balijepalli *et al.*, 2006) and some to the contrary (Calaghan and White, 2006); however, it is clear that caveolae-based signalling complexes modulate Ca^{2+} signalling in cardiomyocytes. Caveolin-3 is the major structural protein of caveolae. Interestingly, it is known that caveolin-3 deficient mice develop HF (Woodman *et al.*, 2002).

β AR localisation is critical to the functional signalling properties of the intracellular signalling molecule cAMP (Nikolaev *et al.*, 2010) (Figure I-8). In normal cells, β_1 -ARs appear to be functionally homologous across the external and the t-tubule membrane, and are not spatially localised to any specific site. However, the β_2 -ARs are spatially localised to the t-tubule in normal cells. In HF, this spatial localisation is lost, with a relative redistribution to the cell surface. The normally tight spatial localisation of the β_2 -AR response depends on its colocalisation with protein kinase A

(PKA), which limits the spread of the cAMP response. The normally striated pattern of PKA is lost in HF. This 'globalisation' of the β_2 -AR response may partly mediate the loss of its cardioprotective effects and its role in driving the disease process in HF. The full implications of this remain to be tested.

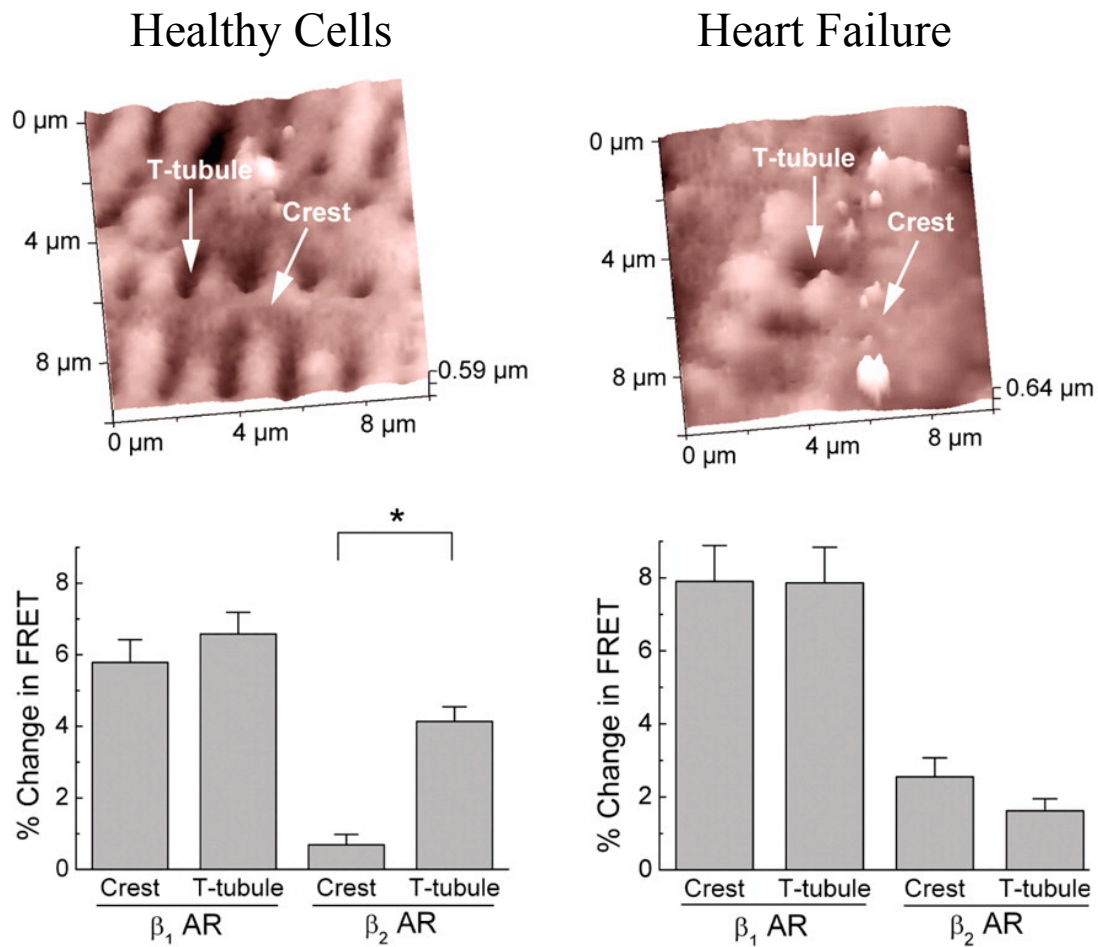


FIGURE I-8: B-ADRENERGIC RECEPTOR DISTRIBUTION IS ALTERED IN HEART FAILURE

The left panel shows the cell surface structure (top) and β_2 -AR distribution in healthy cells. Normally, β_2 -AR are concentrated in the t-tubules, whereas β_1 -AR are spread across both membrane fractions. In HF, t-tubule abnormalities are associated with a reversal of normal β_2 -AR distribution. Adapted from (Nikolaev *et al.*, 2010).

1.7.2 T-Tubule Regulation

The t-tubule network is extremely dynamic; it appears after birth (e.g. (Haddock *et al.*, 1999)), when the ventricular pressures rise, and disappears when cardiomyocytes are placed in culture (Mitcheson *et al.*, 1996), or in HF (see later). T-tubules are also

absent in stem cells and in neonatal cardiomyocytes. The exact mechanisms responsible for these changes remain unknown. However, they appear to be regulated by both biochemical (Table I-4) and biophysical factors (e.g. load and heart rate). Both these factors appear to interact under both physiological and disease conditions and govern the t-tubule's structure and function.

Protein	Major finding	
BIN1	Involved in tubule formation. Mutations in BIN1 in skeletal muscle myopathies.	(Fugier <i>et al.</i> , 2011,
	Shuttles LTCCs to the t-tubule.	Hong <i>et al.</i> , 2012, Hong <i>et al.</i> , 2010, Lee <i>et al.</i> , 2002)
Myotubularin	Mutations are associated with x-linked myotubular myopathy, and result in severe structural and functional disruption to the t-tubules. There is a possible common amphiphysin-myotubularin pathway regulating t-tubule biogenesis. Overexpression causes accumulation of membrane saccules. May also directly modulate RyR function and alters the expression of DHPR and RyR.	(Al-Qusairi <i>et al.</i> , 2009, Buj-Bello <i>et al.</i> , 2008, Dowling <i>et al.</i> , 2009)
Tropomyosin	Absence of certain isoforms of tropomyosin (an actin binding protein) disrupts t-tubules, suggesting the myofilaments may be involved in maintenance of T-tubule system.	(Vlahovich <i>et al.</i> , 2009)
Telethonin	Mutations cause muscular dystrophy-like phenotype with associated t-tubule disruption. Forms part of stretch sensitive centre, which is defective in some cardiomyopathies eg HOCM.	(Knoll <i>et al.</i> , 2011, Zhang <i>et al.</i> , 2009)
Junctophilin	Critical for accurate association of T-tubule and SR membrane. Downregulated in HF. Knockdown in culture causes disrupted t-tubule structure.	(Ito <i>et al.</i> , 2001, Komazaki <i>et al.</i> , 2002, Komazaki <i>et al.</i> , 2003, van Oort <i>et al.</i> , 2011, Wei <i>et al.</i> , 2010)
Mitsugumin	Mice lacking mitsugumin-29 display significant ultrastructural abnormalities to the t-tubule membranes but only have limited myopathy. This indicates some of these genetic abnormalities may be partly due to alterations in the function of the constituent ion channels as well as structural changes.	(Nishi <i>et al.</i> , 1999)
Triadin	Triadins are structural proteins which bind RyR. Triadin null mice have normal life spans and preserved contractility despite a significant reduction in the amplitude of evoked Ca ²⁺ release and disrupted t-tubule structure in skeletal muscle.	(Shen <i>et al.</i> , 2007)
Obscurin	Depletions in zebrafish cause profound disruption to the t-tubules as well as many other structural and functional features of both skeletal and cardiomyocytes.	(Raeker <i>et al.</i> , 2006)

TABLE I-4: MOLECULES INVOLVED IN T-TUBULE REGULATION

Among the biochemical factors, amphiphysin 2 (BIN1) has a fundamental role in t-tubule formation (Lee *et al.*, 2002). When ectopically expressed in non-muscle cells, it results in tubular formation, and is concentrated at the sites of developing membrane striations in muscle (Lee *et al.*, 2002). It is also essential for the trafficking of LTCC to the t-tubule membrane, along microtubules (Hong *et al.*, 2010). Total knockdown of BIN1 is lethal in perinatal life, with a DCM phenotype. Hong *et al.* showed that BIN1 expression is reduced in the failing human heart and that while total protein expression of LTCC is unchanged, in failing cardiomyocytes LTCCs are not concentrated at the t-tubule (Hong *et al.*, 2012, Hong *et al.*, 2010). Lentiviral knockdown and replenishment of BIN1 are able to modulate the surface expression of LTCCs, with a major impact on the whole cell ICa,L. Thus, reduced BIN1 expression in the failing heart could result in a failure to direct LTCC to the membrane. The major consequence of detubulation in diseased cardiomyocytes is a loss or uncoupling of LTCCs, and changes to BIN1 expression may play a role in mediating changes to both gross t-tubule structure and correct protein localisation.

Junctophilin-2 (JPH2) has been suggested to have an important role in promoting junction formation between the sarcoplasmic membrane and the t-tubule (Takeshima *et al.*, 2000). It has recently been reported to be downregulated proportionally during the progression from hypertrophy to HF (Wei *et al.*, 2010). This may partly mediate the uncoupling observed between the t-tubule and SR in cardiac muscle during pathological remodelling. JPH2 has emerged as a candidate molecule because of work that shows it is a membrane-linking protein and is reduced in expression in HF (Wei *et al.*, 2010). JPH2 germline knock-out (KO) results in perinatal death with a HF phenotype (Ito *et al.*, 2001). This initially hindered understanding its mode of action.

Van Oort and colleagues show that loss of JPH2 in the adult mouse hearts leads to DCM without gross remodelling (van Oort *et al.*, 2011), with pronounced early mortality. Surviving mice have cardiomyocytes with reduced CICR gain due to reduced colocalisation of LTCC and RyR without changes in amount of protein expression. They also document dyadic remodelling and increased SR Ca^{2+} leak. The authors also show that these defects are not present when JPH2 over-expressing animals are crossed with conditional short-hairpin RNA animals (which reduce JHP2 expression), demonstrating that the effects are specifically associated with loss of JPH2, rather than an unknown secondary effect. Thus JPH2 has a role in mediating pathological remodelling at the level of local CICR.

Telethonin (Tcap) was identified as a part of the stretch-sensitive complex in myocardium in previous studies (Hayashi *et al.*, 2004). The effect of Tcap mutations on t-tubule structure in cardiac muscle is unknown; disruption of Tcap in zebrafish produces a form of muscular dystrophy, suggesting that it is important in force production (Zhang *et al.*, 2009). Lack of Tcap is associated with disrupted t-tubule development and abnormal muscle function. Tcap expression is increased by stretch and appears to correlate with the level of t-tubule development, suggesting the novel possibility that Tcap promotes t-tubule formation in response to increased stretch. Tcap mutations can cause DCM or hypertrophic cardiomyopathy (HOCM) which could be related to the differential response of the myocardium to stretch (Hayashi *et al.*, 2004). In a small group of patients with DCM or HOCM, Hayashi *et al* found that DCM-associated Tcap mutations impaired the interaction of Tcap with its partners in the stretch-signalling complex, whereas HOCM-associated mutations promoted the interaction of these molecules (Hayashi *et al.*, 2004). They suggest that these different

Tcap mutations were consistent with an impaired stretch-response in DCM patients, and a hyper-reactive stretch response in HOCM patients, possibly affecting the clinical manifestation of the cardiomyopathy. The findings from zebrafish discussed above raise the possibility of a role for Tcap in t-tubule structural disruption in the mechanisms of this subset of rare cardiomyopathies, but this remains to be tested. Importantly, Tcap binds minK (K^+ channels which reside in the t-tubule) (Furukawa *et al.*, 2001). This provides a possible link between the stretch-sensing complex of the cardiomyocyte and the t-tubule membrane. Recent work using a Tcap KO shows that under basal conditions, there is no spontaneous cardiac dysfunction, but when subjected to mechanical overload, cardiac failure develops rapidly (Knoll *et al.*, 2011). In this setting, there is marked apoptosis and fibrosis. Tcap expression was recently found to be depressed in HF and enhanced following SERCA2a gene therapy, which recovered the t-tubule network and many elements of myocardial structure and function (Lyon *et al.*, 2012).

1.7.3 T-Tubule Dysregulation In Heart Failure

1.7.3.1 Animal Models

The presence of t-tubular changes in HF has been demonstrated in both human studies and animal models (e.g. (He *et al.*, 2001, Benitah *et al.*, 2002, Louch *et al.*, 2004, Louch *et al.*, 2006, Balijepalli *et al.*, 2003). In animal models, HF is associated with t-tubule disarray which appears to be caused by a number of myocardial insults including sustained tachycardia (Benitah *et al.*, 2002), chronic mechanical overload (Wei *et al.*, 2010) and myocardial infarction (Heinzel *et al.*, 2008). T-tubule changes appear to occur early in the transition from hypertrophy to HF (Wei *et al.*, 2010), suggesting they could be driving the pathogenic mechanism. T-tubule density loss

appears to be the major mechanism in larger species, with loss of t-tubule regularity in smaller species.

Work using a pig post-ischaemic cardiomyopathy model suggests that t-tubule dysfunction is causally related to the contractile dysfunction of the failing heart (Heinzel *et al.*, 2008). The impaired contractility of the failing heart was associated with a reduced Ca^{2+} release synchronicity, a delayed Ca^{2+} release and a smaller Ca^{2+} transient. The authors of this study documented a significant reduction in t-tubule density, while the ICa_L and SR Ca^{2+} content were not deleteriously affected. They therefore suggest that the flaw is in the gain of the CICR process, which implicates the structural dysregulation of the t-tubules in the pathophysiology of the failing cardiomyocyte. Failing rat cardiomyocytes have an increased Ca^{2+} spark frequency, which is consistent with an uncoupling of the Ca^{2+} release machinery mediated by structural disruption to the t-tubules. The mechanisms mediating this increased Ca^{2+} spark frequency are likely to be multifaceted, including changes to the regulation of the RyR. Further work suggests that the level of detubulation correlates with the degree of HF, and regions of delayed Ca^{2+} release are spatially localised to regions of disrupted t-tubular structure (Heinzel *et al.*, 2008). Structural uncoupling of the Ca^{2+} release machinery may be a final common pathway in HF, as it appears to be a mechanism common to diabetic cardiomyopathy (Stolen *et al.*, 2009), hypertensive cardiomyopathy (Song *et al.*, 2006) and ischaemic HF (Louch *et al.*, 2006).

The possible causal role of t-tubule dysregulation in the impaired contractility of HF is supported by evidence from experiments where the t-tubules are artificially disrupted (discussed in detail below). Artificial detubulation (Brette *et al.*, 2002)

mimics some of the changes in Ca^{2+} handling observed in HF, with a reduced Ca^{2+} transient synchronicity leading to a slow Ca^{2+} transient, associated with diminished but prolonged contraction (Brette *et al.*, 2004a, Brette *et al.*, 2004b). Isoproterenol activation can improve the Ca^{2+} transient dynamics and amplitude of detubulated cells (Brette *et al.*, 2004a). In these circumstances, wave-like propagation of the Ca^{2+} transient from the sarcolemma to the interior is observed. In culture, Louch *et al* showed that Ca^{2+} transient propagation is initially triggered at t-tubules and propagates to detubulated regions (Louch *et al.*, 2004). Litwin *et al* first showed that failing cardiomyocytes had dysynchronous Ca^{2+} release (Litwin *et al.*, 2000), and this was shown to be related to detubulation (Louch *et al.*, 2006). Double staining with Ca^{2+} -sensitive and membrane-binding dyes shows that areas of dysynchronous Ca^{2+} release correspond to gaps in the t-tubule network (Louch *et al.*, 2006). Such dysynchronous Ca^{2+} transients results in overall broadening and slowing of the Ca^{2+} transient.

The notion of reduced CICR gain (i.e. less SR Ca^{2+} release for the same ICa_L) during detubulation was first shown by Gomez *et al* and later shown to be related to detubulation (Gomez *et al.*, 1997). Detubulation leads to orphaned RyRs, unstimulated by ICa_L (Song *et al.*, 2006). Other changes may also be important including LTCC activity or number, RyR number, organisation or functional status. Prolongation of the AP in failing cells reduces the driving force for Ca^{2+} entry, which can reduce ICa_L (Mork *et al.*, 2009). Switching the AP stimulus morphology from normal to failing results in loss of Ca^{2+} transient synchrony. Such Ca^{2+} transient dysynchrony is different to the dysynchrony found after detubulation because it is highly variable beat to beat, whereas that found after detubulation is consistent over

multiple beats (Harris *et al.*, 2005).

In failing cells which show t-tubule loss, the upstroke of the Ca^{2+} transients shows a pronounced lag after the upstroke of the AP, and this may correspond to expansion of the dyadic cleft (Louch *et al.*, 2006). Importantly, such changes may be obtained by relatively minor changes to the t-tubules, which could expand the dyadic cleft sufficiently to slow LTCC-RyR signalling. Xu *et al* showed that the response of RyR to the opening of a single LTCC is delayed, which is consistent with dyadic expansion (Xu *et al.*, 2007).

Most investigators report unchanged ICa_L density in HF (Bers, 2006a), due to a combination of reduced LTCC number but increased activity (Schroder *et al.*, 1998). The mechanisms mediating this could include phosphorylation of LTCC by PKA or Ca^{2+} /calmodulin-dependent protein kinases II (CAMKII) (Chen *et al.*, 2002). Brette *et al* 2004 showed that detubulation with formamide (discussed later) reduced ICa_L (Brette *et al.*, 2004b). Whether LTCC redistribute and the precise mechanisms of their regulation in the failing heart is not yet known.

There is also substantial evidence that RyR function is altered in HF. In isolated bilayer experiments, Marx *et al* showed that RyR had greater activity and suggested that such increased activity could deplete the SR (Marx *et al.*, 2000). Marx suggested that hyperphosphorylation of RyR by PKA causes dissociation of the regulatory element FKBP12.6 (FK506 binding protein) and this enhances RyR activity, destabilising it and causing uncoupling of neighbouring RyR. This could lead to increased diastolic activity and also to loss of synchrony. Other groups have been

unable to demonstrate FKBP12.6 dissociation from RyR (Xiao *et al.*, 2005). RyR activation may arise from phosphorylation by CaMKII (Ai *et al.*, 2005) or other changes to RyR regulatory proteins (Kubalova *et al.*, 2005). Increased SR leak, apart from reducing SR content and therefore blunting contractility, may also be pro-arrhythmic because NCX-mediated extrusion can trigger extra APs (Bers, 2006a). The issue of the nature, conditions, mechanisms and implications of RyR phosphorylation are contentious and unresolved (Bers, 2012). RyR phosphorylation-mediated Ca^{2+} leak is a feature of the failing heart, and may be more important than PKA in modulating RyR open probability.

Whether these RyR changes are due to generic changes in the cell including general shifts in phosphorylation patterns or localised changes to the dyad, or are more specific is uncertain. Meethal *et al* showed that increased Ca^{2+} spark frequency occurred at gaps in the t-tubules where diastolic Ca^{2+} levels were higher (Meethal *et al.*, 2007). This suggests that orphaned RyR show increased activity than coupled dyads, possibly pointing to a direct effect of detubulation on local RyR activity.

The degree of t-tubules loss that can be tolerated without functional changes is not clear. APs may access a similar portion of the cell via an axial or transverse t-tubule, possibly providing a safety mechanism. Normally, APs of the surface and t-tubule network are identical (Bu *et al.*, 2009). This tight electrical coupling appears to fail when the t-tubules are disrupted in HF, as AP propagation does not access the t-tubules and spontaneous electrical activity takes hold (Sacconi *et al.*, 2012). Other compensatory mechanisms may initially mitigate the loss of t-tubules, including an increased SR Ca^{2+} content.

SERCA2a depletion is commonly reported in HF (reviewed in (Louch *et al.*, 2010c)). Recent studies of SERCA2a gene therapy in a rodent model of HF show that the t-tubule membrane and many elements of local CICR recover after gene therapy (Lyon *et al.*, 2012). In addition, a study using SERCA2a gene KO animals shows that the t-tubule network grows in response to impaired SR Ca^{2+} release, with the appearance of longitudinal t-tubules which allow NCX-mediated SR Ca^{2+} release to partially compensate for impaired SR Ca^{2+} release (Swift *et al.*, 2012).

1.7.3.2 Artificial Detubulation

While the detubulation following myocardial insults has provided a great deal of information on t-tubule biology, the interpretation of such studies is complicated by the sophisticated response to myocardial stressors, which impinges upon multiple aspects of cardiovascular biology. Artificial detubulation allows a more direct examination of the effects of t-tubule loss alone, enabling an examination of their physiological functions. Artificial detubulation is achieved using the detubulating agent, formamide, which results in osmotic shock, sealing off the t-tubule compartment and disrupting t-tubule structure within the cell.

Detubulation reduces cell capacitance by 30% and $\text{I}_{\text{Ca,L}}$ by approximately 80% (Kawai *et al.*, 1999). There is more $\text{I}_{\text{Ca,L}}$ concentrated in the t-tubule than data from immunocytochemistry experiments would predict, suggesting that the channels in the t-tubule are more active as well as more numerous than those in the surface sarcolemma (Brette and Orchard, 2003). Detubulation reduces transmembrane Ca^{2+} entry by 60% (Brette *et al.*, 2006). The percentage reduction in transmembrane Ca^{2+}

entry corresponds to the percentage of NCX localised in the t-tubule (Despa et al., 2003). Other currents, such as the Na^+ and K^+ currents, are equally distributed across the surface and t-tubule membrane fractions (Komukai et al., 2002, Yang et al., 2002).

Interestingly, inactivation of ICa_L is slower in detubulated cells, although not in the presence of Ryanodine (i.e. when SR Ca^{2+} release is diminished) (Brette et al., 2005). This suggests that SR Ca^{2+} release inactivates ICa_L in the t-tubules more than ICa_L in the surface sarcolemma (Brette et al., 2004a, Brette et al., 2004b, Brette et al., 2006). As noted by Orchard, this has a number of implications (Orchard and Brette, 2008). First, that the t-tubules contain a large and rapidly inactivating ICa_L , ideal for acting as a Ca^{2+} release trigger. Second, that the more slowly inactivating ICa_L arising from the surface sarcolemma may function as an SR Ca^{2+} loading mechanism (Brette et al., 2005). The differential inactivation of ICa_L in the surface sarcolemma and the t-tubules adds weight to the notion of the t-tubule as a specialised site for Ca^{2+} release.

Detubulation studies also shed light on the regulation of Ca^{2+} sparks. Detubulated cells show Ca^{2+} sparks mainly within $2\mu\text{m}$ of the cell edge (Brette et al., 2005). This does not appear to be due to ICa_L , as inhibition of ICa_L does not affect Ca^{2+} spark frequency in control or detubulated cardiomyocytes (Brette et al., 2005). It has been suggested that detubulation makes the t-tubules less organised and disrupts dyads associated with t-tubules, which reduces Ca^{2+} sparks. These results imply that the structural integrity of the dyad influences SR Ca^{2+} release. The effect of dyadic disruption on Ca^{2+} sparks is addressed in the subsequent Chapters.

1.7.3.3 Human Studies

Loss of and disruptions to the regularity of t-tubules occur in human cardiac cells from patients with HF, irrespective of the aetiology (Lyon *et al.*, 2009). Cardiomyocytes from patients with HOCM, DCM and ischaemic HF all show profound t-tubule damage, with a reduction in the t-tubule density and parameters of cell surface regularity (Figure I-9). This mirrors the findings of t-tubule disruption in animal models of HF from an ischaemic, hypertensive or tachycardic phenotype described above and suggests that t-tubule disruption is a common pathway in HF. Failing human cardiomyocytes have t-tubules that run more on the longitudinal axis and are often dilated and bifurcated rather than aligned normally along the radial axis of the cardiomyocyte (Cannell *et al.*, 2006). Recent work has described the detailed structural remodelling of the t-tubules that occurs in human HF (Crossman *et al.*, 2011).

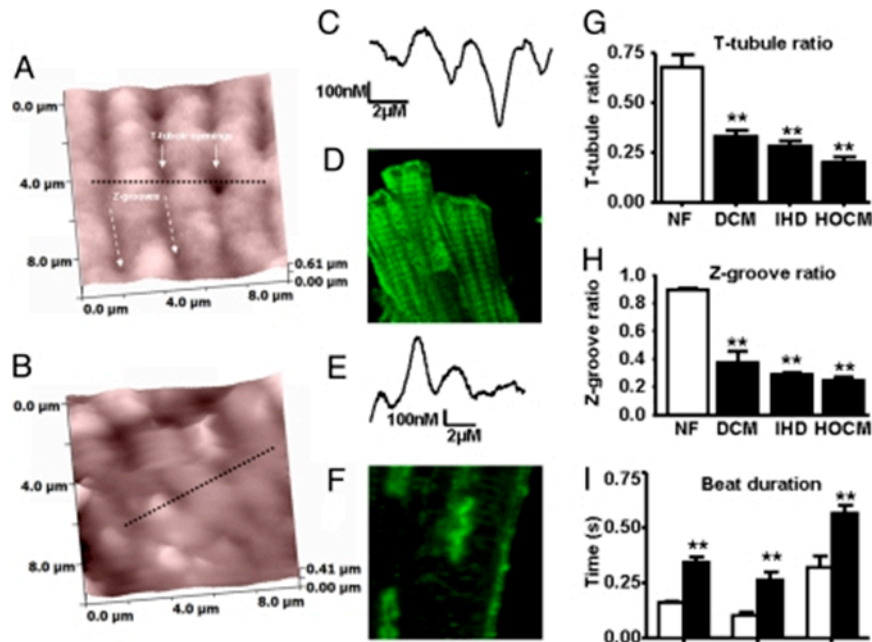


FIGURE I-9: HEART FAILURE, REGARDLESS OF THE AETIOLOGY, DISRUPTS THE T-TUBULE NETWORK

Scanning Ion Conductance Microscope images from the surface of cardiomyocytes isolated from nonfailing (A) and failing (B) human hearts. The black dotted line represents a linear trace presented as a 1-dimensional surface contour map from nonfailing (C) and failing (E) human cardiomyocytes. Confocal images after staining with Di-8-ANNEPPS in nonfailing (D) and failing cardiomyocytes (F). T-tubule (G) and z-groove (H) ratios in cardiomyocytes isolated from patients with DCM, HF secondary to Ischaemic Heart Disease (IHD), or HOCM and non-failing, NF. (I) (from left) Prolonged time to peak and decline times (R50 and R90) in human failing cardiomyocytes (solid bars, n = 12) compared with nonfailing human cardiomyocytes (open bars, n = 6). **, P < 0.01 vs. nonfailing. From (Lyon et al., 2009).

1.7.4 T-Tubule Dysregulation In Atrial Fibrillation

Although atrial cells lack the complex t-tubule structure of ventricular cells, some reports suggest that there is a functional uncoupling of the LTCC and RyR leading to asynchronous, chaotic Ca^{2+} release, which may be implicated in the poor contractility and arrhythmia of experimental models of AF (Bootman *et al.*, 2006). In larger animals, a more extensive t-tubule network has been reported with evidence that the t-

tubules of atrial cells are disrupted in models of HF (Dibb *et al.*, 2009). The loss of the network of t-tubules in atrial versus ventricular cardiomyocytes appears to have different effects on Ca^{2+} handling (Smyrniak *et al.*, 2010). Detubulation of atrial cardiomyocytes appears to have significantly less effect on cellular Ca^{2+} handling than in ventricular cells. Nevertheless, in atrial cells where t-tubules are present, they have a major impact on the Ca^{2+} transient morphology. Whether t-tubular changes play a major role in the pathological remodelling of atrial cardiomyocytes, given their paucity under physiological conditions, is a major question for future work.

1.7.5 Non LTCC-RyR Proteins

NCX expression and activity is increased in HF. Activity of NCX is enhanced by AP prolongation and increased $[\text{Na}^+]_i$ (Bers *et al.*, 2006). Greater NCX-mediated Ca^{2+} influx could stimulate Ca^{2+} release in HF, in the setting of reduced $\text{I}_{\text{Ca,L}}$. For this to be a major effect, NCX would need to be close to RyR. Importantly, Fowler shows that when cells were devoid of t-tubules, increased $[\text{Na}^+]_i$ did not enhance the SR Ca^{2+} load (Fowler *et al.*, 2004). This could be due to loss of concentrated NCX zones in the t-tubule.

Reduced $\text{Na}^+-\text{K}^+-\text{ATPase}$ appear to be important in increasing intracellular Ca^{2+} (Swift *et al.*, 2008, Louch *et al.*, 2010b). The α -2 isoform is localised to the t-tubules, which when disrupted could impair NCX and $\text{Na}^+-\text{K}^+-\text{ATPase}$ cross-talk, resulting in increased local $[\text{Ca}^{2+}]_i$ (Swift *et al.*, 2008). Other factors thought to contribute to elevated cytosolic $[\text{Ca}^{2+}]$ in HF include enhanced late Na^+ current and higher activity of the Na^+-H^+ exchanger (Valdivia *et al.*, 2005). T-type Ca^{2+} channels are normally expressed at low levels, and some studies have shown increased density in hypertrophy and HF, with a Ca^{2+} influx reaching one third of the $\text{I}_{\text{Ca,L}}$ (Jaleel *et al.*,

2008). The role of these channels in disease is poorly understood, but they are unlikely to be localised at the t-tubule. The Plasma Membrane Ca^{2+} ATPase (PMCA) was thought to possibly compensate for reduced NCX expression in HF but recent studies suggest that PMCA is reduced in HF (Mackiewicz *et al.*, 2009). Inositol trisphosphate receptors are upregulated in HF at the junctional SR and have been shown to augment SR Ca^{2+} release (Zima and Blatter, 2004). Their interaction with NCX, the t-tubule and other dyadic proteins is an important topic for future studies.

The t-tubule is thus a highly concentrated zone of electrophysiological activity and changes in all of the elements described could contribute to the changes observed during cardiomyocyte remodelling after alterations in mechanical loading. The t-tubule creates a “neighbourhood” of local, compartmentalised Ca^{2+} signalling.

1.7.6 Reversibility of T-tubule Dysregulation

A recent report by Stolen *et al* using an animal model of diabetic cardiomyopathy demonstrated disrupted t-tubular structure in cardiomyocytes and that interval training can induce improvements to the t-tubule network, contractility and CICR (Stolen *et al.*, 2009). This is an important first report of changes to the t-tubule network that are associated with improved function. Little is known of the exact biophysical regulators of the t-tubule system and this is a missing gap in the knowledge required to attempt reversal of t-tubule dysfunction. A second report recently documented the effect of pulmonary hypertension in inducing progressive t-tubular dysfunction in the heart, using recordings taken at the whole heart level (Xie *et al.*, 2012). Pulmonary hypertension results in increased afterload of the right heart, and t-tubule dysfunction appears to be an early change in the cellular remodelling process. Interestingly, the

use of sildenafil (a phosphodiesterase-5 inhibitor), a drug which acts to reduce pulmonary hypertension, induces reversal of t-tubule dysfunction (Xie *et al.*, 2012). This indicates that modulation of mechanical overload can regulate t-tubule structure, and that normalisation of mechanical overload is able to induce recovery of the t-tubule system. The administration of β -adrenergic blockade shortly after myocardial infarction in rats largely prevented t-tubule disruption many weeks later (Chen *et al.*, 2012). Importantly, the investigators observed maintenance of JPH2 expression, possibly providing a mechanism for preserved t-tubule structure. The biophysical effects of β -blockade could be numerous, including effects on blood pressure (and therefore loading of the ventricles), adrenergic stimulation (driving remodelling), and heart rate. Because of the widespread use of β -blockade clinically, this study would merit further investigation.

SERCA2a gene therapy has been associated with reversal of remodelling of the t-tubule network as discussed earlier (Lyon *et al.*, 2012). One of the emerging therapies for clinical HF is cardiac resynchronisation therapy (CRT), thought to act by promoting synchronous contraction. This has a number of effects, including improving cardiac output and reducing wall stress, and could be said to induce mechanical unloading. A recent study shows that CRT can induce reversal of t-tubule structure and Ca^{2+} handling (Sachse *et al.*, 2012).

All these studies show that the t-tubule network is a highly plastic system, which is regulated by cross-talk between multiple elements of local CICR as well as in response to altered haemodynamic conditions.

1.8 Hypothesis

The evidence presented above suggests that the t-tubule system is a labile network, sensitive to alterations in a number of biophysical properties, including mechanical load (Figure I-10). Alterations in the t-tubule network have a major impact on Ca^{2+} handling. In addition, the growing role of LVAD therapy makes the dissection of the mechanisms mediating the myocardial response to mechanical unloading a priority. Whether the functional benefit of mechanical unloading is mediated by reversal of damage to the t-tubule network is untested.

This thesis addresses the hypothesis that mechanical load affects t-tubule structure and function in a graded and reversible manner via specific molecular mechanisms.

In particular, the following specific hypotheses were tested.

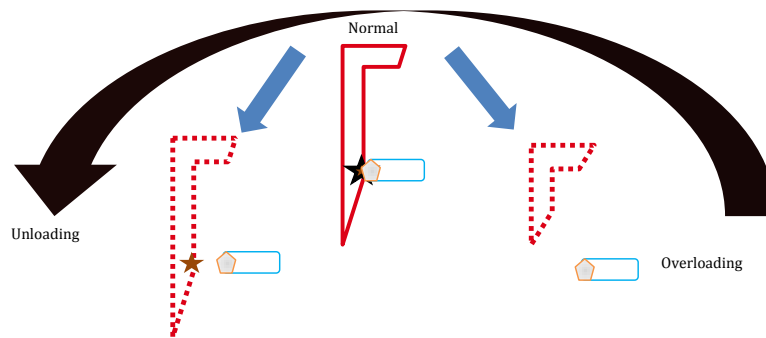


FIGURE I-10: T-TUBULE STRUCTURE IS SENSITIVE TO MECHANICAL LOAD VARIATION

Chronic mechanical overload results in t-tubule loss and irregularities, which uncouple the LTCC and RyR, which are normally tightly coupled. Mechanical unloading could also induce changes to t-tubule structure. If so, this raises the possibility that normalisation of mechanical load could normalise the t-tubule structure.

1. The t-tubule network of LV cardiomyocytes is altered by prolonged mechanical unloading, with consequences for CICR. This hypothesis was tested using a model of prolonged mechanical unloading using heterotopic abdominal heart transplantation (HAHT) in rats. Cells were isolated and studied for cellular and t-tubule structure, and local CICR (Chapter 4).

2. Mechanical unloading of chronically overloaded hearts can restore t-tubule structure with important consequences for local CICR. This hypothesis was tested using a model of mechanical unloading using HAHT following chronic coronary artery ligation (CAL). Cells were isolated and studied for cellular and t-tubule structure, and local CICR in the setting of control, failing and failing hearts following HAHT (Chapter 5).

3. The t-tubule network and local CICR is sensitive to graded variations in the degree and duration of mechanical load. This hypothesis was tested using a model of moderate mechanical unloading using heterotopic abdominal heart-lung transplantation (HAHLT) or following a model of mechanical overload, thoracic aortic constriction (TAC). Results from HAHT were compared to HAHLT. Cells were isolated and studied for cellular and t-tubule structure, and local CICR in the setting of control, HAHT, HAHLT and TAC at different time points (Chapter 6).

4. The molecular mechanisms governing t-tubule structure are not understood. In particular, the molecules that mediate the load-sensitivity of the t-tubules are not

known. Tcap is a member of the stretch-sensitive complex of the cardiomyocyte, binds the t-tubule membrane and its expression is load-sensitive and correlates with the appearance of the t-tubule network, and it was therefore hypothesised that Tcap is a regulator of t-tubule structure, which is especially important during mechanical overload. This was tested using cardiomyocytes from a mouse KO model of Tcap, under spontaneous conditions and following chronic mechanical overload (Chapter 7).

Chapter 2

General Methods

This thesis involves the use of rat and mouse models of human disease and models of clinical interventions. Single cells were isolated from the whole heart at defined time periods following surgery. An array of single cell electrophysiological techniques was used to assess local CICR and t-tubule structure. All solutions are described in detail at the end of this Chapter.

2.1 Animal Models

Four animal models were used throughout the course of this project. One was performed by Mr Manoraj Navaratnarajah (HAHT) and three were performed by myself (HAHLT, CAL, and TAC (mouse TAC surgery in Chapter 7 was performed by Dr Mutsuo Harada)). All animals were supplied by Harlan Laboratories, Blackthorn, UK.

2.1.1 Species

A number of previous studies showed that mechanical unloading in rats produced measurable effects at the level of Ca^{2+} handling after four weeks (Soppa *et al.*, 2008b). The use of a syngeneic strain (inbred Lewis rats) allows transplantation without the need for immunosuppression (Forbes and Guttman, 1984). The isolation of cardiomyocytes is reproducible and provides living cardiomyocytes, which can be assessed in terms of local CICR (Siedlecka *et al.*, 2008). Rat cardiomyocyte electrophysiology is somewhat different to human electrophysiology (Bers, 2001).

Examples of such differences include different t-tubule density, AP morphology, complement of ion channels, and heart rate (Bers, 2001). KO mice (described below) were used to assess the role of Tcap.

2.1.2 Surgical Procedures

Pre-operative preparation of the animals was similar irrespective of the operation to be performed. All operations were performed under licence by the UK Home Office and according to their guidelines for the ethical and humane treatment of animals. The use of animal models is of great use in furthering our understanding of disease, for the advancement of human and animal health. However, it is right to acknowledge that it raises significant ethical questions, and is contentious. All animals were treated with respect, with the aim of minimising suffering and discomfort.

Animals were kept in normal rodent cages in groups of up to five and had free access to food and water. Animals were sedated with 5% Isoflurane in an anaesthetic chamber and then weighed and shaved, leaving a generous border surrounding the incision area. The animals were then returned to the anaesthetic chamber for further light sedation. 1.5% Isoflurane was used throughout the surgical procedures to enable humane surgery but maintain adequate tissue perfusion and enable rapid post-operative recovery. The animals were then placed supine on the operating table and were breathing spontaneously, under Isoflurane delivered by a nose cone, when the chest was not to be opened. If a thoracotomy was to be performed, an endotracheal tube was used and the animal mechanically ventilated. The endotracheal tube was a modified 20-gauge adult peripheral venous cannula (NHS Supply Chain, UK). Using a standard paediatric laryngoscope, the larynx was visualised from the mouth, and the tongue displaced upwards using forceps. A light was used to visualise the opening of

the vocal chords, and the modified endotracheal tube was placed, as described previously for mice (Tarnavski *et al.*, 2004). Prior to ventilation, correct placement was confirmed by checking for exhaled condensation through the cannula, using a scalpel blade. Ventilation was provided by a Model 683 Rodent Ventilator (Harvard Apparatus Ltd, UK), attached to a standard gas anaesthetic rig with medical oxygen and Isoflurane (National Veterinary Services, UK). Mechanical ventilation was set to 85 breaths per minute with a tidal volume of 1.5ml. All animals received 10-15mg/kg amoxycillin trihydrate (Clamoxyl LA®, Pfizer, USA) as antibiotic prophylaxis and 10-20µg/kg buprenorphine hydrochloride (Vetergesic®, Reckitt & Colman, UK) for pain prevention. An iodine-containing solution (10% Povidone-iodine, NHS Supply Chain, UK) was applied to the skin. To maintain hydration, a bolus dose of 3ml warm saline was given subcutaneously, at the start of the operation.

All techniques were performed solo using sterile technique. Operations were performed under 3.5X magnifying loupes (Designs for Vision, London, UK).

2.1.3 Permanent Coronary Artery Ligation (CAL)

Permanent occlusion of the left anterior descending artery produces a model of acute myocardial infarction and can progress to chronic HF. This model has a number of important anatomical, physiologic and clinical considerations.

2.1.3.1 Anatomical Considerations

Coronary artery anatomy is highly variable in humans and rats. Bloor *et al* analysed 4500 rat coronary trees and found large variations which are consistent with polygenetic and environmental determination (Bloor *et al.*, 1967). The basic coronary anatomy of the rat is as follows: a left coronary originates from the left sinus of

Valsalva and passes to the anterior surface of the LV. Before descending toward the apex of the heart, it branches to supply the lateral border of the LV. The point at which it branches is variable as indicated by the variable pattern of blanching observed during coronary artery occlusion. This is the single biggest practical consideration when attempting CAL, as the presence of a variable branch point also means that a different amount of the LV will be affected after CAL. Another important consideration concerns the presence and quality of an anastomosis between the posterior descending artery and the left anterior descending artery at the apex of the heart. In the presence of a widely patent apical anastomosis, ligation of the anterior descending artery results in infarction of less myocardium, as there is more overlap in vessel supply territory. These factors increase the heterogeneity of the animal model. Therefore, echocardiography to select failing hearts is mandatory.

2.1.3.2 Physiological Considerations

CAL leads to the rapid death of the dependent myocardium due to ischaemia. The subsequent necrosis sets in motion a cascade of pathological sequelae including inflammation and ultimately leads to the replacement of cardiomyocytes with scar tissue. Since cardiac preload and afterload are not chronically reduced, the remaining myocardium must increase its work to maintain the CO. Adaptive mechanisms promote a hypertrophy in the remaining cardiomyocytes, which initially results in maintained CO. Chronic overloading of this healthy myocardium depletes the adaptive reserve and results in pathological remodelling and HF (Anversa *et al.*, 1986).

2.1.3.3 Clinical Considerations

CAL is typically described as being a model of ischaemic cardiomyopathy. In principle, it is similar to clinical ischaemic cardiomyopathy in that a coronary insufficiency leads to death of the dependent myocardium. However, it has a number of important differences. First, the natural history of coronary artery disease is gradual and involves the whole coronary tree to a greater or lesser degree. This results in some damage to the total extent of the myocardium and not an isolated region. Second, after clinical myocardial infarction, the remaining myocardium which must compensate for the loss of cell number is diseased. Therefore, the sudden, isolated loss of myocardium obtained by coronary ligation is different to clinical myocardial infarction in important respects. CAL models may be said to achieve volume and pressure overload of healthy surviving myocardium, due to an ischaemic trigger.

2.1.3.4 Operative Technique.

After anesthesia and intubation, draping and preparing the skin, a left lateral thoracotomy was started by incising the skin overlying the left 4th intercostal space using a number 22 scalpel (NHS Supply Chain, UK). Blunt dissection of Pectoralis major allowed its retraction from the operative field using a stay suture or retracting clip. Latissimus dorsi was dissected from the chest wall and retracted away from the operative field. The intercostal space to be targeted was confirmed and a needle was introduced into the thoracic cavity and a cut extended with a number 22 or number 11 blade along the intercostal space away from the sternum. The cut must not extend too medially, otherwise the internal thoracic artery can be torn which results in potentially life-threatening bleeding. Needle holders were used to widen the thoracotomy further

and the incision was held open using a rodent thoracotomy retractor (World Precision Instruments, UK).

The heart was now visible inside the pericardium, which may be divided using forceps to expose the anterior surface of the LV. In order to access the proximal portion of the left anterior descending artery, a small swab was placed over the left atrium, which was thus retracted laterally. Once the outline of the left coronary artery was visible, a 6-0 Prolene suture was placed around it at the level of the lower border of the left atrium and permanently tied. Adequate bite size was needed to ensure that the suture does not tear the myocardium while tying down. Blanching to the level of the apex was seen, which was usually blue or white in colour and associated with akinesia of the anterior LV wall. If no blanching was observed a second suture, taking a wider bite of the myocardium was taken. The suture was cut and the chest was closed with a single wide 4-0 Prolene mattress suture. The lung was re-expanded by increasing end-expiratory pressure by clamping the expiration line of the ventilator. The muscles were replaced and a saline-iodine wash of the chest was performed prior to closing the skin. The skin was closed with straight running suture using 2-0 or 3-0 silk. The animal was ventilated until it roused and then extubated. The animals were allowed to recover in a quiet, well-oxygenated and warm environment. Close observation is required to ensure smooth post-operative recovery. Monitoring thereafter consists of weekly weighing, clinical examination (respiratory rate, absence of piloerection, examination of the scar) and echocardiography.

2.1.4 Permanent TAC

Placing a band to induce a stenosis of the ascending aorta produces myocardial pressure overload, by increasing cardiac afterload. Greater LV pressures are needed to drive blood past the stenosis, therefore increasing wall stress and myocardial workload. This technique has a number of anatomical, physiological and clinical considerations.

2.1.4.1 Anatomical Considerations

This technique provides much more consistent anatomical targets than coronary ligation, but involves a much riskier operative pathway. This technique has two major variants, depending on whether the band was placed distal to, or proximal to the brachiocephalic artery. Placing the band distal to the brachiocephalic artery provides normal brain perfusion but less afterload increase. Placing the band proximal to the brachiocephalic artery produces severe overload but likely induces some cerebral hypoperfusion. This was the approach used here because of the marked increase in afterload.

2.1.4.2 Physiological considerations

In order to generate consistent cardiac remodelling, two factors must be kept constant: the size of the stenosis and the size of the aorta. In this way, the relative reduction in aortic diameter will be constant. In order to produce a fixed size of the aorta, a needle of 0.9mm external diameter was used. In order to attempt to use animals of constant aortic diameter, animals of weights of 200-300g only were used.

2.1.4.3 Clinical Considerations

TAC is often compared to clinical aortic stenosis, but there are a number of important differences. The cardinal difference is that clinical aortic stenosis (at the level of the aortic valve) involves reduced coronary perfusion, mediating part of the dysfunction, as flow is reduced prior to the pulse wave passing the coronary ostia. Therefore, TAC provides a model of chronically raised afterload alone without ischemia.

2.1.4.4 Operative Technique

In rats, an approach via the right thoracotomy was found to provide better exposure of the ascending aorta. Preparation and access was as for CAL, except on the right. Once the chest was opened, the thymus lobes were divided to provide a view of the ascending aorta. The superior vena cava (SVC) and surrounding structures were bluntly dissected from the ascending aorta. Curved forceps were passed under the aorta, followed by a 3-0 silk tie. The tie was constricted around the needle overlying the aorta. Further ties were rapidly laid down, and the needle removed. End-expiratory pressure was increased to reinflate the lungs and reduce any pulmonary oedema, by increasing alveolar pressure. The chest and skin were closed routinely.

For the Tcap experiments, in mice, a similar approach was taken except that the second rib was partially excised and the loop was placed between the brachiocephalic and left subclavian arteries. The loop was tied around a 26 gauge needle using 7-0 Prolene. Otherwise, all elements of the procedure were identical.

2.1.5 Heterotopic Abdominal Heart Transplantation For Severe Mechanical Unloading.

This operation was first described by Ono and Lindsey (Ono and Lindsey, 1969). This operation achieves virtually complete unloading of a transplanted donor heart by anastomosis of the donor aorta to the recipient aorta, and anastomosis of the donor

pulmonary artery to the recipient inferior vena cava (IVC) (Figure M-1). Blood flows from the recipient aorta into the aortic root of the donor, and as the donor aortic valve is competent, perfuses the coronaries without entering the LV. Coronary flow is returned to the right atrium (RA) through the venous coronary circulation and the coronary sinus. This blood is ejected by the right ventricle (RV) through the Pulmonary artery (PA) and into the venous circulation of the recipient via the IVC. This produces a donor LV that is virtually completely mechanically unloaded. Under the conditions of mechanical unloading in the LV, Thebesian veins in the LV wall itself open and allow a small amount of blood to directly enter the LV cavity. This blood is occasionally ejected through the aortic valve. This subset of operations were performed by Mr Manoraj Navaratnarajah, whereas I performed all the heart-lung transplantations (later). This model has a number of anatomical, physiologic and clinical considerations.

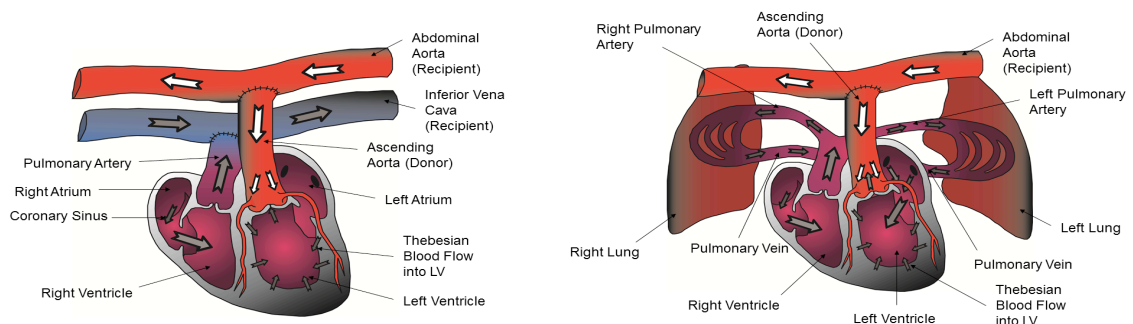


FIGURE M-1: HETEROTOPIC ABDOMINAL HEART (LEFT) AND HEART-LUNG TRANSPLANTATION (RIGHT)

These two models of provide for severe (HAHT) (left) or moderate (HAHLT) (right) mechanical unloading. Arrows within structures show direction of blood flow.

2.1.5.1 Anatomical Considerations

Matching of the donor and graft aortic vessels' size is imperative for proper anastomotic technique. The most critical component of this operation is to avoid kinking of the aorta and inferior vena cava (IVC).

2.1.5.2 Physiological Considerations

This operation achieves preload reduction by reducing the amount of blood flow into the LV. The LV occasionally fills with Thebesian blood flow which is ejected, and thus the LV does not experience constant afterload reduction. In performing the operation, the most important physiologic consideration is to avoid myocardial damage by achieving rapid technique and adequate cardioprotection.

2.1.5.3 Clinical Considerations

The unloading achieved by this operation is different to that achieved by LVADs in several important respects. LVADs act on an intra-thoracic and innervated heart, whereas the heterotopically transplanted heart is deinnervated and subjected to intra-abdominal, not intrathoracic pressures. LVADs enhance coronary blood flow whereas heterotopic abdominal transplantation does not impact greatly on coronary blood flow.

2.1.5.4 Recipient Preparation

To minimise the ischaemic time of the donor heart, the recipient animal was prepared first. Animals were shaved from penis to xiphisternum and iodine was applied to the shaved skin. Animals were placed supine and limbs and tail taped down onto a warming blanket. Anaesthesia (1.5% Isoflurane) was administered via nose cone.

1. A midline laparotomy from xiphisternum to above the pubic vein was made.
An abdominal retractor was placed.
2. Using cotton wool buttons, the bowel was carefully retracted from the abdomen, and wrapped in wet gauze.
3. Using cotton wool buttons, the retroperitoneal fascia was dissected, to reveal the great vessels (aorta and IVC) for a length of approximately 4cm. Using non-toothed Debaquey forceps (World Precision Instruments, USA), the vessels were lifted and bluntly dissected free from surrounding structures. Any lumbar branches were ligated using fine sutures such as 4-0 Mersilk sutures (World Precision Instruments, USA).
4. Bulldog microclamps were applied to the top and bottom of these vessels, leaving as much space between clamps as possible. A small hole in the aorta (using 30 gauge needle) was made in the middle of the longitudinal section of clamped aorta.
5. The IVC was perforated.
6. Using straight microscissors with blades of 5mm length (World Precision Instruments, USA) the needle holes were extended to leave an aortotomy and venotomy, both of which were approximately 4mm in length and were in the midline of the vessels.
7. The bowel was returned to the abdomen, and wet again with saline. The level of Isoflurane anaesthesia was reduced to around 1% and the heating mat was switched off (thereby reducing the likelihood of spinal cord ischaemia) whilst moving onto the donor harvesting operation.

2.1.5.5 Harvesting The Donor Heart.

1. An incision was extended from the jugular notch to the level of the pubic vein. The abdominal muscles were divided, and the great vessels were exposed as before. Similarly the heart was exposed in the thorax by cutting the sternum. A long silk suture was tied around both the abdominal aorta and IVC, which can be used to lift them with ease.
2. 4000 units of heparin (LEO Labs LTD, Bucks, UK) was injected into the IVC via a 30-gauge needle and allowed to circulate for 1 minute. Clamping the great vessels ensures the abdominal aorta was distended, facilitating subsequent aortotomy. Then a transverse cut was made over the anterior surface of the abdominal aorta, sufficient to introduce a 25mm radial artery cannula and guide wire. The cannula was advanced to the level of the aortic root. The heart was arrested by administration of at least 50ml of cold cardioplegic solution (St Thomas II) slowly through the abdominal aortic cannula, avoiding LV distension.
3. Blunt dissection of the IVC, left and right SVC was performed. Exposure of the left SVC can be challenging, as it lies beyond the azygous vein and just beyond the LA. Special care must be taken here to avoid the PA and LA. Once all cavae were exposed they were permanently ligated using 4-0 Mersilk (World Precision Instruments, USA).
4. The aorta was transected, just before the brachiocephalic trunk, in one cut. The same applies for the PA, which was divided just below the bifurcation.
5. Dissection in the aorta-PA window with cleaning of fat and fibrous tissue was helpful at this stage and aids later implantation.
6. The pulmonary veins were then tied off en masse and the heart removed.
7. The heart was kept in ice cold saline until ready for reimplantation.

2.1.5.6 Heterotopic Transplantation Of The Donor Heart

1. The recipient vessels should not be over-cleaned, as a little fascia can be used to support the suture line and reduce the risk of bleeding. For the anastomosis, the cut ends of the donor vessels should be similar in size to the recipient aortotomy and venotomy. If the donor vessels were very small, a diagonal incision of the donor vessels was made to enlarge the relative circumference. If the donor vessels were excessively large, then an incision on the recipient vessels would be extended. Alternatively, the relative proportion of donor: recipient vessel circumference taken with each bite was increased, thereby adjusting for the discrepancy.
2. The donor heart was positioned on the left side of the abdomen, correctly oriented such that the RA and RV appeared anteriorly. Correct orientation was crucial in order to avoid aorta and PA kinking following anastomosis.
3. 8-0 Prolene with a 5mm round-bodied needle (Ethicon, Johnson and Johnson UK) was used throughout. One Prolene suture was placed at the heel and one at the toe of the anastomosis. A continuous suture line was made all the way to the opposite anchor suture and eventually tied to the short end of the opposite anchor suture, taking care not to purse-string the anastomosis. The easiest method was to suture one half of the anastomosis and then 'flip' the heart to the opposite side (i.e. from the left side of the abdomen to the right) and then complete the other half of the anastomosis. The ideal aortic anastomosis in rats of this size results in an everted intima-intima edge of approximately 0.5mm. Bite size should be approximately 1.5mm.
4. Before commencing the PA-IVC anastomosis, the heart was covered in a fresh cold swab. The PA should be clearly visible, and the anastomosis was then completed as before.

5. Once the anastomosis was complete, it was examined for any gaps, which can be obliterated using a single interrupted suture. The gauze covering the heart was removed. First, the distal clamp was removed and the anastomoses were examined for any bleeding (bright red indicating arterial and dark purple indicating venous bleeding).
6. The top clamp was then removed and the heart should ideally commence beating within 5 seconds.

The abdomen was closed using continuous 5-0 Vicryl (NHS Supply Chain, UK) and the animal allowed to recover alone as for other surgical procedures.

2.1.6 Heterotopic Abdominal Heart-Lung Transplantation For Moderate Mechanical Unloading

When the lungs are left connected to the heart via the pulmonary circulation (PA and pulmonary veins remain connected), the LV receives blood from the pulmonary veins. This produces a LV which is moderately loaded with the small volume of coronary sinus blood returning to the left heart via the pulmonary circulation. The two models of heterotopic heart transplantation are shown in Figure M-1.

This operation involves a single aorto-aortic anastomosis in an end-to-side fashion as described for the HAHT procedure. The lungs remain connected to the donor heart by the pulmonary artery and pulmonary veins, but do not require ventilation as they merely act as a vascular conduit to get the coronary sinus blood from right heart to the LV. It does not require operation on the IVC and PA, which are relatively fragile structures. However, it does require the careful handling of the pulmonary circulation and lungs which are prone to bleeding. In addition, it is preferable to tie off the

airways to avoid the collection of fluid which may lead to an inflammatory response.

The heart-lung block should be wrapped in wet gauze during the operation.

2.1.6.1 Recipient Preparation

This is in principle the same as for the total unloading operation. Important differences are:

1. No IVC incision was performed.
2. The IVC and aorta were separated from one another by blunt dissection for greater access to the aorta and to reduce the risk of injuring the IVC during suturing of the aorta. This also allowed the vascular clamps to be placed only on the aorta.

2.1.6.2 Harvesting The Heart-Lung Block

Steps 1-3 were identical to the steps required to harvest the heart alone (dissect vessels; give Heparin through IVC; give cardioplegia through abdominal aorta). Dissection of the left SVC should be performed carefully to avoid damage to the PA. Once the heart was free from the cavae, the aorta was cut. The PA and pulmonary veins were left connected to the block.

1. The trachea was cut as high as possible and pulled on whilst sharply dissecting behind the trachea. This frees the heart-lung block, leaving only the oesophagus connecting it to the animal, which should be cut.
2. With the block on ice, any thymic tissue or fat was cleaned from the block.
3. Because the right heart will pump against pulmonary vascular resistance, meticulous protection was mandatory.

2.1.6.3 Reimplantation Of The Heart-Lung Block.

1. For the aortic anastomosis, and with the lungs and heart wrapped in gauze with ice, two anchor sutures were placed at the heel and toe of the anastomosis. The anastomosis was completed as described for the total unloading procedure.
2. The lungs should rapidly become pink, and the heart should beat. Rarely, it was necessary to give short-term cardiac massage, especially to the right heart.
3. The abdomen was closed in layers as before.

2.1.7 Transplantation Of The Failing Heart

Apart from the use of these models to understand the consequences of mechanical unloading on the normal heart, they have also been used to understand the effect of mechanical unloading on the failing heart. There are a number of considerations when attempting this operation.

Adhesions may complicate the harvesting of the failing donor heart, following chronic CAL. This necessitates careful dissection which avoids trauma to the atria and the aorta. Adhesions may also make the aortic edge less defined, which can compromise the quality of the anastomosis. Elevated right heart pressures can lead to dilation of the pulmonary artery, which should be taken into account during the PA-IVC anastomosis.

The failing heart is hypertrophic and requires a greater degree of cardioprotection. This may be achieved by addition of more cardioplegia during the harvesting operation, in conjunction with greater care in keeping the heart cool.

2.1.8 Echocardiographic Assessment

To assess the advent of myocardial hypertrophy and subsequent HF, transthoracic echocardiography was used. The animal was anaesthetised as before and maintained on 1.5% Isoflurane. The chest hair was clipped and warm ultrasound transmission gel was applied to the skin surface gel (Sonogel®, AnaWiz Ltd, UK). An ACUSON Sequoia™ C256 (Siemens Medical Solutions, USA) echocardiography machine with a 14MHz phased-array transducer (15L8-S, Siemens Medical Solutions, USA) was used.

Two modes of operation were used: two-dimensional mode and motion (M)-mode (Figure M-2). Scanning in the two-dimensional mode allows visualisation of the LV cavity and walls. It is imperative that a similar segment of the ventricle is selected for assessment to ensure fair comparison. To do this, a level just above the papillary muscles was selected. Using the draw mode, a line was placed at the border of the LV cavity in diastole and again in systole. This allows comparison for the calculation of ventricular volumes and EFs.

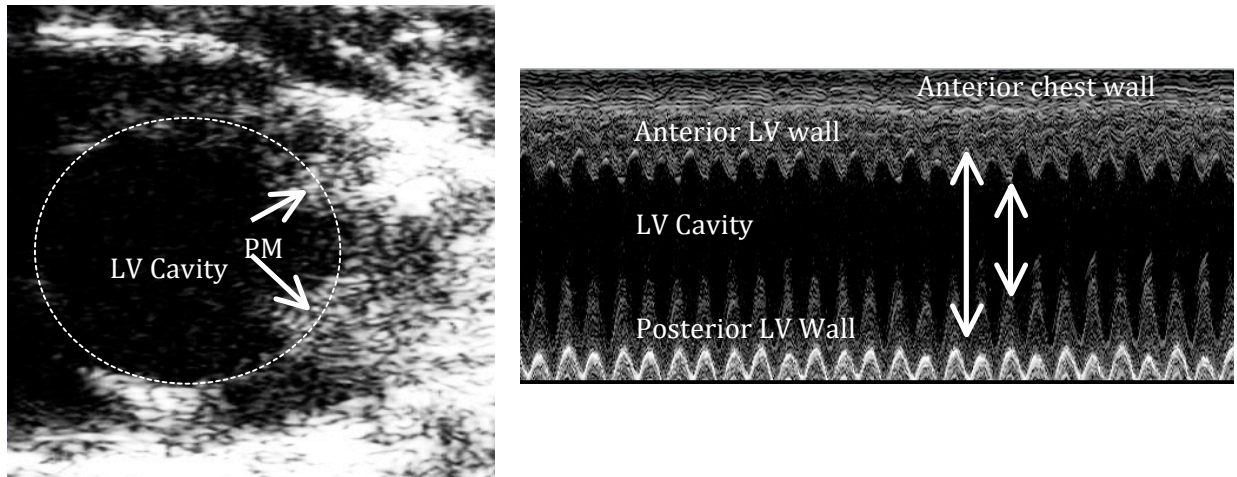


FIGURE M-2: TWO DIMENSIONAL (LEFT) AND M-MODE (RIGHT) ECHOCARDIOGRAPHY

Left, two dimensional echo involves tracing the LV cavity in diastole (shown) and then in systole (not shown). Right, M-mode involves tracing the anterior LV wall, LV cavity and posterior LV wall in systole and diastole. This allows calculation of the values related to the structure and function of the LV. The longer line represents the LV cavity in diastole and the shorter line represents the LV cavity in systole. PM – Papillary muscles.

M-mode echocardiography consists of scanning a line across the ventricles, which was tracked in time (Figure M-2). Animals undergoing CAL and TAC underwent echocardiography before harvesting. Echocardiography of the transplanted heart was technically prohibitive and the values obtained do not provide an accurate assessment of LV function, as the values are load-dependent.

The plane of view used should be consistent because the wall dynamics differ significantly at different myocardial levels following myocardial infarction (Rottman *et al.*, 2007). A two-dimensional short axis view just above the papillary level was selected to assess LV function and confirm presence of scar in the anterior wall for experiments involving CAL. Images were acquired at end-diastole and end-systole from a cine-loop and EF was calculated. M-mode images of the same level were

acquired for measuring LV wall thickness (LV posterior wall in systole (LVPWs) and diastole (LVPWd)), LV chamber diameter (in diastole (LVd), systole (LVs)), and LV function. EF was the percentage of the end-diastolic volume of blood which was ejected (in this case, by the LV) at end systole. The formulae which derive these measurements (based on M-mode echocardiography) are as below³.

$$\text{LV End Diastolic Volume (LVEDV)} = (7.0/(2.4 + \text{LV d})) \times \text{LVd}^3$$

$$\text{LV End Systolic Volume (LVESV)} = (7.0/(2.4 + \text{LV s})) \times \text{LVs}^3$$

$$\text{LV EF} = ((\text{LVEDV} - \text{LVESV}) / \text{LVEDV}) \times 100$$

$$\text{LV \% Fractional Shortening (FS\%)} = ((\text{LV d} - \text{LVs}) / \text{LVd}) \times 100$$

2.1.9 Knock-out Telethonin Mouse Model

Telethonin-deficient animals were generated by replacing the coding regions of exon 1 and 2 by a lacZ-neomycin cassette to monitor endogenous gene expression using homologous recombination. These animals were generated by the group of Professor Ralph Knoll, at Imperial College London. Genotyping was performed in that institution (Knoll *et al.*, 2011).

2.1.10 Controls

For transplanted normal hearts, the recipient's own heart was used as a control. For CAL and TAC, Sham-operated sex and age-matched hearts were used. For KO animals, non-littermate age, sex and strain-matched animals were used.

³ Echo Made Easy. Sam Kaddoura. Churchill Livingstone, Second Edition, 2009.

The protocol for sham surgery was identical to TAC/CAL except for the critical step of tying the suture down (whether around the left coronary artery) or the ascending aorta.

2.1.11 Harvesting The Operated Heart

Whichever model was used, the heart must be carefully removed. This involves repeat anaesthesia, shaving of the animal and surgical access to the thorax or abdomen. Careful dissection of adhesions allows a clean cut of the aorta. This facilitates aortic cannulation for enzymatic digestion for the isolation of single cardiomyocytes. When the heart was removed, it was placed in a warm normal tyrode (NT) solution containing 1000 units of heparin per ml, to avoid clot formation. Subsequently, the heart was transferred to ice cold NT (again containing heparin) to arrest the heart.

2.2 Isolation Of Single Living Cardiomyocytes

Living single cardiomyocytes were isolated by enzymatic digestion after perfusing the heart with enzymes using a Langendorff setup. This involves the use of Collagenase (type 2), an enzyme which breaks down collagen - the major constituent of the extracellular matrix. In addition, Hyaluronidase was also used which degrades hyaluronate, another major constituent of the extracellular matrix. In these studies, cardiomyocyte isolation was carried out using a modification of standard enzymatic digestion protocols described previously (Tytgat, 1994). The aorta was cannulated using a 16-gauge cannula and fixed securely with one or two ligatures. The heart was retrogradely perfused with NT solution at 37°C, bubbled with 100% oxygen. Perfusion was achieved using a pump (MasterFlex® L/STM Economy Drive, Cole-Parmer® Instrument Company Ltd., UK) and flow rate gradually increased until the vessels were cleared of blood and the heart was contracting well. The heart was then arrested by perfusion with low Ca^{2+} solution for 5 minutes. The hearts were then perfused with Buffer A, containing Type-2 collagenase (1mg/ml, Worthington Biochemical Corporation, USA; Product code: CLS2, 292 units/mg), and

hyaluronidase (0.6mg/ml, Sigma®, Sigma Aldrich Company Ltd, UK; Product code: H-3506, 451units/mg) for 8 minutes. Hearts were then taken off the Langendorff system and the atria and right ventricle were removed.

For non-CAL hearts, the entire LV was used. For CAL-hearts, the scar was excised with a border zone and viable LV was cut into cubes, approximately 2mm³. These cubes were resuspended in fresh Collagenase and Hyaluronidase solution and agitated for five minutes in the presence of 100% oxygen in a waterbath at 37°. The digested tissue was gently agitated to give a single cell suspension, which was filtered through a nylon mesh with an average pore size of 300µm. The residue was then resuspended in Buffer A with fresh Collagenase and Hyaluronidase for another five minutes. The cell suspension was centrifuged at 500 rotations per minute (rpm) for one minute (Sorvall® LegendRT, Kendro Laboratory Products, Germany), and the supernatant was discarded and cell pellet resuspended in Buffer A without any enzymes and re-centrifuged at 500 rpm for one minute. The supernatant was discarded again and cell pellet resuspended in Buffer A without enzymes, stored at room temperature and all cellular experiments were performed within six hours of cell isolation. This method provided reliable isolation of rod-shaped cardiomyocytes.

For mice isolations, a smaller 24-gauge cannula was used. In addition, Proteinase enzyme (2.7mg in 20ml Buffer A) was added to enhance enzymatic digestion at the Langendorff enzyme-perfusion stage for one minute prior to Collagenase/Hyaluronidase digestion. Collagenase/Hyaluronidase digestion lasted five minutes.

A photograph of the cell isolation setup used is shown in Figure M-3.

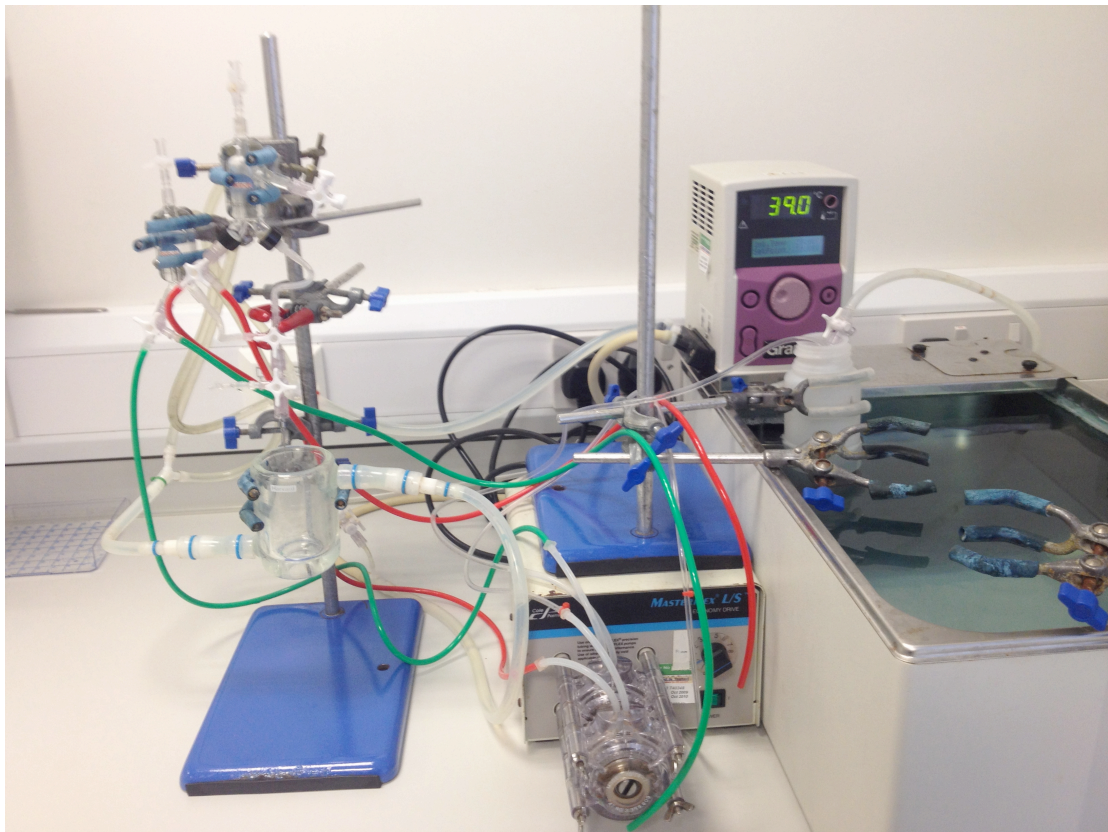


FIGURE M-3: CELL ISOLATION SETUP

The waterbath contains solutions with three lines going to each solution. An output line to the Langendorff setup, an input line to receive the solutions recycling in the Langendorff and an oxygenation line. The input/output lines are controlled by the pump. Each solution goes to its own bubble trap to ensure that any air bubbles do not perfuse the heart, which could cause air embolus in the coronaries. The heart is attached (not shown) to a cannula within the water jacket, which maintains physiological temperature.

2.3 Confocal Microscopy and Fluorescence

Confocal microscopy and fluorescence form a central part of this thesis and deserve explanation. Fluorescence is the property of emitting a different wavelength of light than that at which the sample was excited. By using dyes that target specific molecules or structures, it is possible to detect these specific emitted fluorescence signals and therefore identify those structures.

Confocal microscopy was invented by Minsky in 1955⁴. He proposed a method to image samples on a pixel by pixel basis. The confocal microscope involves several key components to achieve this. First, a series of lenses focus light onto a focal point. To promote the collection of this light, a screen with a pinhole is added, which allows only light at the desired focal point through. The pinhole is **conjugate** to the **focal** point of the lens, providing a “confocal pinhole”. Other important components are the dichroic mirrors, which reflect light below a specific wavelength. The benefit of being able to select the light detected in this way has a drawback, in that the light which is let through is of low intensity. The solution is to use a laser which has sufficient intensity to enable detection of highly selected light. The point which is imaged is dictated by internal mirrors which are motorised. In this way, laser confocal microscopy enables point illumination and rejection of out of focus light.

The Laser Scanning Microscope (LSM 510) Zeiss Axiovert inverted confocal microscope was used for all experiments. Cardiomyocytes were observed through a Zeiss EC Plan-NeoFluarTM ×40 oil-immersion objective (numerical aperture = 1.3). The pinhole was set to 1 Airy unit in all experiments. One Airy unit generally gives the best signal to noise ratio. Data was collected in 12-bit mode, with unidirectional

⁴ US Patent Number 3,013,467 in 1961

scanning for Di-8-ANEPPS protocols and bidirectional scanning mode for Fluo-4 experiments to reduce the scan time. The gain and offset of the channel were kept as constant as possible: detector gain was set between 700-800 in all experiments, amplifier offset was set to 0.04, and amplifier gain was 1. Excitation was using a 488nm Argon Laser set at 5% transmission. Emission was collected through a 505nm long-pass filter.

2.3.1 Imaging Of The T-Tubule Network

The t-tubules were assessed in the following ways:

-Using Di-8-ANEPPS and confocal microscopy: during these experiments, the cell was imaged throughout its depth to enable three-dimensional reconstruction for the assessment of the density of the t-tubule network and also to calculate the cell volume. One plane of the cell was selected for high resolution scanning, which allowed assessment of the regularity of the t-tubule network.

-EM: cells were fixed and embedded to allow sectioning to image the t-tubules.

-Scanning Ion Conductance Microscopy: performed on single cardiomyocytes to provide an image of the surface of the cell membrane.

2.3.2 Di-8-ANEPPS

Di-8-ANEPPS (di-8-aminonaphthylethylenylpyridinium) (Molecular Probes, Invitrogen, Oregon, USA) is a fluorescent, voltage-sensitive membrane binding dye. Di-8-ANEPPS is a zwitterionic molecule and therefore is less susceptible to internalization than Di-4-ANEPPS, permitting extended observation of the cell

membrane. The excitation maxima is 465nm and the emission maxima is 635nm. Di-8-ANEPPS is non-fluorescent until bound to membranes.

The cell membrane was stained with Di-8-ANEPPS using the following protocol. A cell suspension was loaded with 10 μ M Di-8-ANEPPS (solved in DMSO, dimethyl sulfoxide, Sigma-Aldrich, UK) and left for ten minutes. The supernatant was removed from the cell pellet and fresh Buffer A was added to wash the cells. A few drops of cells were added to a glass-bottom 35mm Petri dish coated with Laminin (5 μ l of 1 mg/ml Laminin solution (Sigma-Aldrich, UK)) and left to attach for five minutes.

Cells were selected in an orderly fashion, starting from the centre of the dish and working outward in a circular fashion. Any cell, irrespective of size or appearance was imaged, unless it met any of the following rules:

- touches another cell
- was not rod-shaped
- was contracting
- was not stuck flat

The microscope was then switched to laser scanning mode, and a digital crop along the longitudinal axis of the cell was made. The focal plane of the cell was manipulated in 1 μ m steps until a plane was found which did not show nuclei. An initial scanning stack was used to define the top and bottom of the cell. Then, another scan was taken, where each optical slice was recorded four times and averaged to enhance the signal to noise ratio. After this, a single high resolution image through a chosen plane, averaged 16 times, was recorded.

Offline analysis of the t-tubule density and cell volume was performed simultaneously in ImageJ (using a custom-written macro (provided by Dr Mark Stagg) (U.S. National Institutes of Health; <http://rsb.info.nih.gov/ij/>)) and previously described (Stagg *et al.*, 2008). The steps of this macro are:

- Conversion to 8-bit images
- Autothresholding of the images to generate binary images. This employs serial divisions of the “top” and “bottom” ends of the range of foreground and background pixel intensities.
- Calculation of the average percentage area of staining at every optical slice. These values were further divided into ten regions along the length of the cell (z1-z10). z1 and z10 were not included as these regions show dense staining, related to the cell edge.
- The mean of these values excluding the cell edge was taken as the average Di-8-ANEPPS staining, a marker of the t-tubule density.

Using a second macro, the whole intracellular space of every optical slice was stained and used to generate a three-dimensional reconstruction, where all optical slice volumes were added to provide a total cell volume.

Assessment of the t-tubule regularity was performed using a custom-written macro in Matlab. Initially, images were prepared in ImageJ. This involved rotation of the image to ensure the t-tubules were aligned in the vertical direction. Using the autothreshold function of ImageJ, a binary image was created. A central portion of the cell membrane was selected, always of fixed dimensions (140 pixels in length and 25

pixels wide) to allow comparison between cells and cell groups of differing sizes. A plot profile was then generated from this binary image and exported to Matlab, where a standard Fourier transform was performed. This plotted the underlying frequencies in the image and provided a power-frequency curve. The dominant peak always occurred at the t-tubule frequency, because the images were dominated by the regular t-tubules. The power of that peak, at approximately 0.5 cycles per micron (therefore occurring every 2 microns, approximately the length of a sarcomere) provided a t-tubule regularity index. This methodology was adapted from published reports (Swift *et al.*, 2008).

2.3.3 Electron Microscopy

Isolated cardiomyocytes were attached to coverslips using a Shandon Cytospin 2 ® centrifuge (Wolf labs, York, UK). This allows all the cells to align longitudinally along the coverslip, preventing random orientation during fixation. Coverslips were washed twice in 0.1M phosphate buffer, post-fixed in 1% osmium tetroxide for one hour and processed for transmission electron microscopy (TEM) using 2.5% glutaraldehyde in 0.1M phosphate buffer for 24 hours at 40°C. Ultra-thin sections were post-stained using 2% aqueous uranyl acetate and lead citrate for ten minutes in each solution and viewed with a JEOL 1200 EX microscope (Herts, UK). Digital micrographs were taken using Gatan digital micrograph software (Gatan, Pleasanton, USA) at 30000X magnification and analysed by measuring the maximum t-tubule diameter of transverse t-tubules (along the long-axis) and the number of t-tubules per optical section.

2.3.4 Scanning Ion Conductance Microscopy (SICM)

SICM uses a micropipette which scans close to the cell surface to provide a non-optical, no-contact method of imaging the cell surface. As the pipette approaches the cell surface, the resistance of the pipette increases, and current falls. This is measured through an amplifier, and a corresponding voltage is applied to prevent collision with the cell surface. Hopping mode was used to enable the imaging of vertical topographical structures (Novak *et al.*, 2009). This means that after each approach to the cell surface, the micropipette returns to a preset point away from the cell surface, at which point a reference current is measured, and then the micropipette descends toward the cell surface until the current falls by 0.5 % of the reference current. The z-position at which this 0.5 % reduction in reference current is achieved is recorded, and thus the changes in current produce a trace outline of the cell surface. As the micropipette navigates the entire cell surface, an image was constructed (Figure M-4). In all SICM experiments, micropipettes and the bath solution contained the same physiological L-15 medium (Life Technologies, Inc., Parsley, UK), so that salt concentration gradient potentials and liquid junction potentials were not generated. To quantify the data obtained during scanning, an index of the completeness of the z-grooves on the surface of cardiomyocytes (z-groove index, Figure M-4) was used (Gorelik *et al.*, 2006). The z-groove describes the domes and troughs of the surface of cardiomyocytes, and t-tubule openings are known to sit in these z-grooves. To calculate the z-groove index, the maximum extent of z-grooves observed on single SICM images was measured and divided by the total estimated z-groove length, as if they extended across the whole surface.

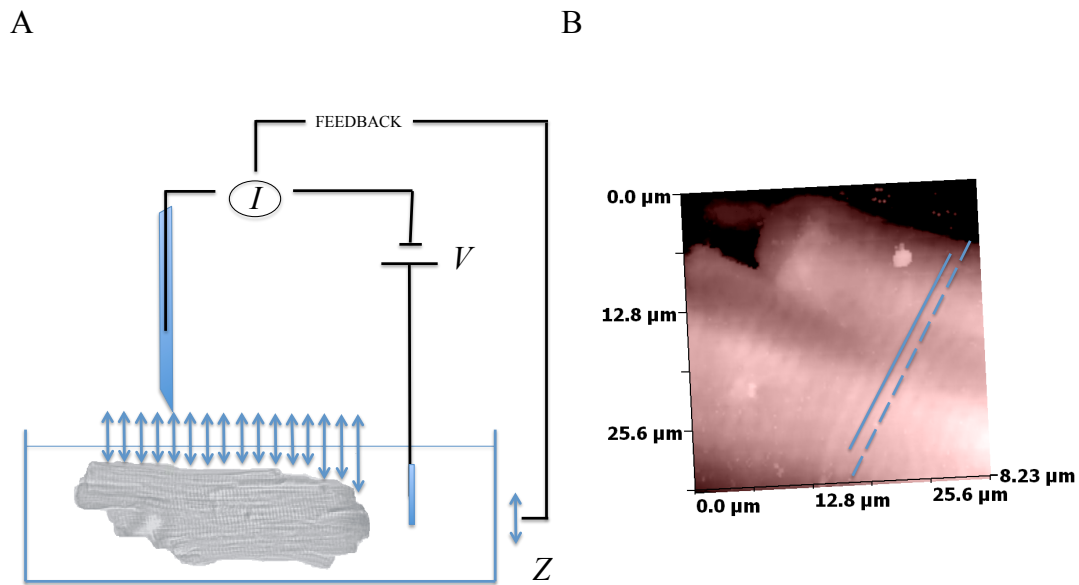


FIGURE M-4: SCANNING ION CONDUCTANCE MICROSCOPY

(A) The cells were attached to a coverslip; a micropipette in hopping mode scans the surface by returning to a preset level and approaching the cell again. The current through the pipette falls as it approaches the cell surface. A corresponding voltage is applied to keep the pipette from touching the cell surface. (B) A single z-groove index is calculated by dividing the measured length of an observed z-groove (solid line) by the predicted length of a z-groove spanning the entire surface (dashed line).

2.3.5 Assessment of Ca^{2+} Handling

Ca^{2+} handling was assessed using the following techniques:

Fluo-4 assessment of Ca^{2+} transients: single stimulated cells loaded with Fluo-4 were imaged in line scanning mode and the change in fluorescence recorded using confocal microscopy.

Fluo-4 assessment of Ca^{2+} sparks: single non-stimulated cells loaded with Fluo-4 were imaged in line scanning mode and the change in fluorescence recorded using confocal microscopy.

Whole Cell Patch Clamping: used to measure the whole cell $\text{I}_{\text{Ca,L}}$. Current-voltage relationship, activation and inactivation properties and cell capacitance can be recorded during these experiments. These experiments were performed by Dr Urszula Siedlecka.

Measurement of SR Ca^{2+} content: Single cells labelled with the Ca^{2+} -sensitive fluorescent dye, Indo-1 were rapidly superfused with caffeine and the peak of the fluorescence rise was taken as a measure of SR Ca^{2+} content.

2.3.6 Fluo-4 assessment of the Ca^{2+} transient

Fluo-4-acetoxymethyl ester (AM) (Molecular Probes, Invitrogen, Oregon, USA) is a non-fluorescent molecule which easily crosses the membrane and then undergoes de-esterification to form the membrane-impermeable fluorescent dye Fluo-4. Fluo-4 fluorescence is Ca^{2+} -dependent and rapid allowing visualisation of the millisecond scale of the Ca^{2+} transient. Single cells were loaded with 10 μM Fluo-4-AM (dissolved in DMSO) for 20 minutes and then washed with Buffer A. 30 minutes were allowed for de-esterification.

Cells were plated as for Di-8-ANEPPS. During Fluo-4 experiments, the cells were perfused with NT containing 1mM Ca^{2+} . The perfusion system consisted of NT perfusing the dish under gravity, and a suction system to remove excess solution. A

custom-made plastic insert was used to support the platinum wires used to stimulate single cells. These wires were connected to a standard bipolar stimulus generator. All experiments used 1V stimulus at 1Hz. The bath was kept at 37° C.

The same confocal settings as for Di-8-ANEPPS were used except that during the Fluo-4 experiments, bidirectional scanning was used to increase the scan speed, and line scanning mode was used. This involves drawing a line across the longitudinal axis of the cell. This line was scanned continually for the duration of the scan. The scan therefore captures changes in time along a specific location in the cell (Figure M-5). The changes in fluorescence provide a measure of the change in Ca^{2+} fluxes and can be analysed to provide information regarding properties of the Ca^{2+} transients. The 100 pixel horizontal bar on all experimental traces corresponds to 22 μm .

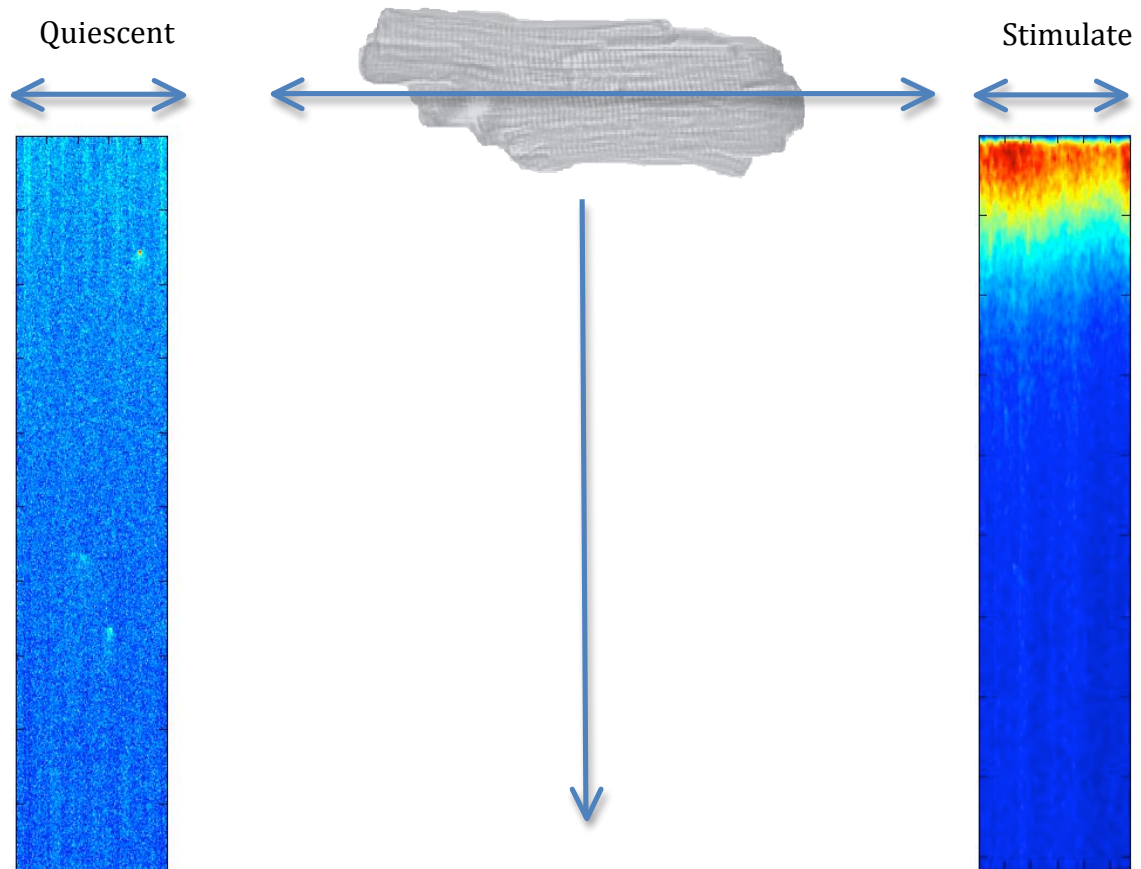


FIGURE M-5: LINE SCANNING CONFOCAL MICROSCOPY OF FLUO-4 LABELLED CARDIOMYOCYTES

A line is drawn across the length of a rod-shaped cardiomyocyte. When stimulated, this line shows the Ca^{2+} transient. When unstimulated, Ca^{2+} sparks are observed.

The analysis of the Ca^{2+} transients was performed according to the following protocol. Images were cropped to remove the cell border component of line scans. Custom-written Matlab Protocols (written by Dr Mark Stagg) were used to calculate the time to peak (from the stimulus to the peak fluorescence at each pixel), time to 50% and 90% decline (from the peak fluorescence) and the peak change in fluorescence (amplitude). The calculation of the time to peak on a pixel-by-pixel basis allows the calculation of the variance of the time to peak. This can be inherently

noisy, due to changes in time of a single pixel (e.g. see two-dimensional traces on pages 150 and 168). However, the overall effect of this noise is limited as the number of transients analysed becomes larger. For this reason, as many transients as possible were analysed.

The input information required for this (and Ca^{2+} spark) analysis was:

- The spacing sample, which is the ratio between a pixel and a micrometer.
- For sparks, the detection criterium is set to 3.8 times the background value.
- The scan time, which provides the total time required to scan one line.

2.3.7 Fluo-4 Assessment Of Ca^{2+} Sparks

The term Ca^{2+} spark originates from Cheng *et al.*, who visualised focal Ca^{2+} release events in rat cardiomyocytes (Cheng *et al.*, 1993). Ca^{2+} sparks are elementary Ca^{2+} release events that are restricted in space and time. They raise the local $[\text{Ca}^{2+}]$ to a peak of approximately 200nM, have a time to peak of approximately 10ms, and decline with a decay time constant (τ) of approximately 25ms (Cheng and Lederer, 2008).

The same cells (and therefore loading conditions) used for Ca^{2+} transients were employed for measurements of Ca^{2+} sparks. After a period of steady-state 1Hz field-stimulation, a period of 30s quiescence was allowed before line scans were collected. Up to 15000 lines ranging from 500 to 963 μs were scanned. Acquired x-t scans were exported as high-resolution tagged image file format (TIFF) digital files.

Similar to the analysis of the Ca^{2+} transients, analysis of the collected TIFF image

files was performed offline using custom scripts written by Dr. Mark Stagg. These were used in Matlab® R2006b (MathWorks Inc. MA, USA) following the algorithm for automatic Ca^{2+} spark detection described by Cheng *et al* (Cheng *et al.*, 1999). As for the Ca^{2+} transient analysis, detection criteria for Ca^{2+} sparks were set at 3.8 times the standard deviation above the mean background (F_0). The frequency of Ca^{2+} sparks was also obtained. During the Matlab macro protocol, images are generated of line scans with sparks labelled for identification. This allows visual assessment in case sparks have been double-counted or if a region of abnormally high Fluo-4 is causing artificial sparks. The amplitude of Ca^{2+} sparks is defined as the peak fluorescence over the background fluorescence (F/F_0). Ca^{2+} sparks were also analysed for their x and y dimensions, providing the full-width at half-maximal (FWHM) and full-duration at half-maximal (FDHM) parameters. The output was a Microsoft Excel file.

2.3.8 Measurement Of The SR Ca^{2+} Content

Measurement of the SR Ca^{2+} content involved the use of the IonOptix system and loading cardiomyocytes with Indo-1-AM. Indo-1 shows maximum absorption at approximately 360nm. Indo-1's maximum emission in zero Ca^{2+} is 495nm and goes toward 405nm when Indo-1 binds with Ca^{2+} . Thus, as the $[\text{Ca}^{2+}]_i$ increases, emission at 405nm rises, but emission at 495nm falls. In this way, the ratio of the emission at 405nm with that at 495nm provides a measure of $[\text{Ca}^{2+}]_i$ (Poenie and Tsien, 1986). This ratiometric property makes Indo-1 a good tool for measuring SR Ca^{2+} content. In addition, it makes the measurements less sensitive to unequal loading or photobleaching. Indo-1 is highly selective for Ca^{2+} but has a low Ca^{2+} affinity, which limits its capacity to buffer Ca^{2+} (Grynkiewicz *et al.*, 1985). One limitation of Indo-1 is that its fluorescence is influenced by caffeine (O'Neill *et al.*, 1990).

Fresh cardiomyocytes were loaded with 10 μ M Indo-1-AM (Molecular Probes, Invitrogen, Oregon, USA) for 20 minutes, and washed with Buffer A and allowed to deesterify for 30 minutes. Cells were then loaded onto a bath and imaged using an Olympus IX-71TM inverted microscope. Approximately 5 μ l of 1 mg/ml laminin (Sigma-Aldrich, UK) was used to promote cell sticking to the microscope dish. The cells were superfused with NT solution at 37°C and stimulated at 1Hz as for Fluo-4 recordings. For each cell, pre-recording corrections for background fluorescence were made. Continuous recordings were used to obtain steady-state fluorescent traces. The stimulator was then switched off and the following perfusion protocol was simultaneously activated: 0 Na⁺/0 Ca²⁺ was applied for 100ms, then 0 Na⁺/0 Ca²⁺ with 20mM caffeine for 1s, followed by NT with 20mM caffeine for 5s. This perfusion protocol was described in (Soppa *et al.*, 2008a).

The data was analysed in IonOptix Ion Wizard 5.0. The peak fluorescence ratio obtained following caffeine application was taken as a measure of SR Ca²⁺ content.

2.4 Whole Cell Patch Clamping For Recording I_{Ca,L}

These experiments were performed by Dr Urszula Siedlecka. I_{Ca,L} was measured using a MultiClamp 700A (Axon Instruments, Union City, CA, USA) in whole-cell patch configuration. The bath of cells was viewed using a Nikon Diaphot 200 inverted microscope, on a vibration-isolation table (Technical Manufacturing Corporation, Peabody, USA). A Faraday cage was used to minimise electrical interference (Technical Manufacturing Corporation). Micropipettes were made using borosilicate glass tubes of 0.86mm inner diameter (Harvard Apparatus, UK) in a microprocessor-

controlled Flaming/Brown pipette-puller (Model P97, Sutter Instruments, CA, USA). The pipette was manoeuvred using a Burleigh PCS-5000TM piezoelectric micromanipulator (EXFO Lifesciences, Ontario, Canada), aiming to place the tip of the pipette close to the cell membrane. The potential difference between the electrode and the ground (pipette offset) was then calibrated to zero. The pipette resistance and pipette capacitance were compensated. Suction was then applied to form a Gigaseal. Following this, the patch was ruptured to obtain the whole-cell configuration of the patch clamping technique (Sakmann and Neher, 1984).

Whole cell patch clamping involves the use of a relatively large pipette, which was connected to the intracellular space. Because the pipette was relatively large, its contents may dialyse the cell over time, changing its electrophysiological properties. The pipette resistance was $\sim 2\text{--}3\text{M}\Omega$, and the pipette-filling solution contained (in mM) 115 Cesium aspartate, 20 Tetraethylammonium chloride, 10 1,2-Di(2-aminoethoxy)ethane-NNN'-tetra-acetic acid (EGTA), 10 HEPES, and 5 MgATP, pH 7.2. The external solution contained the following (in mM): 140 NaCl, 10 Glucose, 10 HEPES, 1 CaCl_2 , 1 MgCl_2 , and 6 CsCl, pH 7.4. Current-voltage relationships for $\text{I}_{\text{Ca,L}}$ were built using 450 ms depolarisation steps from a holding potential of $\sim 40\text{mV}$ (range $+40$ to -40mV , in 5mV increments) at 1Hz . Then $200\mu\text{M}$ Cd^{2+} was applied and the protocol was repeated. Subtracted currents obtained were normalised to cell capacitance. All experiments were conducted at 37°C .

2.5 Immunofluorescence To Examine Colocalisation Between DHPR And RyR

In order to measure changes to the colocalisation pattern of DHPR and RyRs, immunolabelling of these two channels was performed in fixed single

cardiomyocytes. This involved the use of primary antibodies from different species, targeting DHPR and RyR specifically. Two secondary antibodies, each tagged with a different fluorescent probe, against the two primary antibody species were then used to label the protein distribution. The staining protocol involved pre-plating coverslips with laminin (20 μ l of 1mg/ml laminin solution (Sigma-Aldrich, UK)) for one hour. 1ml of cell suspension was then added and allowed to stick for two hours. It was important that the coverslips were kept in a humidifying chamber (a box covered with aluminum foil and with wet filter paper lining the bottom surface) during this process to avoid drying of the cell suspension. Cardiomyocytes were then fixed with 1ml cold acetone (4°, for 10 minutes), which was washed off three times with Buffer A (each wash lasting 1 minute). Cardiomyocytes were then permeabilised using 1ml of 1% Triton and a blocking solution of 1% Bovine serum albumin and 1% Goat serum (for 10 minutes). Blocking solution was then left on the cells for a further one hour. Cells were then incubated with mouse anti-DHPR α -1 antibodies for one hour at room temperature (Abcam, Cambridge, UK) and after washing, were incubated with goat anti-mouse secondary antibodies conjugated with Alexa Fluor 488 for one hour at room temperature (Molecular Probes, Invitrogen, Oregon, USA). Cells were then incubated with rabbit anti-RyR for one hour (Santa Cruz Biotechnology Inc., Santa Cruz, Ca) and after washing were incubated with goat anti-rabbit antibody conjugated with Alexa Fluor 594 for a further hour (Molecular Probes, Oregon, USA). Between each antibody stage, coverslips were washed using Buffer A for 45 minutes in three cycles of 15 minutes each. The whole cell was imaged at high resolution using single direction scanning and 16 frame averaging. For Alexa 488, excitation was at 488 nm, with the laser at 5% transmission and emission was collected through a 500-550 nm band-pass filter (peak emission at 519nm). For Alexa 594, excitation was using a 543

nm laser at 7% transmission and emission was collected through a 535-590 nm band pass filter (peak emission at 617nm). Colocalisation of DHPR-RyR was assessed using the colocalisation threshold plug-in for ImageJ. Automatic thresholding was used, and confocal images were recorded using the same gain and background settings to avoid bias. The coefficient of colocalisation (percentage voxels above threshold which were colocalised) was analysed for red (RyR):green (DHPR) and green (DHPR):red (RyR).

2.6 Statistical Analysis

Data are represented as mean $\pm$ standard deviation. Two groups were analysed using the nonparametric Mann-Whitney test. The analysis was performed using Prism4 software (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was taken as significant. Statistical analysis of more than two groups was performed using non-parametric one-way analysis of variance (ANOVA) (Kruskall-Wallis test). Dunn's post-hoc test was used in conjunction. In graphs, one star indicates a difference of $p < 0.05$, two stars indicates a difference of $p < 0.01$ and three stars indicate a difference of $p < 0.001$.

Non-parametric tests are useful when two populations with unequal variances are compared. To test for unequal variances, the F-test for unequal variance was used. Non-parametric techniques do not rely on assumptions that the data belong to any particular probability distribution.

2.7 Solutions

The following standard solutions were used. They are described in mM (unless otherwise stated). All chemicals were provided by VWR International or Sigma Aldrich.

Normal tyrode (NT): 140 NaCl, 6 KCl, 10 glucose, 10 HEPES (free acid), 1 MgCl₂, 1 CaCl₂, pH 7.4

NT+Heparin: 140 NaCl, 6 KCl, 10 glucose, 10 HEPES (free acid), 1 MgCl₂, 1 CaCl₂, 1000U/ml Heparin, pH 7.4

Low Ca²⁺ solution (LC): 120 NaCl, 5.4 KCl, 5 MgSO₄, 5 Na⁺ pyruvate, 20 glucose, 20 taurine, 10 HEPES (free acid), 5 nitrilotriacetic acid, and 0.04 CaCl₂, pH 6.96
(When isolating mouse cardiomyocytes, 0.045 CaCl₂ was used)

Buffer A: 120 NaCl, 5.4 KCl, 5 MgSO₄, 5 Na⁺ pyruvate, 20 glucose, 20 taurine, 10 HEPES (free acid), and 0.2 CaCl₂, pH 7.4

0 Na⁺/0 Ca²⁺ solution used during measurement of SR Ca²⁺ content: 140 LiCl, 10 glucose; 10 HEPES, 0.75 EGTA, 1 MgCl₂, pH 7.4 (using KOH up to 6mM, thereafter using LiOH if necessary).

St Thomas II Cardioplegia: (From NHS Supply Chain): 110 NaCl, 10 NaHCO₃, 16 , 1.2 CaCl₂, pH 7.8

L-15 Medium: For SICM. Listed in mg/L below.

SALTS

CaCl ₂	140
KCl	400
KH ₂ PO ₄	60
MgCl ₂	93.70
MgSO ₄	97.67
NaCl	8000
Na ₂ HPO ₄	190

OTHER

D+ Galactose	900
Na ⁺ Pyruvate	550
Phenol Red	10

AMINO ACIDS

DL-Alpha Alanine	450
L-Arginine	500
L-Asparagine	250
L-Cysteine	120
L-Glutamine	300
Glycine	200
L-Histidine	250
DL-Isoleucine	250
L-Leucine	125
L-Lysine	75
DL-Methionine	150
DL-Phenylalanine	250
L-Serine	200
DL-Threonine	600
L-Tryptophan	20
L-Tyrosine	300
DL-Valine	200

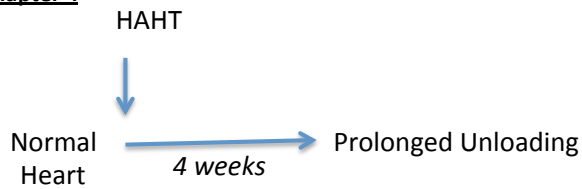
VITAMINS

D-Ca ²⁺ Pantothenate	1
Choline Chloride	1
Folic Acid	1
I-Inositol	2
Niacinamide	1
Pyridoxine Hydrochloride	1
Riboflavin 5'-Phosphate, Na ⁺	0.10
Thiamine hydrochloride	1

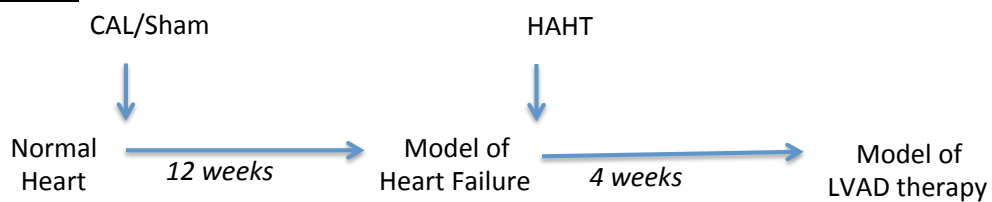
2.8 Experimental Scheme

The general experimental scheme for the subsequent Chapters is described below.

Chapter 4



Chapter 5



Chapter 6

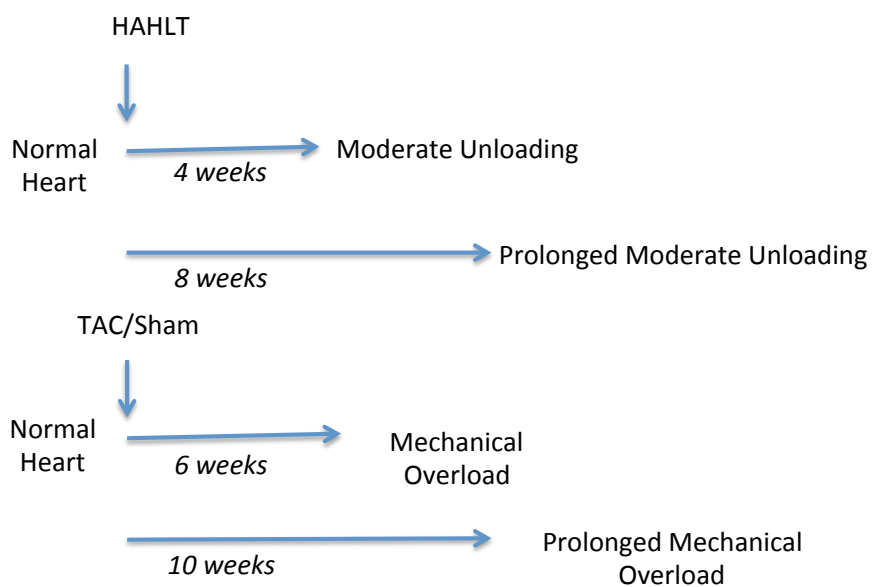


FIGURE M-6: SCHEME OF EXPERIMENTS IN CHAPTERS 4 TO 6

Chapter 7

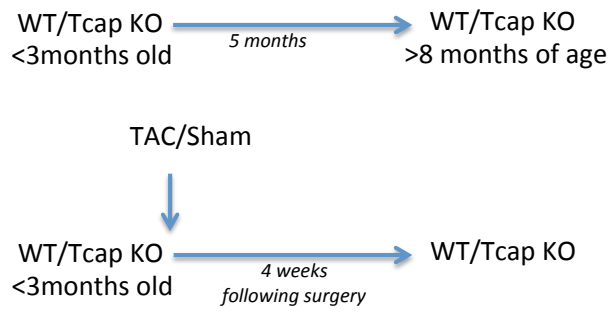


FIGURE M-7: SCHEME OF EXPERIMENTS IN CHAPTER 7

Chapter 3

Characterisation Of Animal Models Of Cardiac Mechanical Load Variation At The Level Of The Whole Heart

In this Chapter, the models of mechanical overload (TAC), unloading (HAHLT) and HF (post-CAL) used in subsequent Chapters were validated using clinical, echocardiographic and heart weight data.

3.1 Introduction

The animal models of myocardial mechanical load variation and HF used in the studies described in the following Chapters are extensively used in the cardiovascular sciences and accepted as suitable substrates for the study of relevant physiological processes (Abarbanell *et al.*, 2010). However, it is possible to produce models of widely different clinical states and physiological conditions depending on the operative technique used and therefore these models require validation and characterisation.

The most practical and effective means to validate these models differ. CAL surgery results in progressive HF as evidenced by echocardiography (Schwarz *et al.*, 1998). TAC surgery produces physiological hypertrophy and eventually HF and can also be assayed using serial echocardiograms (Del Monte *et al.*, 2002). The models of mechanical unloading are more difficult to evaluate. Echocardiographic assessment can be used to exclude graft failure and provide some measure of cardiac dimensions, but cannot be used for functional assessment due to the technical difficulty and because echocardiography provides only load-dependent parameters. LV weight and cardiomyocyte volume provide an index of atrophy (a concept which will be discussed below), indicating the impact of mechanical unloading.

3.2 Materials and Methods

The operative technique for these models, the basis of echocardiographic assessment and general animal husbandry has been detailed in the Methods. The number of animals shown in this Chapter is not always the number of animals used in the experimental groups in following Chapters. The number of animals used per experimental group is detailed in the Methods of each specific Chapter.

3.2.1 Kaplan–Meier Curves

Kaplan-Meier curves provide a running estimate of survival after an intervention (Bland and Altman, 1998). Animals would be withdrawn from the study for a number of reasons, for example by developing a complication that causes them to be incomparable to other members of the experimental group. A typical case is the development of aortic regurgitation in a post-operative HAHT or HAHLT animal. These subjects are censored (removed from the rolling analysis) with a new estimated survival. Censoring is a declaration that the final outcome for that subject is not known and therefore they are excluded from the analysis.

When there is no censoring, the Kaplan-Meier statistic for a given point in time estimates survival beyond that point and is obtained by subtracting the deaths at a time point from the observed survival directly before that time (n), divided by n . When there is censoring, n is adjusted for the lost number of cases no longer observed (Leung *et al.*, 1997).

Kaplan-Meier curves are given for CAL, TAC and HAHLT in Figure C3-1. All these operations were performed by myself. They exclude the series of TAC performed in mice by Dr Mutsuo Harada (Chapter 6), the heart-only transplantations performed by Dr Manoraj Navaratnarajah (Chapter 3 and 4) and the operations performed during the learning curve of these operations. The issue of learning curves is discussed below. There were no deaths for Sham operations in any group.

3.2.2 Heart Weight

The heart weights reported were measured directly after harvesting and removing the oesophagus, lungs, descending aorta and any excess thymic tissue or fat.

3.2.3 Functional Assessment

Hearts were assessed using echocardiography as described in the Methods. Unloaded hearts underwent echocardiography for dimensions but not for functional assessment.

3.2.4 Statistical Analysis

Statistical analysis was performed using non-parametric one-way ANOVA (Kruskal-Wallis test). Dunn's post-hoc test was used to test for differences between groups. The analysis was performed using Prism4 software (GraphPad software Inc., San Diego, CA, USA). $P < 0.05$ was taken as significant.

3.3 Results

3.3.1 Survival

The survival of animals following TAC (Chapter 6), CAL (Chapter 5) and HAHLT (Chapter 6) is shown in Figure C3-1. Early mortality occurred within the first day and usually peri-operatively or immediately postoperatively and accounts for the majority of deaths in all three animal models. This early mortality was largest for CAL surgery and lowest for HAHLT. The causes of deaths for CAL are listed in the first five practice series of experiments in Table C3-1. Table C3-1 also shows the significant learning curve involved in generating CAL models, with a mortality of 50% in the first series compared to approximately 10-20% in later series. All perioperative or day 1 deaths in the TAC group were secondary to severe pulmonary oedema, where animals were unable to tolerate the significant aortic pressure gradient generated and suffered acute HF. All perioperative or day 1 deaths in the HAHLT group were due to bleeding from the anastomosis.

Mortality		Causes of Death
Series 1	7/14	Anaesthetic error (3) Bleeding (2) Surgical Error (2)
Series 2	5/15	Acute HF/VF (3) Bleeding (2)
Series 3	3/16	Acute HF/VF (3)
Series 4	6/23	Acute HF/VF (4) Bleeding (1) Surgical Error (1)
Series 5	2/8	Bleeding (2)

TABLE C3-1: CAUSE OF DEATH IN FIVE INITIAL CAL SERIES

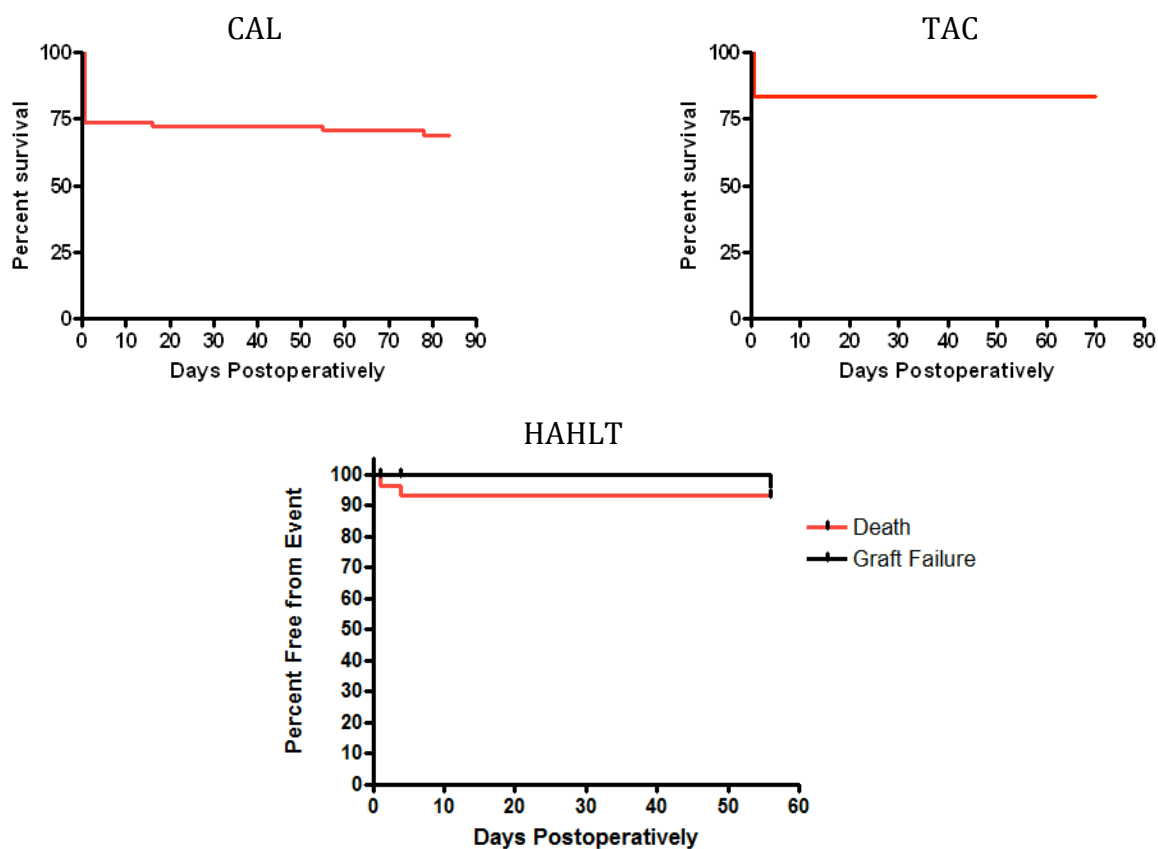


FIGURE C3-1: KAPLAN-MEIER CURVE ESTIMATION OF SURVIVAL IN CAL, TAC AND HAHLT

The figure shows Kaplan-Meier analysis of survival following CAL, TAC and HAHLT.

Late mortality occurred following CAL surgery only. The mechanisms for this are not known but could be ischaemia-related arrhythmia or arrhythmia due to the presence of scar tissue. Other possible causes include HF.

The HAHLT curve also shows the incidence of graft failure, which was low. Graft failure was defined as cessation of contraction, presence of large LV clot, significant aortic valve regurgitation (evidenced on echocardiography or by dilation of the ventricle), or infection.

3.3.2 Heart Weight

Heart weights after CAL and TAC are shown in Figure C3-2. The TAC experiments show that heart weight increased as the duration of overload was increased. This increase in heart weight was associated with cardiomyocyte hypertrophy, as will be demonstrated in following Chapters, but other mechanisms including increases in extracellular matrix components such as collagen may also play a role. This hypertrophic response occurs in response to pressure overload in a number of studies (Wei *et al.*, 2010, Del Monte *et al.*, 2002).

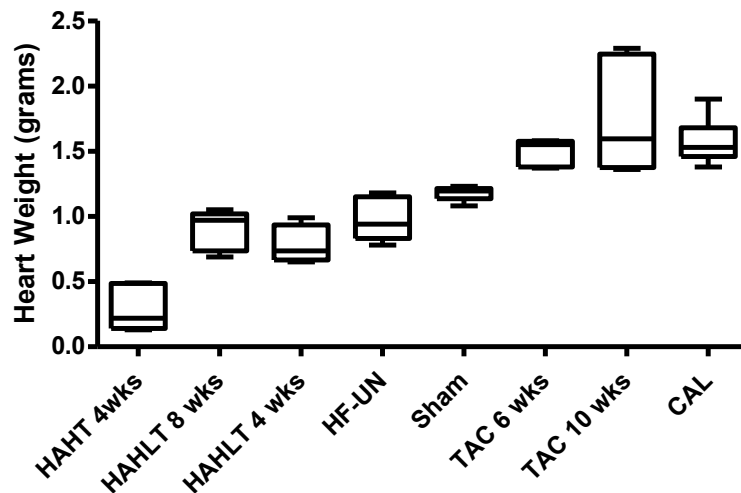


FIGURE C3-2: MECHANICAL LOAD VARIATION AND HEART FAILURE MODULATED HEART WEIGHT

Pre-digestion heart weights are shown. To avoid a large number of statistical comparison bars, significant differences are as follows: CAL vs HF-UN ($p<0.05$), CAL vs HAHLT 4wks ($p<0.01$), CAL vs HAHLT 8wks ($p<0.05$), CAL vs HAHT 4wks ($p<0.001$), HAHLT 4wks vs TAC 6 weeks ($p<0.05$), HAHLT 4wks vs TAC 10 weeks ($p<0.01$), HAHT 4wks vs TAC 6 weeks ($p<0.01$), HAHT 4wks vs TAC 10 weeks ($p<0.001$). Abbreviations: HAHT 4wks - normal heart 4 weeks following HAHT; HAHLT - normal heart following HAHLT; HF-UN - failing (12 week post CAL) heart 4 weeks following HAHT; CAL - 12 weeks post CAL. N numbers are as follows: Sham=8; CAL=8; HF-UN=8; HAHLT 4wks=6; HAHLT 8wks=5; HAHT 4wks=5; TAC 6wks=5; TAC 10wks=6.

Conversely, reductions in mechanical load have been shown to induce reductions in cardiac mass (Klein *et al.*, 1990). In a previously normal heart, mechanical unloading resulted in a time-dependent reduction in heart weight, of approximately 75%, 4 weeks following HAHLT (Figure C3-2). Partial mechanical unloading induced by HAHLT resulted in slower and less profound reductions in heart weight. Mechanical unloading of a failing heart (post-CAL HAHT) induced a regression of whole heart hypertrophy, indicating that the determination of cardiac mass is dependent at least partly on load and is reversible.

3.3.3 Functional Assessment

Functional assessment consisted of echocardiography as described in the Methods. These data provide information on dimensions of the LV cavity and wall as well as LV wall functional properties, except in the case of the mechanically unloaded heart where functional assessment is meaningless as the heart is not equally loaded as a normal heart (discussed below).

Serial echocardiographic assessment of hearts following CAL is shown in Figure C3-3 (single, not averaged values from individual animals). Data obtained at 1, 6 and 12 weeks post-operatively are shown. The initial part of the reduction in contractility is due to loss of cardiomyocytes and later due to remodelling of the chronically-overloaded remaining myocardium (Pfeffer and Braunwald, 1990). At the early stage, it was possible to identify and exclude a population of animals who suffered myocardial infarction that was not extensive enough to result in a large drop in EF (Figure C3-3). Remodelling of the remaining myocardium results in a more slowly progressing reduction in contractility, primarily due to pressure overload. Animals used showed echocardiographic features of HF, including dilation of the ventricle and impaired contractility with an EF of 40%.

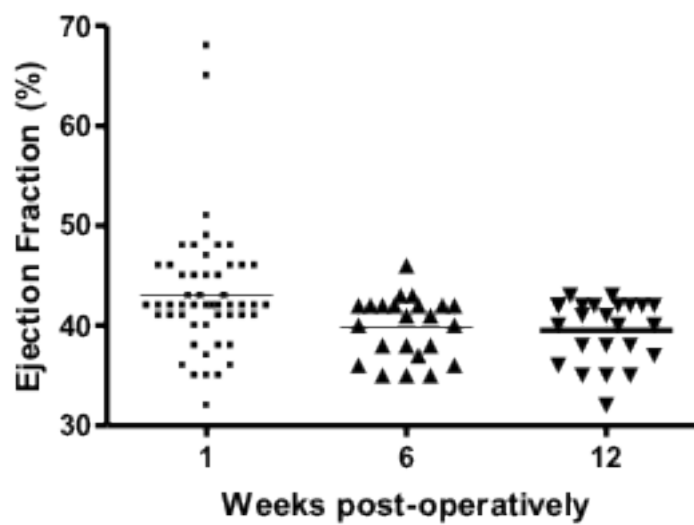


FIGURE C3-3: ECHOCARDIOGRAPHIC ASSESSMENT OF CAL

The figure shows LV EF at 1, 6 and 12 weeks following CAL. 1 wk vs 12 week, $P<0.05$.

Final echocardiograms were performed three times and the average values were used for each single animal. The overall average is provided in Table C3-2.

	EF%	FS%	LVs (cm)	LVd (cm)	LVPWd (cm)
Normal (n=12)	74±8	45±9	0.39±0.08	0.65±0.1	0.22±0.1
HAHLT 4 week (n=4)			0.45±0.13	0.47±0.13*	0.17±0.04
HAHLT 8 week (n=3)			0.34±0.05	0.46±0.04	0.15±0.04
TAC 6 week (n=5)	81±13	47.6±8.4	0.34±0.1	0.53±0.15	0.25±0.3
TAC 10 week (n=6)	82±8.9	46.8±7.8	0.35±0.07	0.55±0.1*	0.28±0.2*
CAL (n=8)	39±2.3*	20.6±4.3*	0.81±4.2*	0.92±3.4*	0.20±3.8

TABLE C3-2: EJECTION FRACTION OF VARIOUS ANIMAL MODELS

This table shows the final average value based on n number of animals in each group. Each animal undergoes three echocardiograms and the average value is used. * indicates a significant difference (p<0.05) from normal. Note that the n numbers here only include those animals who went on to be included in an experimental group, unlike for Figure C3-2.

Echocardiographic assessment of TAC animals shows an increase in EF and FS (Table C3-2). FS was likewise increased. This was associated with a reduction in the ventricular dimensions (LVs and LVd) due to concentric hypertrophy. The LV posterior wall was thicker than normal at 6 and 10 weeks, correlating with the increased heart weight.

The table includes echocardiographic assessment of normal hearts following HAHLT. These show that mechanical unloading was associated with a reduction in the size of the ventricular cavity and thinning of the LV posterior wall, possibly indicating atrophy. This correlates with the reduction in heart weight above.

3.4 Discussion

This validation study shows that models of mechanical overloading and unloading and HF can be generated in a reproducible manner, with low mortality and evidence of clinically-relevant functional characteristics.

3.4.1 Survival

Survival is dependent on a number of factors related to the injury of the surgery itself and the physiological consequences of the model. In the case of CAL, the surgery itself could result in dehydration, bleeding, trauma to the heart or lungs and chest wall trauma, which could result in the animal dying in the peri-operative or early post-operative period. Myocardial infarction in itself has a significant mortality due to low CO, arrhythmia and pulmonary oedema. Likewise, TAC results in pressure overload and can cause lethal pulmonary oedema. The severity of these insults can be modified to achieve a balance between models of advanced severity and a practical survival rate. This was achieved by selecting the level at which to ligate the left coronary artery in the case of CAL, where ligation toward the origin of the left coronary results in a greater loss of myocardium (and a more severe model), but this risks a higher mortality. In the case of TAC, the degree of band tightening can be adjusted to induce more severe overload, but this risks a higher acute mortality. The learning curve involved in generating the animal models affects not only mortality but also the consistency of the model. This highlights the importance of rejecting initial survivors to achieve a consistent animal model.

3.4.2 Heart Weight

Increases in heart weight are an independent risk factor for mortality in man (Lorell and Carabello, 2000). Heart weight is determined by genetic and environmental factors, as well as their interaction through epigenetic changes (McDermott-Roe *et al.*, 2011, Post *et al.*, 1997). Hypertrophy is a uniform response to increases in mechanical load (Gaasch and Zile, 2011) and enables the heart to cope with the increased workload. Likewise, reductions in cardiac mass are thought to be a uniform response to mechanical unloading, and the reduction in cardiac workload (Baskin and Taegtmeier, 2011).

The term *cardiac atrophy* is complex and ill-defined. Strictly, it refers to diminutions of size below the normal range or below the starting, pre-disease value. It may be measured in a number of ways, including in single cells (Yacoub, 2001), standard morphometric analysis of tissue sections (Dipla *et al.*, 1998) and other techniques (Terracciano *et al.*, 2004). The term ‘atrophy’ however, implies a functional deterioration. However, the link between heart weight, cell size and function is not straightforward. Thus, even though hypertrophy is associated with dysfunction (Lorell and Carabello, 2000), increased mass is not always pathological and may be physiological, for example after endurance training (Ellison *et al.*, 2012). Furthermore, LVAD-induced mechanical unloading of the failing heart results in regression of cardiomyocyte hypertrophy, but this occurs in both those patients who show sufficient cardiac improvement to allow explantation of the LVAD without requiring cardiac transplantation, and those who did not and required maintenance of circulatory support until transplantation (Terracciano *et al.*, 2004). Therefore, it is more accurate to describe the relationship between cardiac mass and function as

operating within a range of normal loading conditions. Deviations outside this range at either the positive or negative end may lead to deterioration of function.

3.4.3 Functional Assessment

The purpose of functional assessment of animal models is to understand the extent to which they reflect a specific clinical syndrome. In this case, echocardiography was used.

In the case of CAL, progressive remodelling as well as the initial loss of myocardium drive the response to myocardial infarction (Pfeffer and Braunwald, 1990). Serial echocardiography reveals a large drop in myocardial contractility initially with a slower decline over time. Anversa *et al* showed that acutely after CAL, side-to-side slippage of cardiomyocytes, as well as increases in cardiac dimensions, leads to reduced CO (Anversa *et al.*, 1991). They also showed that the hypertrophic response to CAL in the remaining viable myocardium was insufficient to maintain cardiac function, nor to reduce wall stress. This overload sustains the progression to HF. Only animals with an EF below 40% were included in the final study, indicating a ‘severe’ HF phenotype.

It is important to understand that the diagnosis of HF in man is clinical, based on symptoms and functional abilities, in conjunction with echocardiographic and biomarker assessment. These animals reside in a restricted space with free access to food and water and therefore do not experience stressful exercise. It is impossible to correlate the human syndrome of HF with CAL in rats, but this model does replicate the myocardial changes observed, primarily defective contractility. Previous studies

have documented increased myocardial fibrosis, arrhythmia and death following CAL (Anversa *et al.*, 1991). It is important that this model specifically replicates the derangements in excitation-contraction coupling observed in the failing heart. This will be addressed in Chapter 5.

In the case of TACs, ventricular remodelling is driven by overload, which progresses more slowly than in CAL. This leads to a HF phenotype at approximately 24 or more weeks (Del Monte *et al.*, 2002). However, cellular remodelling, and specifically t-tubule remodelling, precedes whole heart dysfunction (Wei *et al.*, 2010). The mechanisms mediating the compensatory phase of hypertrophy and its transition to cardiac failure are mediated by numerous factors, including the severity and duration of the onset of pressure overload (Frohlich and Susic, 2012). The model used here provides (at 10 weeks) a model of compensated hypertrophy with preserved contractility. The cellular phenotype associated with this is investigated in Chapter 6.

Echocardiography is a convenient method of checking the viability of a transplanted cardiac graft. It cannot be used to assess cardiac contractility, since the heart is mechanically unloaded and lies on a different part of the Starling curve than the normal heart, and therefore has a different mechanical stimulus to contract (Frank, 1990). It is useful to exclude aortic regurgitation, however, as outlined in the Methods. A better method to assess contractility in a load-independent manner would have been the use of pressure-volume analysis (Pacher *et al.*, 2008).

3.4.4 Summary

This Chapter suggests that the surgical models described are able to produce HF and mechanical load variation.

Chapter 4

Prolonged Mechanical Unloading Altered Cardiomyocyte Excitation-Contraction Coupling, T-Tubule And Cell Surface Structure

In this Chapter, the effect of prolonged mechanical unloading on cardiomyocyte t-tubule, cell and surface structure was studied. Ca^{2+} transients, Ca^{2+} sparks, $\text{I}_{\text{Ca,L}}$ and AP morphology were assessed. A summary of the materials and methods is provided.

This Chapter tested the hypothesis that the t-tubule network of LV cardiomyocytes is altered by prolonged mechanical unloading, with consequences for CICR.

4.1 Introduction

The beneficial effects of LVAD therapy on myocardial function appear to diminish after prolonged mechanical unloading and the clinical recovery obtained is insufficient for device explantation in most patients (Maybaum *et al.*, 2007, Mancini *et al.*, 1998). It is thought that this may be due to atrophic remodelling - a set of changes triggered by excessive mechanical unloading.

In experimental models of LVAD therapy, prolonged mechanical unloading can result in detrimental effects on cardiac function (Oriyanhan *et al.*, 2007) and this may be partly mediated by deranged excitation-contraction coupling (Soppa *et al.*, 2008b). Soppa *et al* showed that prolonged mechanical unloading in normal hearts resulted in impaired single cell contractility, mediated by reduced myofilament sensitivity to Ca^{2+} and reduced SR Ca^{2+} content due to lower SR Ca^{2+} reuptake. This points to changes in excitation-contraction coupling being a major feature of the remodelling which occurs after prolonged mechanical unloading.

There is abundant evidence that alterations in t-tubule structure are an important cause of deteriorations in cardiomyocyte function in a range of cardiac disease states (reviewed in (Brette and Orchard, 2003)). In agreement with their role in guaranteeing efficient Ca^{2+} release, areas of impaired Ca^{2+} release are spatially colocalised to regions of t-tubular disruption (Meethal *et al.*, 2007) and the degree of t-tubular disruption correlates with the severity of impaired Ca^{2+} release (Louch *et al.*, 2004). There is evidence that this derangement is caused by a structural uncoupling of LTCC and RyR due to defects in the t-tubule network (Song *et al.*, 2006).

It is not known whether the t-tubule network is sensitive to mechanical unloading, but a recent report suggests that the t-tubules are regulated by stretch-sensitive molecules (Zhang *et al.*, 2009). This suggests that t-tubule defects may underlie the functional impairment of the chronically unloaded heart. The aim of this Chapter was to characterise the effect of chronic mechanical unloading on t-tubule and cell surface structure and on local CICR. To test the hypothesis that the t-tubule network of LV cardiomyocytes is altered by prolonged mechanical unloading, with consequences for CICR, hearts were mechanically unloaded using HAHT and single cells were characterised for local CICR and t-tubule structure.

4.2 Materials and Methods

4.2.1 Animal Models

Syngeneic male Lewis rats (10 - 12 weeks old, approximately 220g) were used in all the experiments. Four animals were used in each group.

HAHT. The heart was harvested from the thorax and heterotopically transplanted into the abdomen of an age-matched syngeneic recipient, as described in the Methods. Hearts were harvested four weeks following transplantation.

After cell isolation, the following experiments were performed:

- Ca²⁺ transient and Ca²⁺ spark recordings (with help from Dr Abeer Al Masri)
- Cell and t-tubule imaging using Di-8-ANEPPS
- Cell surface imaging using SICM (with help from Dr Alexey Moshkov)
- ICa_L recordings (Dr Urszula Siedlecka)
- AP Recordings (Dr Cesare Terracciano)

4.2.2 Statistical Analysis

Statistical analysis was performed using non-parametric one-way ANOVA (Kruskal-Wallis test). Dunn's post-hoc test was used to test for differences between groups. The analysis was performed using Prism4 software (GraphPad software Inc., San Diego, CA, USA). P<0.05 was taken as significant.

4.3 Results

4.3.1 Prolonged Unloading Reduced Cell Size

The most profound effect of mechanical unloading is a reduction in cell volume. Using three-dimensional volume reconstruction of Di-8-ANEPPS-stained cells, an estimate of cell volume was obtained. The volume of cardiomyocytes from unloaded hearts was 56.5% smaller than control. This reduction in cell size was confirmed by a reduced cell capacitance measured during the electrophysiological experiments (Figure C4-1). This result indicates that absolute cell surface area was reduced with a reduction in cell size.

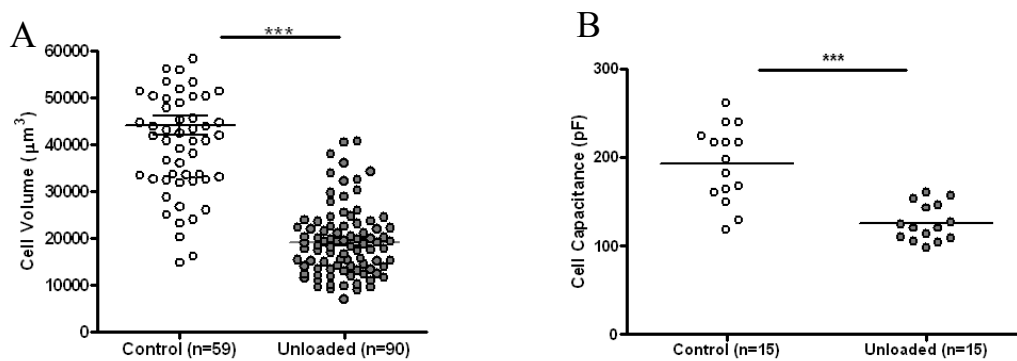


FIGURE C4-1: PROLONGED MECHANICAL UNLOADING REDUCED CELL SIZE

(A) Unloaded cell volumes were significantly smaller compared to control cells. (B) Cell capacitance measurements showed a reduction in cell capacitance after chronic unloading.

4.3.2 The Ca^{2+} Transient Was Altered By Prolonged Unloading

The peak amplitude of the whole cell Ca^{2+} transient was significantly smaller in cells from the chronically unloaded heart compared to control cells (Figure C4-2). In addition, the average time to peak was significantly longer, as was the time to 50% decline, compared to control cells. The variance of time to peak of the Ca^{2+} transient was significantly increased in cardiomyocytes from the unloaded heart, suggesting that the synchronicity of Ca^{2+} release was disrupted. Possible causes for this impaired

Ca^{2+} release include altered ICa_L , altered RyR function or an uncoupling of the CICR machinery. These possibilities are examined in subsequent sections.

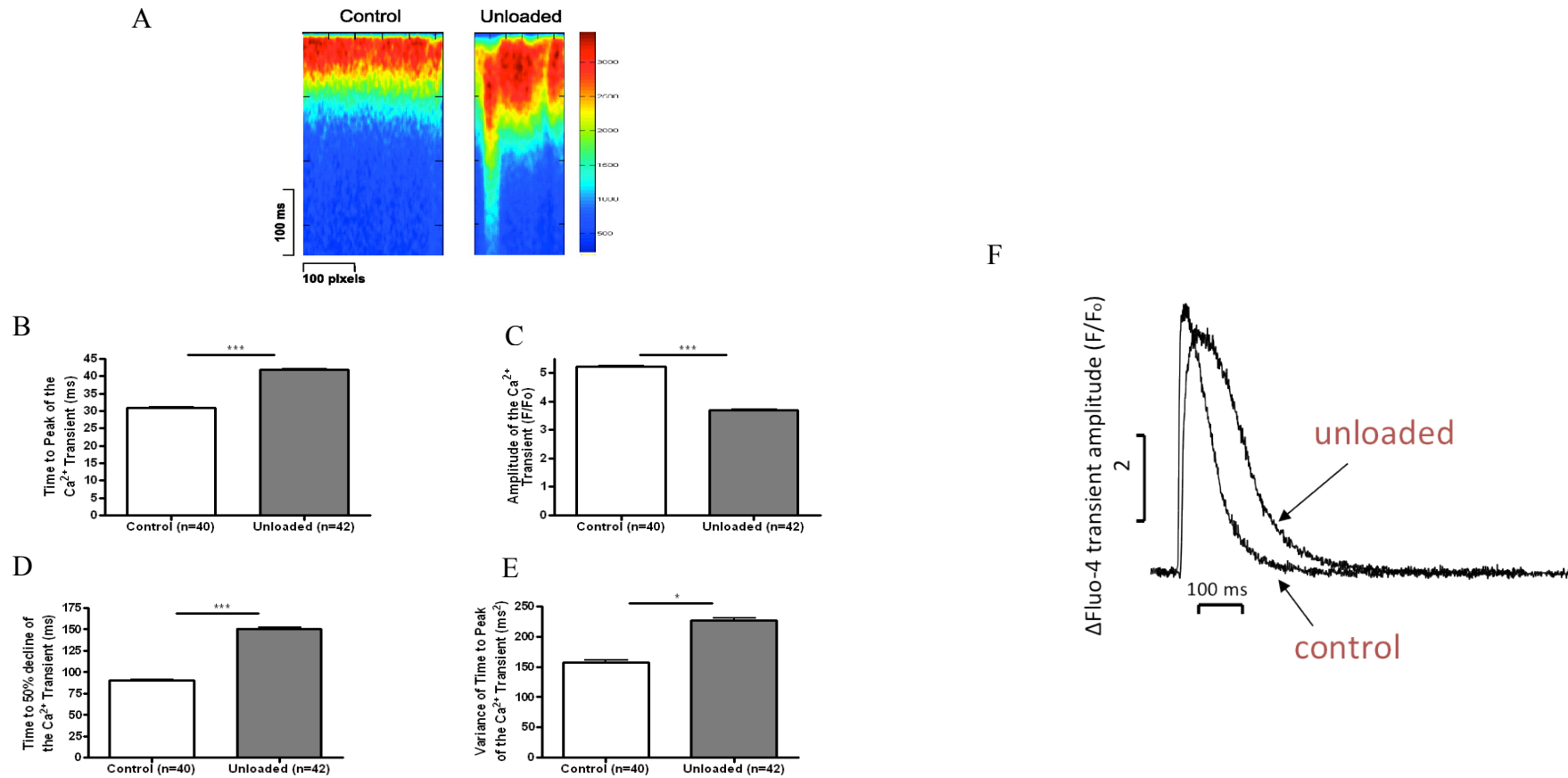


Figure C4-2: Prolonged Mechanical Unloading Disrupted The Ca^{2+} Transient

(A) Two representative traces showing that the whole cell Ca^{2+} transient was less synchronous in unloaded compared to control cells. (B) The time to peak of the Ca^{2+} transient is increased in unloaded cells. (C) The amplitude of the Ca^{2+} transient is decreased in unloaded cells. (D) The time to 50% decline in the Ca^{2+} transient is increased in unloaded cells. (E) The variance of the time to peak of the Ca^{2+} transient is increased in unloaded cells. The colour chart is a normalised scale of fluorescence intensity. The vertical scale bar represents 100 ms and the horizontal scale bar represents 100 pixels. (F) Two dimensional representation of the Ca^{2+} transient of control and unloaded cells.

4.3.3 I_{Ca,L} Was Unaffected

There was no difference in the I_{Ca,L} density between cardiomyocytes from control or unloaded hearts (Figure C4-3). However, the fast-inactivation of I_{Ca,L} was significantly slower in unloaded cardiomyocytes compared to control. Thus the trigger for SR Ca²⁺ release was not altered by prolonged mechanical unloading under voltage-clamping conditions.

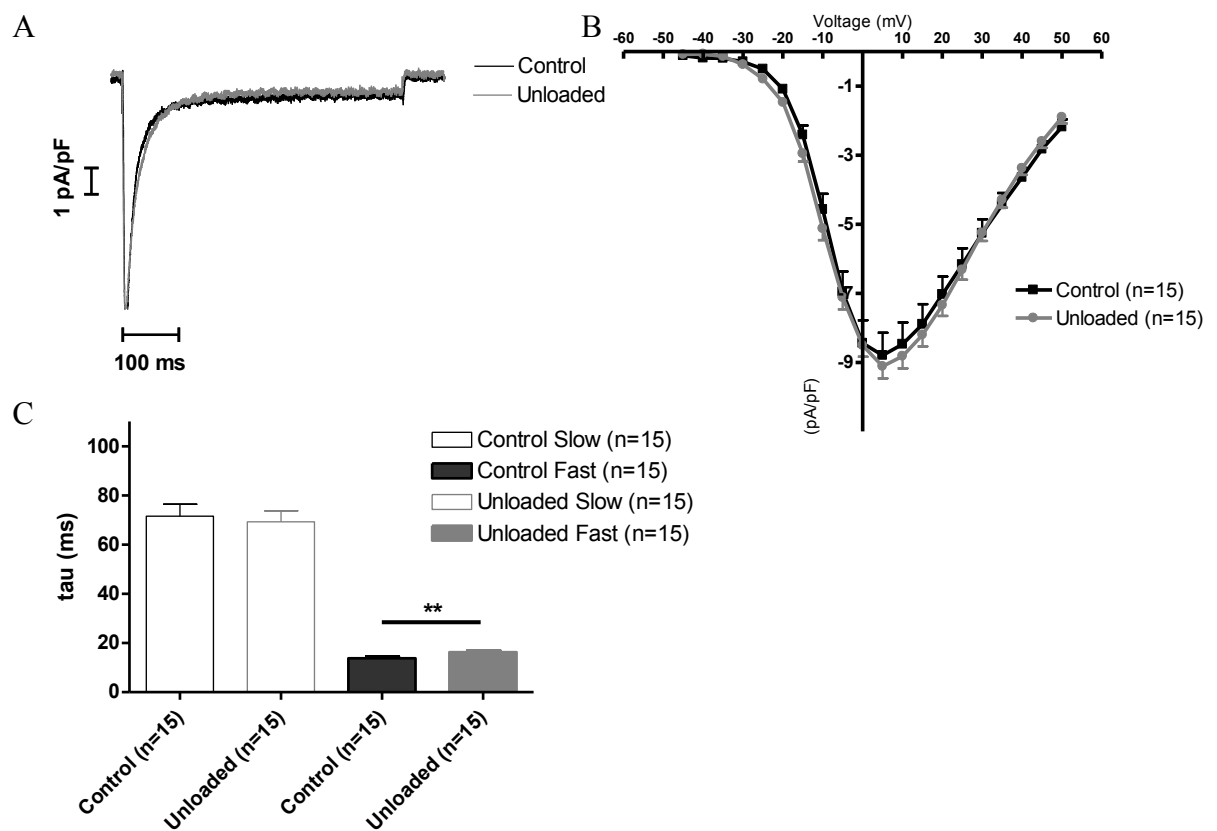


FIGURE C4-3: PROLONGED MECHANICAL UNLOADING DID NOT ALTER THE I_{Ca,L} DENSITY

(A) Raw traces of I_{Ca,L} elicited by a voltage clamp step from -40 to 0 mV. (B) I_{Ca,L} density was unaffected by mechanical unloading. (C) The fast phase of I_{Ca,L} inactivations was significantly slower in cardiomyocytes from the unloaded heart.

4.3.4 The Action Potential Was Unaffected

As the synchronicity of the Ca^{2+} transient may be affected by AP morphology, the AP was recorded from cardiomyocytes from unloaded and control hearts. The resting membrane potential was unaffected by prolonged mechanical unloading. The time to 50% repolarisation was similarly unchanged, as was the time to 90% repolarisation. Thus chronic mechanical unloading did not alter the AP of LV cardiomyocytes (Figure C4-4).

Taken together, these results suggest that there is a local disruption of CICR in unloaded cardiomyocytes, which is not due to altered $\text{I}_{\text{Ca,L}}$. To investigate the basis of this impaired Ca^{2+} release, the components of the local CICR process, which include RyR and the t-tubular structure, were assessed.

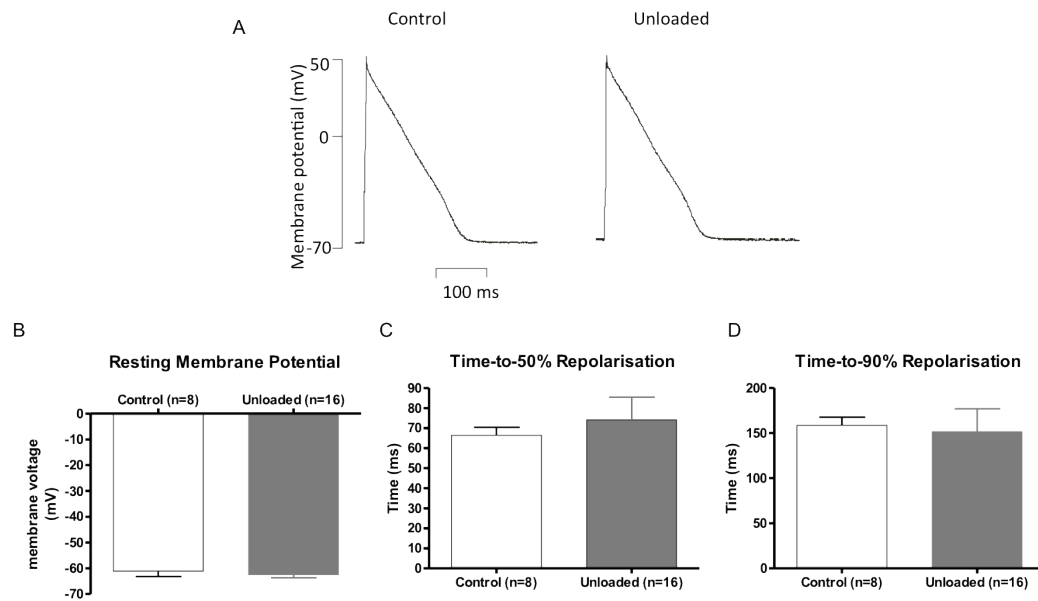


FIGURE C4-4: THE ACTION POTENTIAL WAS UNAFFECTED BY MECHANICAL UNLOADING

(A) AP morphology was unchanged after unloading. (B). Resting membrane potential was unchanged after unloading. (C) Time to 50% repolarisation was unchanged after unloading. (D) Time to 90% repolarisation was unchanged after unloading.

4.3.5 Prolonged Mechanical Unloading Altered Ca^{2+} Spark Frequency And Morphology

Cardiomyocytes from the unloaded heart showed a significantly higher Ca^{2+} spark frequency than control cardiomyocytes (Figure C4-5). Mechanically unloaded cardiomyocytes also showed increased Ca^{2+} spark width and duration, and decreased Ca^{2+} spark peak. These results indicate an altered activity of the RyR. It has been previously reported that the SR Ca^{2+} content is unchanged after prolonged mechanical unloading for four weeks in rats (Soppa et al., 2008b). These changes to Ca^{2+} sparks might be due to alterations at the level of the structural coupling of the RyR and LTCC at the t-tubules.

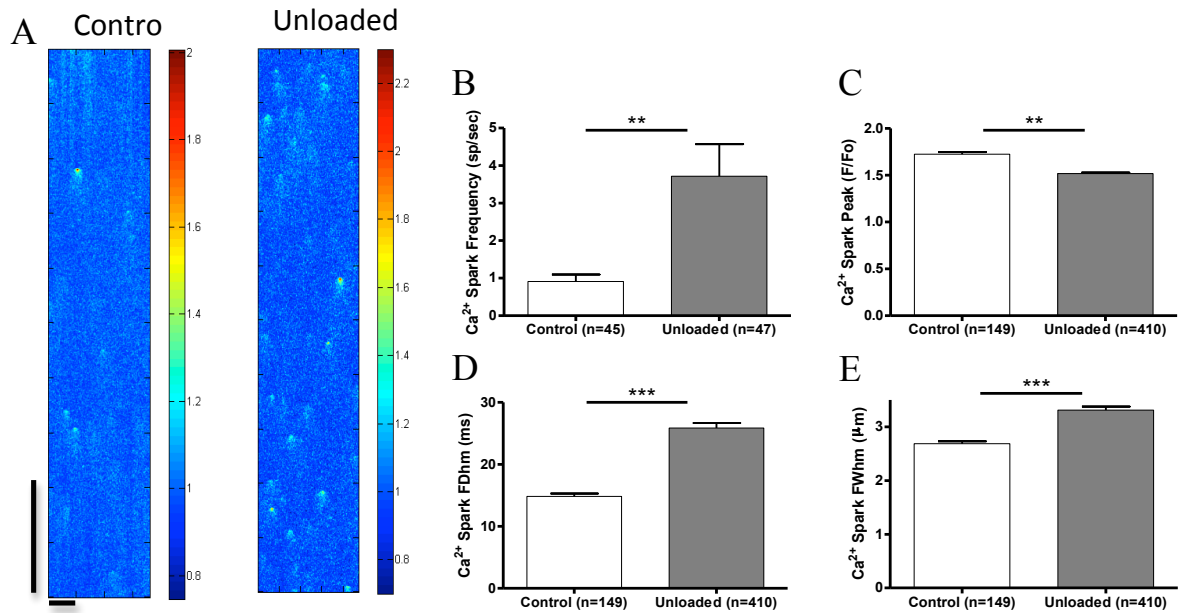


FIGURE C4-5: Ca^{2+} SPARK PROPERTIES WERE ALTERED BY PROLONGED MECHANICAL UNLOADING

(A) Representative images of Ca^{2+} sparks. Cells from the chronically unloaded heart had more frequent Ca^{2+} sparks (B) which were smaller in amplitude (C) longer in duration (FDHM) (D) and wider (FWHM) (E). The vertical scale bar represents 768ms. The horizontal scale bar represents 100 pixels. The colour chart is a normalised scale of fluorescence intensity.

4.3.6 Prolonged Mechanical Unloading Disrupted The T-Tubules

The t-tubule network in cells from the chronically unloaded heart was investigated because changes in SR Ca^{2+} release have been associated with t-tubule disruption (as extensively reviewed in Introduction). To do this, cardiomyocytes were stained with the membrane-staining dye Di-8-ANEPPS (Figure C4-6). Unloading did not alter the density of the t-tubule network, consistent with an unchanged ICa_{L} , but significantly reduced its regularity, with fewer t-tubules lying in their ordinary position.

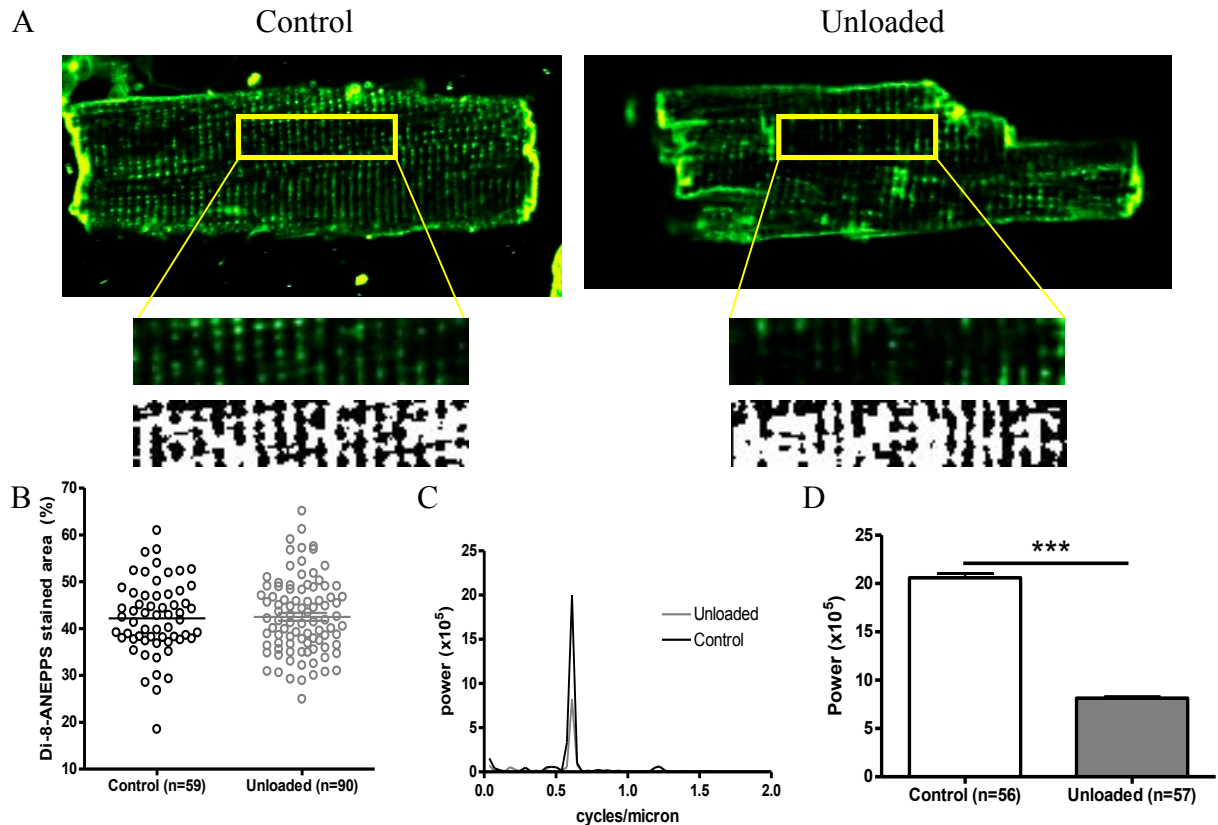


FIGURE C4-6: PROLONGED MECHANICAL UNLOADING DISRUPTED T-TUBULE STRUCTURE

(A) Representative images of Di-8-ANEPPS stained cardiomyocytes from chronically unloaded and control hearts. Binary images of the central area indicated that the t-tubules of unloaded cells were significantly disrupted. (B) The density of the t-tubule network was unchanged by unloading. (C and D) Fourier analysis of the binary images of Di-8-ANEPPS images indicated that the t-tubules were irregular. The length of the yellow box is 25 μ m.

4.3.7 Prolonged Mechanical Unloading Disrupted The Cell Surface

To image the external membrane structure and its relationship with the t-tubule network at high resolution in living cells, SICM was used. SICM imaging of unloaded cells showed changes on the cardiomyocyte surface with a decrease in the z-groove index compared with control cells (Figure C4-7), as previously described (Gorelik *et al.*, 2006).

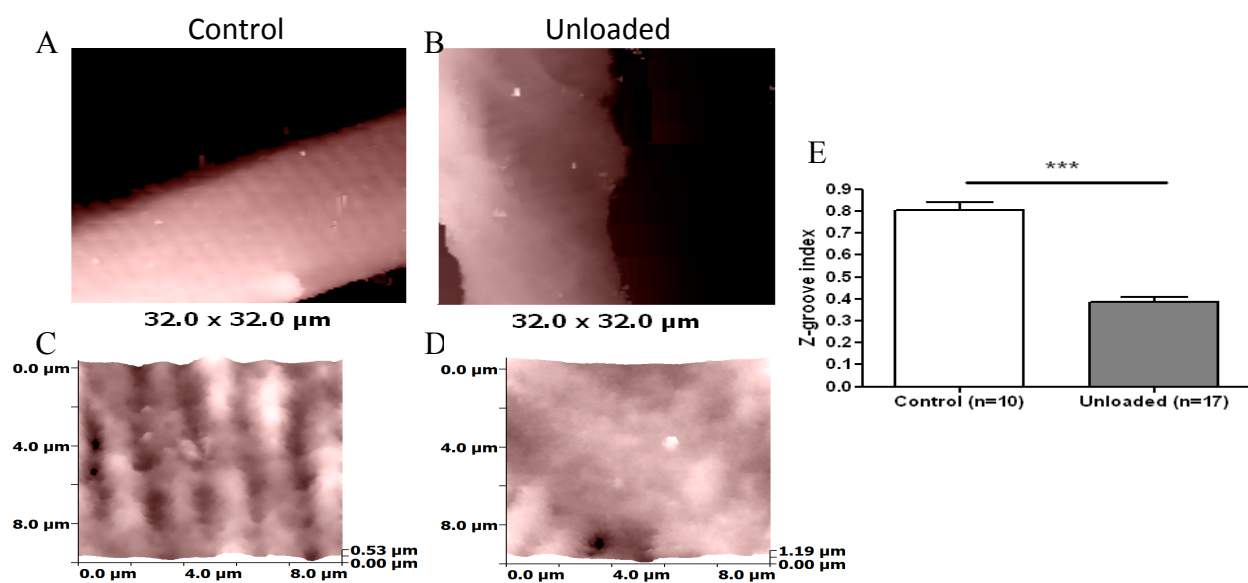


FIGURE C4-7: CARDIOMYOCYTE SURFACE STRUCTURE WAS DISRUPTED BY PROLONGED MECHANICAL UNLOADING

SICM images from the surface of cardiomyocytes isolated from control (A) and unloaded (B) hearts. (C & D) are high power views of the same cells. (E) z-groove index for cells from control hearts and unloaded hearts, indicating a disrupted cell surface structure.

4.4 Discussion

The results presented here show that cardiomyocytes from chronically unloaded hearts have impaired, markedly asynchronous Ca^{2+} release despite intact Ca^{2+} triggers (normal ICa_L). Cells from chronically unloaded hearts also showed disrupted t-tubule structure, with a normal density but abnormal pattern. The disrupted, asynchronous Ca^{2+} release events can be explained by a functional uncoupling of the RyRs and LTCC (Song *et al.*, 2006). This is a fundamental mechanism for impaired contractility and arrhythmia in HF, and these results show that this is of relevance in the chronically unloaded heart. This mechanism may explain the regression of improved contractility observed initially in patients treated chronically with LVADs.

One unexpected result was that despite alterations to the Ca^{2+} transient, the AP was not changed (duBell *et al.*, 1991). In addition, ICa_L is a major determinant of phase two of the cardiac AP. Prolonged Ca^{2+} dependent fast inactivation might also be expected to prolong the AP and Ca^{2+} transient. As the changes observed to the Ca^{2+} transient were small, they may have been too small to alter the AP.

4.4.1 CICR Uncoupling

There is accumulating evidence that t-tubule disruption produces an uncoupling of LTCCs and RyRs during mechanical overload (Song *et al.*, 2006, Gomez *et al.*, 1997, Brette and Orchard, 2003, Louch *et al.*, 2004, Heinzel *et al.*, 2002, Heinzel *et al.*, 2008, Lenaerts *et al.*, 2009). The ‘orphaned’ RyRs no longer respond to LTCCs opening as they are functionally uncoupled by their distance, which leads to lower ICa_L -induced activation. The present results indicate that this is also the case in the chronically unloaded heart. This uncoupling is evidenced by the increased variance in

the time to peak of the Ca^{2+} transient and the increased Ca^{2+} spark frequency. A structural basis is suggested by the disruptions to the t-tubule network. The changes to the parameters of Ca^{2+} spark mass (FWHM and FDHM) may be dependent on a number of mechanisms, including properties of Ca^{2+} diffusion through the cell and altered opening features of individual RyR in each cluster (Zima *et al.*, 2008, Xu *et al.*, 2007, Song *et al.*, 2006, Sham *et al.*, 1998).

The suggestion that the deranged Ca^{2+} release is due to a structural abnormality in the t-tubules is supported by evidence that the points of delayed Ca^{2+} release spatially relate to defects in the t-tubule network in other studies (Meethal *et al.*, 2007). Whether there is RyR-LTCC uncoupling at those points is unknown but this is likely to be the case (Song *et al.*, 2006). Interestingly, when the t-tubule network is disrupted, increased Ca^{2+} spark frequency occurs in areas of delayed activation, indicating possible RyR-LTCC uncoupling (Biesmans *et al.*, 2011).

An important question is how t-tubule disruption leads to increased Ca^{2+} spark frequency, since displacement of the LTCC might be expected to reduce (not enhance) RyR activation. First, it is important to note that while spontaneous sparks localise to the t-tubules (Meethal *et al.*, 2007), this does not appear to be due to the presence of LTCC, as block of the ICa_L in rat atrial cardiomyocytes does not diminish the elevated Ca^{2+} spark frequency close to the membrane (Woo *et al.*, 2003). Moreover, the addition of BayK (an LTCC agonist) in the absence of Ca^{2+} influx, activates Ca^{2+} sparks (Katoh *et al.*, 2000). Indeed, the addition of cardiac DHPR loop II-III peptide to the patch pipette solution lowers Ca^{2+} spark frequency by 50%. This indicates that the normal relationship between the t-tubule membrane, LTCC and Ca^{2+}

spark frequency is due to factors more complex than simple Ca^{2+} influx through ICa_L (Li and Bers, 2001). Rather, the interaction between LTCC and RyR, regulated heavily by the t-tubules, appears to be critical. Thus, complex consequences of t-tubule irregularities might be expected, and not necessarily reduced Ca^{2+} spark frequency. Following myocardial infarction, Ca^{2+} spark frequency appears to increase in areas of delayed activation during the Ca^{2+} transient, where t-tubules would be disrupted (Biesmans *et al.*, 2011). Irregularities in the t-tubule membrane might result in mislocalisation of NCX also, which could locally elevate $[\text{Ca}^{2+}]_i$ and promote Ca^{2+} sparks through either directly activating RyR or via CaMKII (Song *et al.*, 2008). This mislocalisation of NCX could also account for the increased length of Ca^{2+} sparks (Biesmans *et al.*, 2011).

ICa_L was unchanged in density but fast (Ca^{2+} dependent) inactivation was prolonged. The mechanisms mediating fast Ca^{2+} dependent inactivation are complex. ICa_L inactivates with two time constants, one fast (between 7-50ms, Ca^{2+} dependent) and one slow (65-400ms, voltage-dependent) constant (Grandi *et al.*, 2010). The fast Ca^{2+} dependent inactivation depends on both the Ca^{2+} entering through the channel and on the Ca^{2+} released from the SR. One possible cause for delayed fast inactivation could be slower Ca^{2+} release from the SR.

There might be a number of other factors, other than t-tubule irregularity and dyadic uncoupling, which could result in altered Ca^{2+} spark frequency and diminished Ca^{2+} transient amplitude. Foremost amongst these is altered phosphorylation. Schwoerer *et al* reported that mechanical unloading reduced phosphorylation of Phospholamban (Phospholamban is an inhibitor of SERCA2a in the unphosphorylated state, but

inhibition is reduced upon Phospholamban phosphorylation) by approximately 50% (Schwoerer *et al.*, 2008). This could inhibit SERCA2a activity, which ought to reduce the SR Ca^{2+} content, and would be expected to reduce the Ca^{2+} transient amplitude and spark frequency. Further studies are needed to address this issue.

4.4.2 T-Tubule Physiology

The t-tubule is the structural basis of LTCC-RyR coupling. T-tubule disruption also correlates with the degree of HF (Wei *et al.*, 2010). There appears to be a tight connection between disrupted t-tubule structure and defective Ca^{2+} handling. This has been demonstrated as an important mechanism after pathological insults to the myocardium (reviewed in the Introduction). These results in this Chapter suggest chronic unloading could be added to the list of stressors to which the t-tubule structure is sensitive.

The pathological alterations to the t-tubules observed here as a result of prolonged mechanical unloading appear to be different in nature to those after myocardial overload and HF. Mechanical unloading appears to induce a relatively limited loss of regularity, rather than the loss of t-tubule density observed in HF (Louch *et al.*, 2010c). This could be related to the time-point, as t-tubule remodelling begins with irregularity and progresses to frank t-tubule loss during overload (Wei *et al.*, 2010, Ritter *et al.*, 2000). Given that these results suggest that load variation alters t-tubule structure, further studies are needed to examine the nature of that load sensitivity with respect to alterations to the degree and duration of load variation on t-tubule structure and local CICR. This will be addressed in the following Chapters.

4.4.3 Short-Term And Long-Term Unloading

Short-term mechanical unloading has been associated with improved myocardial function in normal (Ritter *et al.*, 2000) and failing hearts (Oriyanhan *et al.*, 2007). Improved myocardial function has also been demonstrated clinically (Maybaum *et al.*, 2007, Mancini *et al.*, 1998, Birks *et al.*, 2006). The mechanisms mediating improved function span the spectrum of cardiac physiology and include improved Ca^{2+} handling (Terracciano *et al.*, 2004). The following Chapter will address whether the initial functional improvements after mechanical unloading are mediated in part by effects on the t-tubule network.

Prolonged unloading is associated with impaired function and Ca^{2+} handling in the normal (Ito *et al.*, 2003) and failing heart (Oriyanhan *et al.*, 2007, Ogletree-Hughes *et al.*, 2001). These data correlate with the observed biphasic response of cardiac function after LVAD implantation in HF patients (Maybaum *et al.*, 2007). Maybaum *et al* show that the cardiac function (EF) is initially increased, as the overload is removed. However, with prolonged unloading cardiac function begins to decrease again. The mechanisms mediating this are not clear, but a clinical report highlights initial improvements in Ca^{2+} cycling (after LVAD therapy in patients with HF), including the expression of a number of Ca^{2+} handling proteins, followed by subsequent impairment in Ca^{2+} homeostasis with prolonged unloading (Ogletree-Hughes *et al.*, 2001).

It is difficult to compare the period of mechanical unloading investigated here with those that would be used clinically, as the timecourse of the response is very different. Previous studies indicated that an atrophic response was produced in four weeks, using HAHT (Soppa *et al.*, 2008b). Soppa *et al.* described the functional impairment

of chronically unloaded normal hearts and identified reduced myofilament sensitivity to Ca^{2+} as an important mechanism (Soppa *et al.*, 2008b).

4.4.4 Clinical Implications

These results provide support to the hypothesis that chronic mechanical unloading alone results in an atrophic derangement of contractile function in cardiomyocytes. T-tubule disruption appears to play a role in this phenomenon. How this interacts with the remodelling described in HF will be addressed in the following Chapter (Chapter 5).

4.4.5 Summary

Prolonged mechanical unloading of normal hearts impairs CICR in cardiomyocytes due to impairment of the SR Ca^{2+} release process. T-tubular disarray may be the structural basis of the impaired CICR observed. These results may explain the deleterious effects of prolonged mechanical unloading in experimental studies and the regression of functional improvements in clinical studies.

Chapter 5

Mechanical Unloading Reversed T-tubule Remodelling and Normalised Local Ca^{2+} -induced Ca^{2+} Release in a Rodent Model of Heart Failure

In this Chapter, the effects of treating failing hearts with mechanical unloading on CICR were investigated. Cellular Ca^{2+} transients, Ca^{2+} sparks, cell and t-tubule structure were studied. To assess DHPR-RyR colocalisation directly, immunofluorescence was used.

The experiments described in this chapter test the hypothesis that mechanical unloading of chronically failing hearts can restore t-tubule structure with important consequences for local CICR.

5.1 Introduction

The evidence presented in the Introduction suggests that t-tubule loss is a common mechanism of cellular dysfunction in HF. Furthermore, the experiments described in Chapter 4 suggest that the t-tubules are also sensitive to mechanical unloading; specifically, they suggest that prolonged mechanical unloading can disrupt the t-tubule system. This raises the possibility that there is a physiological range of loads which are compatible with normal t-tubule structure and CICR. Assuming that mechanical load variation activates specific subcellular signalling pathways which result in t-tubule structural abnormalities, normalising mechanical load could result in ‘retubulation’ with improved Ca^{2+} handling. This may be one mechanism mediating the improved function of failing hearts treated with LVADs, as discussed in the Introduction.

Chapter 4 showed that prolonged mechanical unloading of normal hearts distorted the t-tubules, which resulted in dysfunctional Ca^{2+} handling. Clinical trials suggest that mechanical unloading induces initial functional improvements in the failing heart, which regress after prolonged mechanical unloading (Maybaum *et al.*, 2007). LVAD therapy improves a number of features of cellular structure and function, but whether reverse remodelling of the t-tubules is an element of this is not known.

To test the hypothesis that t-tubule dysfunction of HF is reversible by mechanical unloading (HF-UN) and that this improves cellular Ca^{2+} handling, the structural and functional properties of failing rat cardiomyocytes were studied four weeks after HAHT.

5.2 Materials and Methods

Syngeneic male Lewis rats (10 - 12 weeks old, approximately 220g) were used in all the experiments. Four animals were used in each group.

5.2.1 HF Model

HF was induced by ligation of the left coronary artery. HF was defined as an EF of 40% or less at 12 weeks. Hearts were harvested at 12 weeks after ligation. Sham-operated animals were used as controls.

5.2.2 Heterotopic Abdominal Heart Transplantation.

The failing heart was harvested from the thorax and heterotopically transplanted into the abdomen of an age-matched syngeneic recipient, as described in the Methods (HF-UN). Briefly, the donor aorta was anastomosed to the recipient abdominal aorta and the donor pulmonary artery to the recipient inferior vena cava. Hearts were harvested 4 weeks following transplantation. LV cardiomyocytes were isolated and the following experiments were performed:

- Ca²⁺ transient and Ca²⁺ spark recordings
- SR Ca²⁺ content measurements
- Cell and t-tubule imaging using Di-8-ANEPPS
- Cell surface imaging using SICM (with help from Dr Alexey Moshkov)
- ICa_L recordings (Dr Urszula Siedlecka)
- Immunofluorescence imaging of RyR-DHPR colocalisation

5.2.3 Statistical Analysis

Statistical analysis was performed using non-parametric one-way ANOVA (Kruskal-Wallis test). Dunn's post-hoc test was used to test for differences between groups. The analysis was performed using Prism4 software (GraphPad software Inc., San Diego, CA, USA). $P < 0.05$ was taken as significant.

5.3 Results

5.3.1 Ca^{2+} Transient Features Were Improved After Mechanical Unloading

Single cardiomyocyte Ca^{2+} transients were studied in Sham, HF and HF-UN.

Compared to Sham, HF cells showed a depressed Ca^{2+} transient amplitude, which improved in HF-UN to Sham levels (Figure C5-1). An important determinant of the Ca^{2+} transient amplitude is SR Ca^{2+} content. This parameter was depressed in HF compared to Sham and increased towards Sham values in HF-UN ((Indo-1 ratio units) Sham 0.212 ± 0.07 $n=53$, HF 0.162 ± 0.04 $n = 20$, HF-UN 0.238 ± 0.05 $n=47$, Sham vs HF, $p=0.01$, HF vs HF-UN, $p=0.001$). HF cells showed a prolonged time to peak of the Ca^{2+} transient compared to Sham. Mechanical unloading achieved a significant reduction in time to peak. The time to 50 and 90 % decline in the Ca^{2+} transient were prolonged in HF compared to Sham. Mechanical unloading reduced these values towards Sham levels (Figure C5-1). The variance of the time to peak of the Ca^{2+} transients measured at each pixel was taken as an index of CICR synchronicity. HF cells showed a significant decrease in synchronicity of Ca^{2+} release (increase in variance of the time to peak), compared to Sham cells (Wasserstrom *et al.*, 2009, Kapur *et al.*, 2010) (Figure C5-1). Mechanical unloading induced a normalisation of the Ca^{2+} transient synchronicity (Figure C5-1). This suggests that the stimulus activates Ca^{2+} release throughout the cell uniformly. There are a number of possible causes for this normalisation of CICR including changes to ICa_L or normalised RyR function. Another possibility is that coupling between these trigger and release sites, which partly sets the gain of the positive feedback component of CICR, is reduced after HF and improved by mechanical unloading.

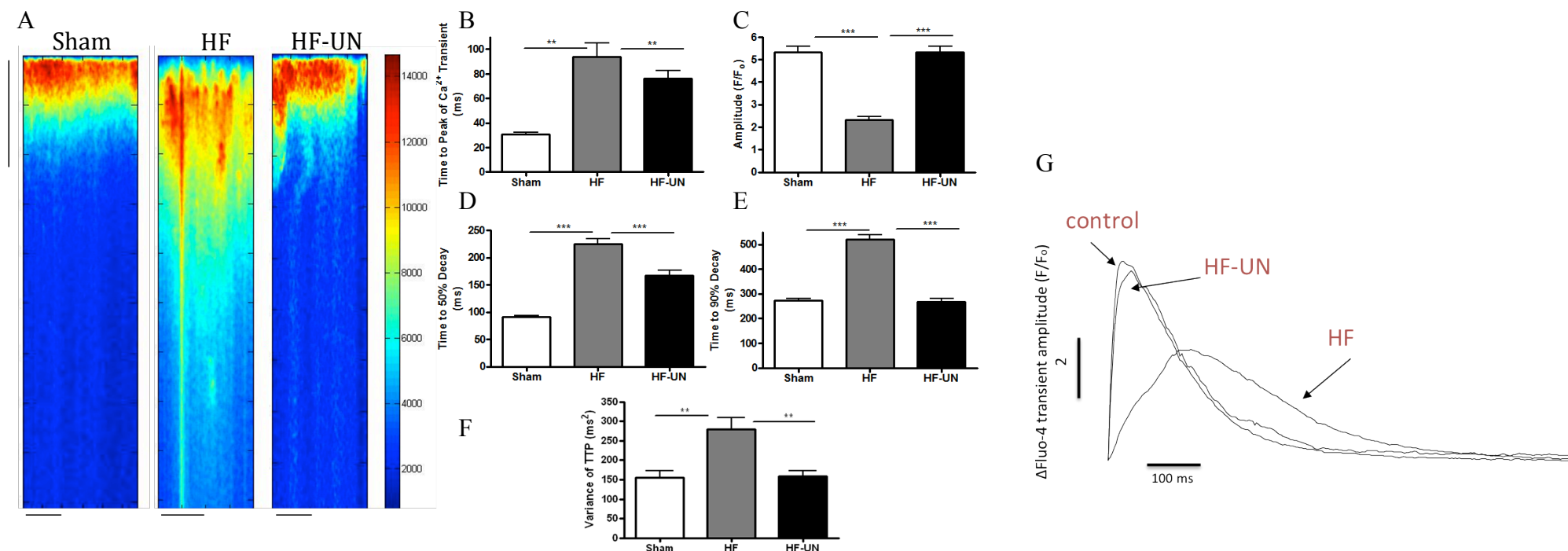


FIGURE C5-1: MECHANICAL UNLOADING INDUCED A NORMALISATION OF Ca^{2+} TRANSIENT PARAMETERS

(A) Line scans of Sham, HF and HF-UN cells. (B-E) The time to peak, decay to 50% and decay to 90% of the Ca^{2+} transient were prolonged in HF. Mechanical unloading normalised these parameters. The amplitude of the Ca^{2+} transient was depressed in HF and normalised by mechanical unloading. (F) The variance of the time to peak, an index of CICR synchronicity, was increased in HF and normalised by mechanical unloading. The vertical scale bar represents 200ms. The horizontal scale bar indicates 100 pixels (Sham n=40, HF n = 34, HF-UN n=29). The colour chart is a normalised scale of fluorescence intensity. (G) Two dimensional representation of Ca^{2+} transients from Sham, HF and HF-UN cells.

5.3.2 Raised Ca^{2+} Spark Frequency In HF Was Reduced By Mechanical Unloading

To assay SR Ca^{2+} release function, Ca^{2+} sparks were recorded. Ca^{2+} spark frequency was increased in HF cells compared to Sham (Figure C5-2). Mechanical unloading reduced Ca^{2+} spark frequency to Sham levels. HF cells had significantly higher Ca^{2+} spark peak amplitude compared with Sham cardiomyocytes, but unloading did not change Ca^{2+} spark peak amplitude. Ca^{2+} spark width and duration were increased by HF and increased further by mechanical unloading (Figure C5-2).

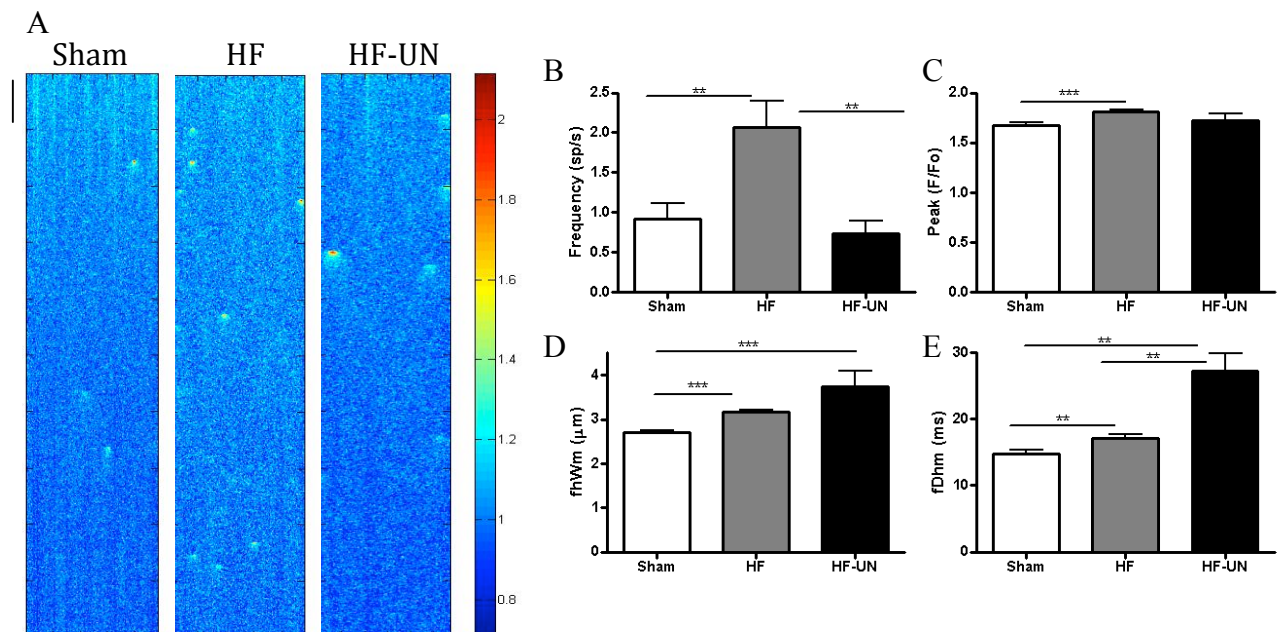


FIGURE C5-2: MECHANICAL UNLOADING REDUCED Ca^{2+} SPARK FREQUENCY

(A) Quiescent Fluo-4 labelled LV cardiomyocytes were imaged in line scans. (B) HF cells showed a higher Ca^{2+} spark frequency, (C) peak, (D) width and (E) duration. Mechanical unloading normalised Ca^{2+} spark frequency and increased width and duration. Mechanical unloading did not influence Ca^{2+} spark peak. The vertical scale bar indicates 280ms, and the horizontal scale bar indicates 100 pixels (Sham n=45, HF n = 34, HF-UN n=36). The colour chart is a normalised scale of fluorescence intensity.

5.3.3 Mechanical Unloading Caused A Regression Of Cellular Hypertrophy

To assess the volume of single cardiomyocytes, three-dimensional reconstructions of Di-8-ANEPPS stained cardiomyocytes were obtained. HF cells were larger than Sham on average, although the overall range of sizes was similar. Mechanical unloading induced a regression of hypertrophy, with average cell volume below that of Sham. In HF-UN, a new population of smaller cells appeared and the population of larger cells was lost (Figure C5-3, left panel). The appearance of a new population of smaller cells has led some investigators to suggest that mechanical unloading leads to the

appearance of a cardiac stem cell pool (Yacoub and Terracciano, 2011). This will be addressed in the discussion below. Cell capacitance provides a measure of cell area and was recorded during the electrophysiological experiments to record the $I_{Ca,L}$ density (Figure C5-3, right panel). The value of the cell capacitance changes as a result of altered surface sarcolemma and and/or t-tubule area. The increase in cell capacitance in HF can be attributed to an increased surface sarcolemma area accompanying hypertrophy. This regressed with mechanical unloading and was sufficient to decrease the cell capacitance.

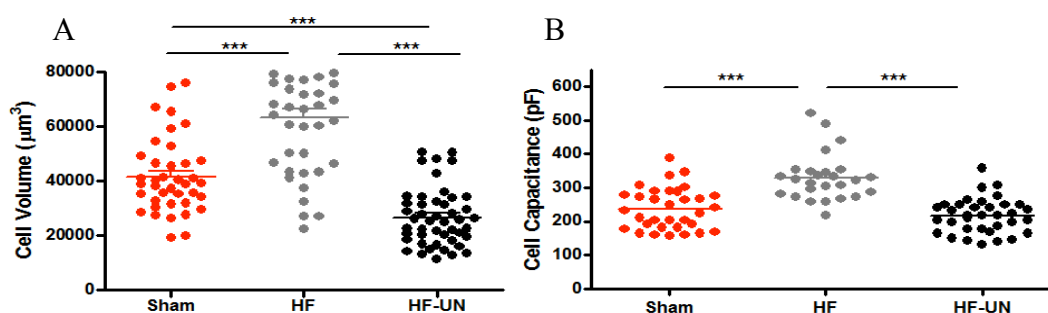


FIGURE C5-3: MECHANICAL UNLOADING CAUSED A REGRESSION OF CELLULAR HYPERTROPHY

(A) Cell volumes were analysed from three dimensional reconstructions of isolated LV cardiomyocytes stained with Di-8-ANEPPS. Mechanical unloading caused a regression of the cellular hypertrophy observed in HF, to below Sham levels. (B) The cell capacitance, a measure of cell area, was recorded during the recordings of $I_{Ca,L}$. The cell capacitance was increased in HF and reduced by mechanical unloading. (For cell volume, Sham n=39, HF n = 40, HF-UN n=47; For cell capacitance, Sham n=33, HF n = 26, HF-UN n=35).

5.3.4 Depressed $I_{Ca,L}$ Was Rescued By Mechanical Unloading

HF showed depressed $I_{Ca,L}$ compared to Sham (Figure C5-4). HF-UN normalised the $I_{Ca,L}$ density to Sham levels (Figure C5-4). The rate of activation of $I_{Ca,L}$ was unaffected by either HF or HF-UN (data not shown). The fast, Ca^{2+} -dependent

inactivation of $I_{Ca,L}$ was faster in HF and showed to Sham levels after mechanical unloading.

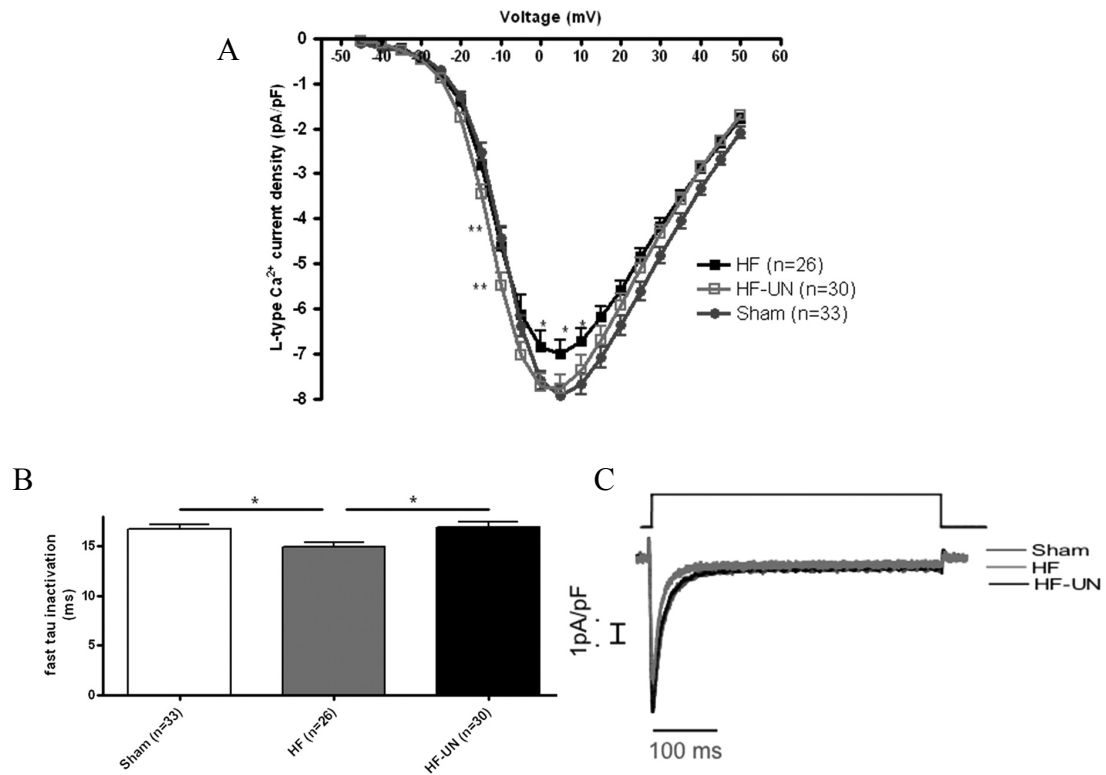


FIGURE C5-4: MECHANICAL UNLOADING RECOVERED THE DEPRESSED $I_{Ca,L}$ DENSITY OBSERVED IN HEART FAILURE

(A) The $I_{Ca,L}$ density was reduced in HF and normalised by mechanical unloading (Sham $n=33$, HF $n=26$, HF-UN $n=35$). (B) Fast inactivation was faster in HF and not different compared to Sham after mechanical unloading. (C) Raw traces of $I_{Ca,L}$ are shown. Cells were held at -40 mV and voltage clamped to $+5$ mV.

5.3.5 Mechanical Unloading Restored Normal T-Tubule Structure

HF reduced the t-tubule density significantly compared to Sham, with the appearance of a new population of cardiomyocytes with very low t-tubule density and the loss of the middle and top of the range of cardiomyocyte t-tubule densities (Figure C5-5). HF-UN increased the t-tubule density to Sham levels (Figure C5-5). The deterioration

in the regularity of the t-tubule system in HF, as measured by the power of the dominant frequency of the Fourier transform, was also partially normalised by HF-UN (Figure C5-5). These results indicate that mechanical unloading can normalise t-tubule structure. The impact of this on DHPR-RyR coupling and microarchitecture was investigated below.

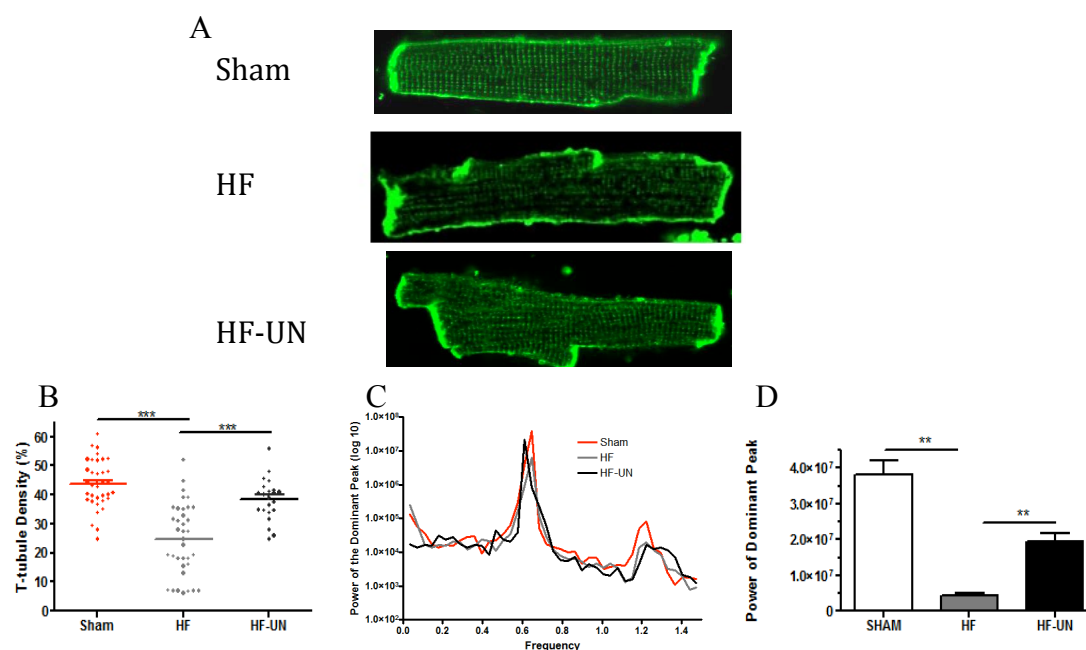


FIGURE C5-5: MECHANICAL UNLOADING RESTORED THE T-TUBULE DENSITY AND REGULARITY

(A) Single LV cardiomyocytes stained with Di-8-ANEPPS are shown. HF resulted in a reduced t-tubule density and (B & C) a lower power of the dominant peak of the Fourier transform of the t-tubules. Mechanical unloading improved t-tubule density and regularity. (Sham n=40, HF n = 32, HF-UN n=27).

5.3.6 T-Tubule Improvements After Mechanical Unloading Were Accompanied By Improved Cell Surface Structure

In Sham cells, clearly defined z-grooves were present which contain the t-tubule openings. This is in common with all ventricular mammalian cardiomyocytes studied to date. The cell surface is characterised by a high z-groove index, which provides a

measure of the actual z-groove over the predicted z-groove. The cell surface was flattened and distorted in HF, with a reduction in the z-groove index. SICM of cells from failing hearts after HF-UN showed the reappearance of a normal cell surface structure with clear striations (Figure C5-6). This was accompanied by a normalisation of the z-groove index. Therefore the cell surface undergoes profound remodelling in HF and reverse remodelling following mechanical unloading.

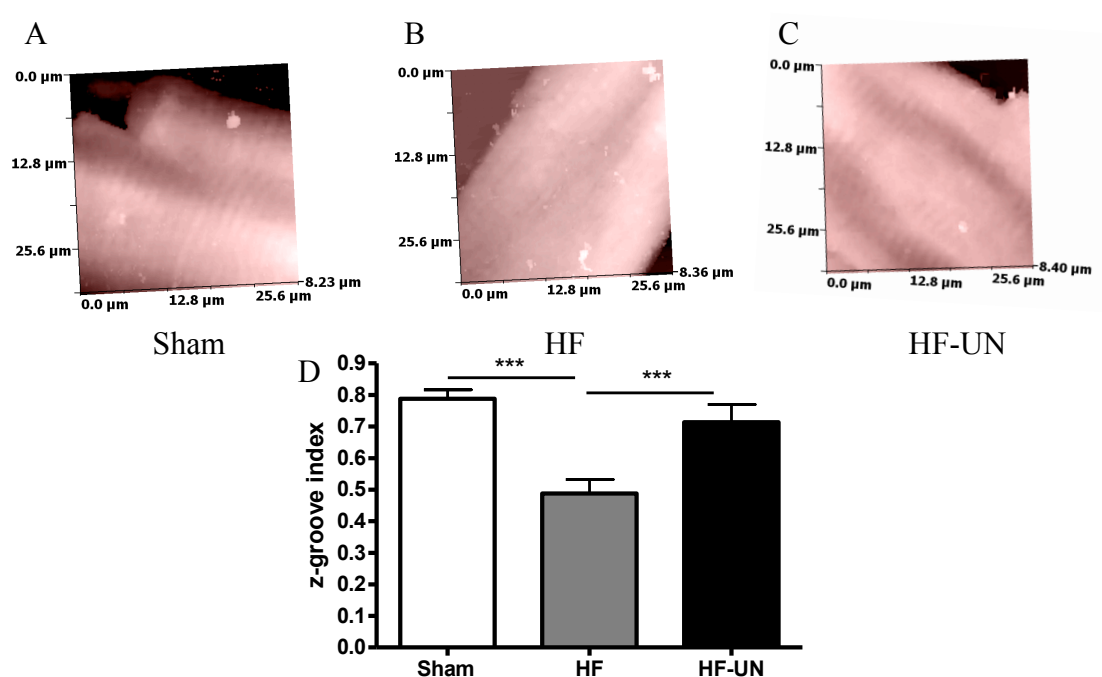


FIGURE C5-6: MECHANICAL UNLOADING RESTORED NORMAL CELL SURFACE ARCHITECTURE

(A-C) SICM shows the cell surface of isolated ventricular cardiomyocytes. (D) The z-groove index is an index of the regularity of the cell surface. This was reduced in HF and normalised by mechanical unloading. (Sham n=14, HF n = 25, HF-UN n=17).

5.3.7 Mechanical Unloading Restored Normal T-Tubule Microarchitecture

T-tubule microarchitecture was assessed using transmission EM of single cardiomyocytes, which were stuck flat along their long-axis using a Cytospin system,

described in the Methods. Only optical sections in which the sarcomere length was between 1.6-2.0 μm were used. The lumen of single t-tubules could be observed close to the z-disk. T-tubule lumen were reduced in density (measured as number of lumen per optical section) and were dilated in HF. Mechanical unloading normalised these parameters towards Sham levels (Figure C5-7).

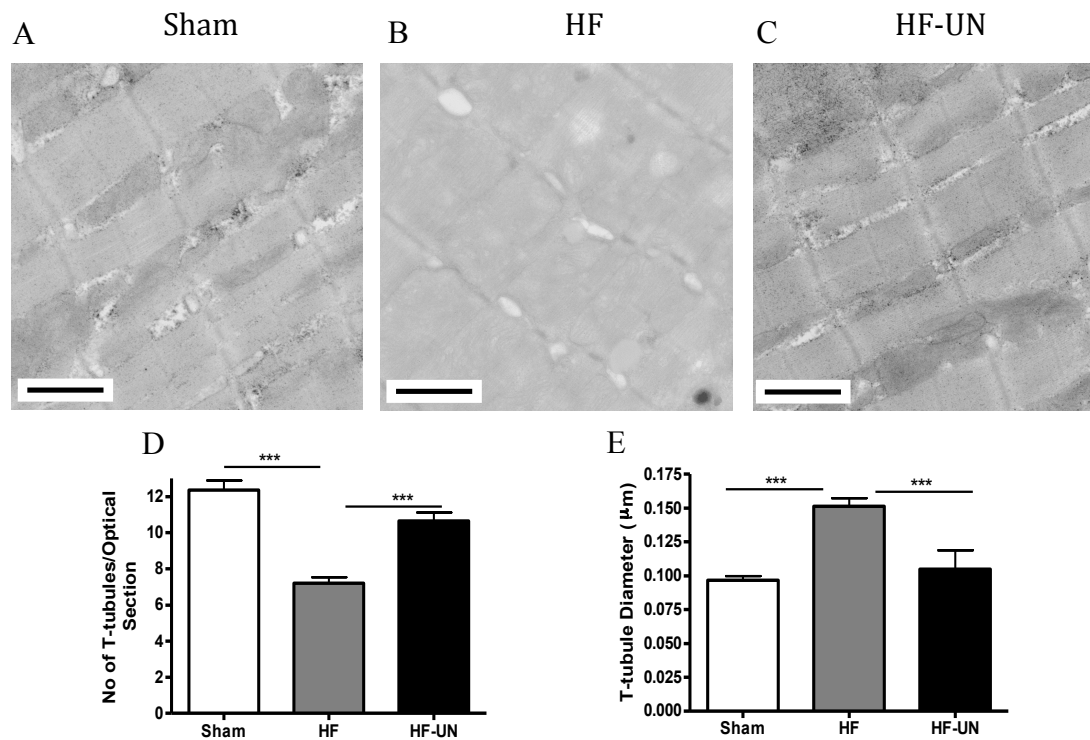


FIGURE C5-7: MECHANICAL UNLOADING RESTORED T-TUBULAR MICROARCHITECTURE

(A-C) Optical sections of TEM of isolated ventricular cardiomyocytes are shown. (D) HF cells showed fewer total t-tubules per optical section which were dilated. (E) Mechanical unloading caused a regression of t-tubular dilation, and a restoration of their normal density. Black scale bars indicate 1 μm (80 optical sections were analysed in each animal in each group).

5.3.8 Mechanical Unloading Restored DHPR-RyR Coupling

To test the hypothesis that the Ca^{2+} handling improvements observed here were the result of recoupling of DHPR-RyR, immunofluorescence was used to quantify the

degree of DHPR-RyR overlap. In cells from Sham-operated animals, the DHPR-RyR staining overlapped, resulting in a pattern of colocalised pixels which was ordered into regular striations. In HF, the degree of colocalisation was reduced as the t-tubular disruption caused loss of and disruption to the DHPR-RyR staining. Unloading normalised the pattern of DHPR and RyR staining and increased DHPR-RyR colocalisation, towards Sham levels (Figure C5-8).

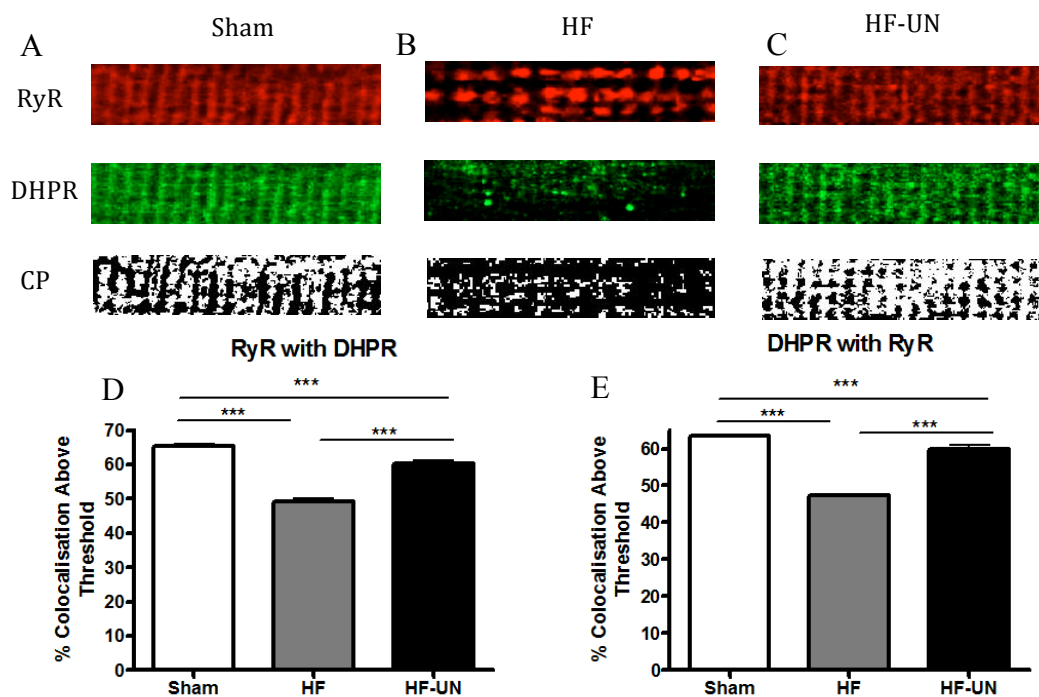


FIGURE C5-8: MECHANICAL UNLOADING RECOUPLED ORPHANED RYR WITH DHPR

(A-C) Examples of DHPR (green), RyR (red) and colocalised pixels (CP, white) are shown. In normal ventricular cardiomyocytes, there is a high degree of DHPR-RyR colocalisation with a clear striated pattern of DHPR-RyR interaction. (B,D,E) In HF, DHPR-RyR colocalisation is reduced with a loss of the striated pattern of colocalised pixels. Mechanical unloading significantly improves DHPR-RyR colocalisation. (Sham n=63, HF n = 49, HF-UN n=42).

5.4 Discussion

The experiments described in this Chapter suggest that mechanical unloading of failing hearts results in regression of a number of pathologic cellular properties observed in HF including the amplitude, time to peak and decay of the Ca^{2+} transient and the synchronicity of the Ca^{2+} release from the SR. Mechanical unloading normalised the Ca^{2+} spark frequency, t-tubule density and regularity all of which were altered in HF. Unloading also normalised ICa_L and SR Ca^{2+} content. Chronic mechanical unloading recovered the t-tubule system structure with improvements in CICR, and recoupling of DHPR-RyR. Such DHPR-RyR recoupling likely underlies the cellular Ca^{2+} handling improvements identified.

5.4.1 Ca^{2+} Handling Improvements

There is considerable evidence that HF cardiomyocytes have disrupted synchronicity of Ca^{2+} release during the Ca^{2+} transient (Song *et al.*, 2006, Louch *et al.*, 2010c, Louch *et al.*, 2006, Louch *et al.*, 2004). This Chapter showed similar results, with Ca^{2+} transients severely disrupted in HF and with multiple points of delayed SR Ca^{2+} release across the cell compared to Sham. This study did not attempt to colocalise defects in the t-tubule system and the Ca^{2+} transient, but other studies show that points of delayed SR Ca^{2+} release are localised to gaps in the t-tubule system (Song *et al.*, 2006, Louch *et al.*, 2004). SR Ca^{2+} release is initiated at t-tubules but propagates more slowly to activate RyR in regions devoid of t-tubules (Louch *et al.*, 2010c). The RyR distribution was partially affected in HF, although to a significantly lesser degree than for DHPR. The reappearance of regular t-tubules following mechanical unloading likely accounts for the improvements in Ca^{2+} release synchronicity by improving DHPR-RyR coupling.

SR Ca^{2+} content, which was also found reduced in HF, is a critical determinant of Ca^{2+} transient amplitude and rate of release. The SR Ca^{2+} content increased after mechanical unloading, which may contribute to the improvement in the Ca^{2+} transient amplitude. Recent evidence from studies in HF rats treated with SERCA2a adenovirus is helpful in understanding what impact this alone could have (Lyon *et al.*, 2012). Lyon *et al.* also showed that cell surface and t-tubule density underwent significant reverse remodelling after SERCA2a adeno-associated virus infection. This resulted in β_2 -AR redistribution and recompartmentalisation of cAMP signalling. Ca^{2+} transient synchrony was also enhanced after SERCA2a therapy, in a similar fashion to the results observed here. Lyon *et al.* also showed that SERCA2a gene therapy, for a period of 4-6 weeks, was sufficient to improve LV systolic function measured by pressure-volume loop analysis. Another important study in this regard is an investigation of the electrophysiological properties of cardiomyocytes from patients who recovered following LVAD implantation and those who did not. Terracciano *et al.* showed that clinical recovery was associated with an enhanced SR Ca^{2+} content specifically (Terracciano *et al.*, 2004). Whether the increase in SR Ca^{2+} content brought about by gene therapy approaches is sufficient to induce functional recovery is not known in the clinical setting. Ogletree-Hughes *et al.* (Ogletree-Hughes *et al.*, 2001) showed that short term LVAD support resulted in enhanced SERCA2a expression and improved contractility, but that longer duration support resulted in a reversion of these properties to failing levels. This supports the notion that physiological cellular Ca^{2+} handling is dependent on a normal range of mechanical load and that deviation in either positive or negative directions results in subnormal performance.

Changes to the RyR phosphorylation status and AP morphology are features which were not investigated, but which may contribute to the changes in the Ca^{2+} transient. With respect to AP changes, Chapter 4 suggests that mechanical unloading alone does not influence the AP morphology but whether this is true in the context of the failing heart is not known. Recent computational studies (Louch *et al.*, 2010a) suggest that AP changes per se are not primarily responsible for dyssynchronous Ca^{2+} release in HF, and that other changes, including t-tubule abnormalities, may be more important for small species; in man, however, AP morphology certainly would play a role in dyssynchronous Ca^{2+} release.

One way of investigating RyR function is to examine Ca^{2+} spark properties. In HF, spontaneous Ca^{2+} sparks occur more frequently at gaps in the t-tubules, suggesting that they are generated by uncoupled RyRs (Meethal *et al.*, 2007). This uncoupling is caused by disruptions to the t-tubule system (Heinzel *et al.*, 2002, Heinzel *et al.*, 2008, Song *et al.*, 2006) and could explain the finding that global Ca^{2+} spark frequency was raised in HF, and that it was normalised by the reappearance of t-tubules after unloading.

Evidence was presented in the previous Chapter to support the notion that coupling between DHPR and RyR stabilises the RyR and reduces Ca^{2+} spark frequency. This is supported by this Chapter, which shows that uncoupled, relatively detubulated cardiomyocytes show increased Ca^{2+} spark frequency, and that Ca^{2+} spark frequency was reduced when the t-tubules reappear. Exposure of RyR to the cardiac DHPR II-III loop peptide can inhibit RyR open probability in bilayers (Li and Bers, 2001). When

included in a dialysing patch pipette this peptide reduces Ca^{2+} spark frequency by 50% in voltage clamp mode (Li and Bers, 2001). Thus it could be possible that retubulation results in recoupling, which directly inhibits RyR. More work is needed to clarify this question.

The Ca^{2+} spark amplitude, duration and width are determined by mechanisms which are incompletely understood, but include the SR Ca^{2+} content, the functional status of RyR, the buffering properties of the cytoplasm and possibly the activity of NCX (Jordan *et al.*, 2010, Neco *et al.*, 2010). Recent evidence suggests that the increase in Ca^{2+} spark duration observed in HF might be due to misplacement of NCX, which leads to persistently raised local Ca^{2+} (Biesmans *et al.*, 2011). β -adrenergic stimulation may also affect the Ca^{2+} spark amplitude (Ramay *et al.*, 2011). All these elements are affected in HF and their interaction sets the Ca^{2+} spark amplitude, which was increased in this model, despite reduced SR Ca^{2+} content. Direct effects of phosphorylation on RyR open probability, due to involvement of CaMKII (Mishra *et al.*, 2010) or accessory proteins (Marx *et al.*, 2000) in HF, may occur but whether these aspects contribute to the Ca^{2+} spark changes observed here is not known, and further studies are required. Chapter 4 demonstrated that mechanical unloading of normal hearts resulted in cardiomyocytes with longer and wider Ca^{2+} sparks, which suggests that mechanical unloading in either the setting of HF or normal hearts could result in increased Ca^{2+} spark width and duration. No previous studies have examined the effect of unloading on Ca^{2+} spark properties and future studies should examine whether this could be due to altered cytoplasmic buffering, or post-translational regulation of RyR, for example by phosphorylation.

5.4.2 Regression Of Cellular Hypertrophy

Mechanical unloading induces a regression of cellular hypertrophy (Zafeiridis *et al.*, 1998). Compared with mechanical unloading of normal hearts described in Chapter 4, mechanical unloading of failing hearts does not cause as large a reduction in cell size. Although profound reductions in cell volume, atrophy, may induce dysfunction, there is no direct relationship between cell size and CICR, although it is clear that profound increases or reductions in cell size are associated with altered function (Oriyanhan *et al.*, 2007, Welsh *et al.*, 2001). Importantly, in a study of the effect of LVAD therapy on failing cardiomyocytes, cells from patients who showed clinical recovery showed enhanced SR Ca^{2+} content, reduced AP duration and improved inactivation properties of ICa_L (Terracciano *et al.*, 2004). That is to say that the regression of cellular hypertrophy occurred in both those who recovered and those who did not, while the cardiomyocytes of those who showed clinical recovery showed specific electrophysiological features.

5.4.3 ICa_L Changes

ICa_L density is reduced in some HF models (Soppa *et al.*, 2008b), while other studies do not show a depressed ICa_L (Schroder *et al.*, 1998). ICa_L depends on both the number and the activity of LTCCs. Since there was a reduction in the t-tubule density in our model, it is likely that the number of channels was reduced. Typically, investigators have explained reports of unchanged ICa_L between Sham and HF as due to increased single channel activity and likely to be altered by the overall severity of the model of HF (Brette *et al.*, 2004b, Brette *et al.*, 2006, Brette *et al.*, 2004a, Chen *et al.*, 2002, Yang *et al.*, 2000). This study shows normalisation of ICa_L with unloading, which suggests that either the new t-tubules contain LTCCs or the same

channels have improved conductance. Further studies are required to clarify these issues.

Fast, Ca^{2+} dependent inactivation time was reduced (faster) in HF and normalised after mechanical unloading. In Chapter 4, mechanical unloading alone resulted in a slowing of fast, Ca^{2+} dependent inactivation. The mechanisms for this may be linked to SR Ca^{2+} release and merit further study.

5.4.4 T-Tubule Physiology

This study confirms the deterioration in t-tubule structure observed by many investigators (reviewed in (Louch *et al.*, 2010c)) in models of HF (He *et al.*, 2001, Gomez *et al.*, 1997, Song *et al.*, 2006) and in diseased human myocardium (Lyon *et al.*, 2009). The nature of this disruption appears to be different depending on the animal model. HF models in large animals appear to show drastic reductions in the t-tubule density, also confirmed in man, and are associated with a reduction in the regularity of the remaining t-tubules. In small animal models, the dysfunction is dominated by a reduction in the regularity of this system, in the absence of large reductions in t-tubule density. However, in our model there is a modest but significant reduction in t-tubule density, which likely reflects the severity of HF obtained in these animals.

This Chapter is the first study to show a recovery of the t-tubule system in post-ischemic cardiomyopathy by reducing mechanical load. Only one study, using exercise in murine diabetic cardiomyopathy, has previously shown that this recovery is possible (Stolen *et al.*, 2009).

The mechanisms mediating the load-dependence of the t-tubule structure are poorly defined, and further studies are needed to dissect the molecular mechanisms. These studies will assess the therapeutic potential of the t-tubules. Such molecules include Tcap, which has recently been shown to be both stretch-sensitive and involved with orderly t-tubule formation (Zhang *et al.*, 2009). The regulators of other proteins, such as BIN1, which is involved in t-tubule biogenesis and the shuttling of LTCCs to the membrane, is not known (Lee *et al.*, 2002, Hong *et al.*, 2012). It was also recently demonstrated that JPH2, a protein thought to promote dyad coupling, was reduced as cardiac hypertrophy developed into HF (Wei *et al.*, 2010). How these molecular pathways interact in health and disease is a central question for future studies. Recent evidence has come from the study mentioned previously on SERCA2a gene therapy in the failing heart (Lyon *et al.*, 2012). This study showed that the recovery of t-tubule density and cell surface structure was associated with enhanced expression of BIN1 and Tcap protein, but not JPH2. Whether these are causally related to the recovery of the t-tubule system is untested. Chapter 7 will address the role of Tcap in t-tubule structure and function.

5.4.5 Summary

This Chapter shows that mechanical unloading of failing hearts produces recovery of the t-tubule structure with improvements to local CICR, likely as a result of enhanced DHPR-RyR coupling. These changes may constitute a subcellular mechanism for the initial improvements in cardiac function seen after LVAD therapy in patients with HF.

Chapter 6

Cardiomyocyte Ca^{2+} Handling And Structure Are Regulated By Degree And Duration Of Mechanical Load Variation

In this Chapter, the effect of chronic mechanical load on local CICR and t-tubule structure is examined using a model of chronic mechanical overload (TAC) and moderate mechanical unloading (HAHLT). Results are then compared with those presented in Chapter 4, which employed a model of severe mechanical unloading, heterotopic abdominal heart-only transplantation. Cellular Ca^{2+} transients, Ca^{2+} sparks, cell and t-tubule structure were studied.

These experiments assessed the following hypothesis: the t-tubule network and local CICR are sensitive to the degree and duration of mechanical load.

6.1 Introduction

T-tubule dysfunction is a major feature of HF in human cardiomyocytes (Lyon *et al.*, 2009), and in animal models of overload, myocardial infarction and arrhythmia (reviewed in the Introduction), which was also found in Chapter 5. HF is driven by a wide range of insults to the myocardium, including inflammation and altered systemic signalling, and it is advantageous to study the effect of isolated chronic mechanical overload. Chronic mechanical overload alone, prior to the advent of HF, may lead to disruption of the t-tubule system (Wei *et al.*, 2010). Prolonged mechanical unloading of the normal heart can also disrupt t-tubule structure and impair local CICR, as found in Chapter 4. In addition, mechanical unloading can promote reverse functional and ultrastructural remodelling in HF (Chapter 5) but unloading-induced improvements regress and prolonged unloading can cause further dysfunction (Oriyanhan *et al.*, 2007). Reducing the degree of unloading could prevent these long-term negative effects and possibly favour a stable myocardial recovery, but this has not been thoroughly investigated (Wang *et al.*, 2008, Wang *et al.*, 2009). These studies raise the question of the nature of the load sensitivity of the myocardium. Specifically, it is not clear whether there is an optimal range of degrees and durations of load variation, and the nature of the transition to a non-physiological range is not understood.

6.2 Materials and Methods

Syngeneic male Lewis rats (10 - 12 weeks old, approximately 220g) were used in all the experiments.

6.2.1 Mechanical Unloading

Moderate mechanical unloading (UN) was obtained by transplanting a heart-lung block from a donor animal into the abdomen of a syngeneic recipient (HAHLT). Briefly, the ascending aorta of the donor was anastomosed to the recipient abdominal aorta. Coronary blood flow is directed to the right heart via the coronary sinus, through the pulmonary circulation and then to the LV. Therefore, the LV only ejects the coronary and not the systemic return, and is moderately mechanically unloaded. Severe, full unloading (S-UN) (as used during Chapters 4 and 5) was obtained by using the heterotopic abdominal heart only transplantation, which completely abolishes blood return to the LV via the pulmonary veins. Data from the experiments performed in Chapter 4 are compared with the moderate unloading data generated in these experiments in Table C6-1. The recipient's native heart acted as a control in these experiments.

6.2.2 TAC Model

The TAC model was used to overload the heart by increasing afterload. A right thoracotomy was made, and the ascending aorta exposed. A suture was looped around the aorta and tied over a 0.9mm bar placed between the ascending aorta and the suture. The suture was tied tightly and the needle removed. Sham-operated rat hearts acted as a control.

6.2.3 Duration Of Mechanical Load Variation And Cell Isolation

UN hearts and normal recipient hearts were harvested after 4 or 8 weeks. The 4-week moderate unloading results are compared with the 4-week severe unloading results generated in Chapter 4. Mechanically overloaded hearts were harvested after 6 or 10 weeks. The number of animals studied in each group were as follows: UN 8 weeks n=3, UN 4 weeks n=4, Control n=7, TAC 6 weeks n=5, TAC 10 weeks n=6.

Single LV cardiomyocytes were isolated as described previously (Siedlecka *et al.*, 2008). All experiments were conducted at 37°C.

Following cell isolation, the following experiments were performed:

- Ca²⁺ transient and Ca²⁺ spark recordings
- SR Ca²⁺ content measurements
- Cell and t-tubule imaging using Di-8-ANEPPS
- Cell surface imaging using SICM (with help from Dr Sergiy Tokar)
- ICa_L recordings (Dr Urszula Siedlecka)

6.2.4 Statistical Analysis

Statistical analysis was performed using Prism4 software (GraphPad software Inc., San Diego, Ca, USA), and the non-parametric Kruskal-Wallis test was used to assess statistical differences. Dunn's post-hoc test was used to compare groups. As no statistical differences were detected between sham-operated controls (for the TAC group) and native recipient heart controls, these two groups were pooled in a single control group for clarity. In the comparison section with Chapter 4, values were

normalised to their controls by dividing each value by the mean control value. These data are reported in ratio units (Table C6-1).

6.3 Results

6.3.1 Chronic Mechanical Load Variation Altered LV Weight and Cardiomyocyte Volume

4 weeks UN significantly reduced cardiomyocyte volume by 25.7%, but did not cause a reduced LV weight (Figure C6-1). After 8 weeks UN, cell volume was further reduced (a 60.1% reduction compared to control, Figure C6-1), as was LV weight. Interestingly, as UN progressed with time, the spread of cell volumes became smaller, as well as the mean. UN at 4 weeks showed a range of cell volumes within that of the control group. At 8 weeks a population of smaller cells, with a cell volume below the minimum range of the control group, were documented.

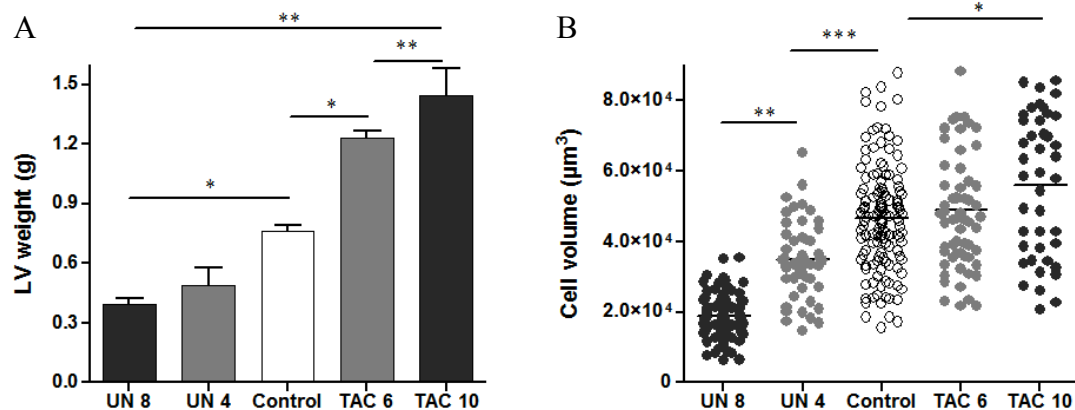


FIGURE C6-1: CHRONIC MECHANICAL LOAD VARIATION ALTERED LV WEIGHT AND CARDIOMYOCYTE VOLUME

(A) LV weight of moderately unloaded and overloaded hearts (UN 8 n=3, UN 4 n=4, Control n=7, TAC 6 n=5, TAC 10 n=6) (B) Cell volume of Di-8-ANEPPS stained LV cardiomyocytes from hearts subjected to chronic mechanical load variation of different periods (UN 8 n=80, UN 4 n=50, Control n=136, TAC 6 n=60, TAC 10 n=42).

Mechanical overload for 6 weeks caused significant LV but not cardiomyocyte hypertrophy (Figure C6-1). Mechanical overload for 10 weeks caused significant LV and cardiomyocyte hypertrophy (23.6% increase in cardiomyocyte volume compared

to Sham, Figure C6-1). The mismatch between LV weight and cell volume might be artefactual and is discussed below. It is notable that the range of cell volumes did not change after mechanical unloading, but the mean did, owing to shifts in the relative distribution of cell volumes within the same range.

6.3.2 The Ca^{2+} Transient Was Affected By Degree And Duration Of Mechanical Load Variation

4 weeks UN did not influence the time to peak of the Ca^{2+} transient, which was prolonged by 17.4% at 8 weeks unloading (Figure C6-2). 4 weeks UN did not affect the amplitude of the Ca^{2+} transient, but 8 weeks significantly reduced this (Figure C6-2). Both 4 and 8 weeks UN increased the time to 50% and time to 90% decline of the Ca^{2+} transient. The variance of the time to peak of the Ca^{2+} transient was increased after 8 weeks but not 4 weeks UN (Figure C6-2).

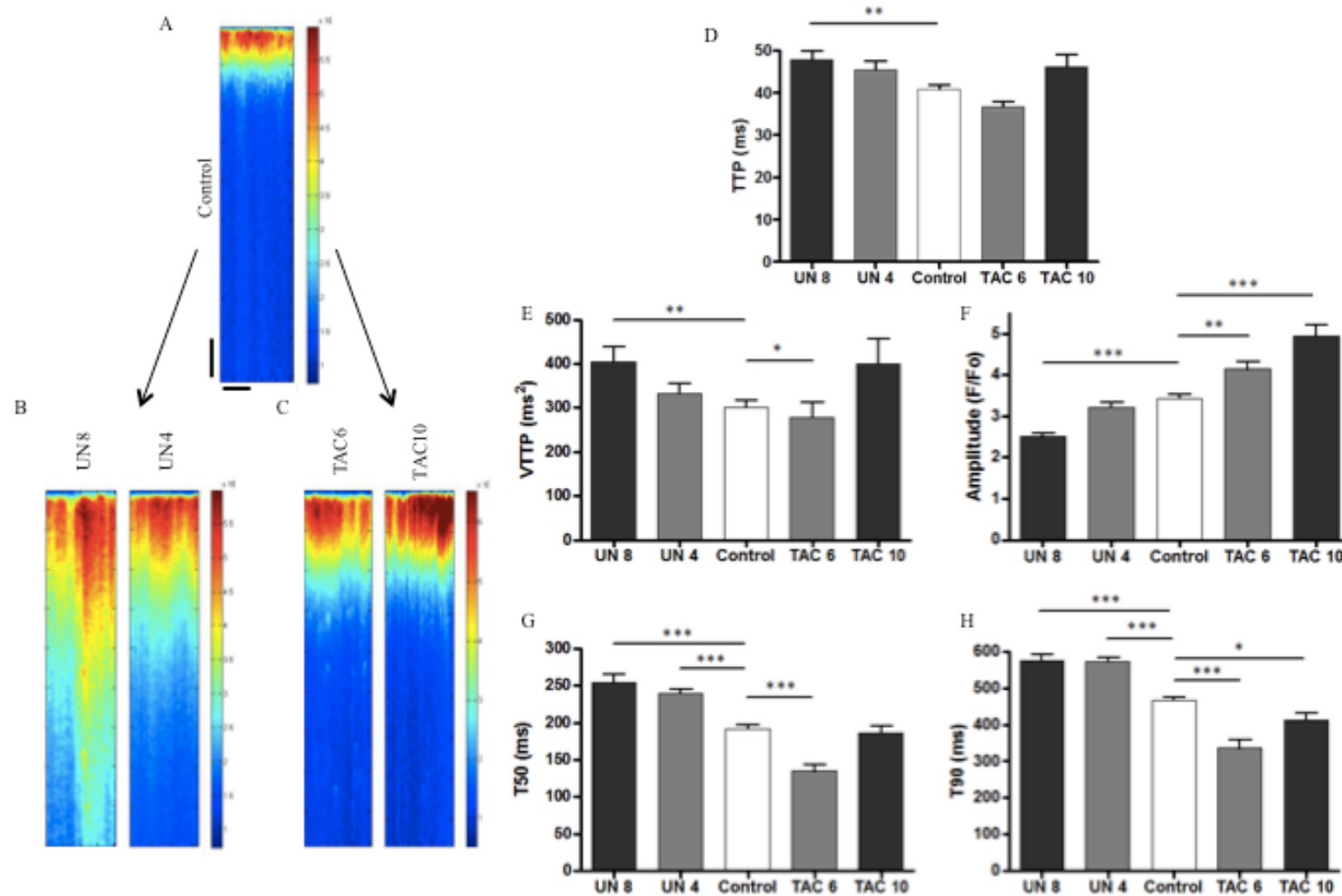


FIGURE C6-2: THE Ca^{2+} TRANSIENT WAS AFFECTED BY DEGREE AND DURATION OF MECHANICAL LOAD VARIATION

(A) Ca^{2+} transients of control, (B) unloaded and (C) overloaded cardiomyocytes. (D) Variance of the time to peak of the Ca^{2+} transient. (E) Time to peak, (F) amplitude, (G) time to 50% decline and (H) time to 90% decline of the Ca^{2+} transient were assessed (UN 8 n=50, UN 4 n=46, Control n=141, TAC 6 n=34, TAC 10 n=21). The vertical scale bar represents 100ms and the horizontal scale bar represents 100 pixels. The colour chart is a normalised scale of fluorescence intensity.

Time to peak of the Ca^{2+} transient was unaffected by the duration of mechanical overload used in this study (Figure C6-2). After 6 and 10 weeks of mechanical overload, the amplitude of the Ca^{2+} transient was larger and the decline of the Ca^{2+} transient was faster compared with controls. 6 weeks of mechanical overload reduced the variance of the time to peak of the Ca^{2+} transient, which was then increased to control levels at 10 weeks (Figure C6-2). SR Ca^{2+} content was augmented at 10 weeks of mechanical overload compared to controls (Control: 0.39 ± 0.1 ratio units, $n=30$ vs TAC 10 weeks: 0.47 ± 0.1 ratio units, $n=29$, $p < 0.0001$). In summary, UN progressively slowed and reduced the amplitude of the whole cell Ca^{2+} transient but the two overloading conditions induced responses which resembled physiological adaptation to stress.

6.3.3 Ca^{2+} Spark Frequency And Morphology Were Affected By Degree And Duration Of Mechanical Load Variation

8 but not 4 weeks UN increased Ca^{2+} spark frequency. 4 weeks UN increased the Ca^{2+} spark peak amplitude, which was reduced at 8 weeks UN compared to control (Figure C6-3). 4 and 8 weeks UN increased Ca^{2+} spark width and duration.

10 but not 6 weeks of mechanical overload increased Ca^{2+} spark frequency (Figure C6-3). 6 and 10 weeks of mechanical overload increased Ca^{2+} spark peak and width. Again, although these were statistically significant, they were small in magnitude and possibly of limited functional relevance. 6 weeks of mechanical overload reduced Ca^{2+} spark duration, which was increased at 10 weeks compared to control. These results indicate that Ca^{2+} sparks are affected by the direction, degree and duration of chronic mechanical load.

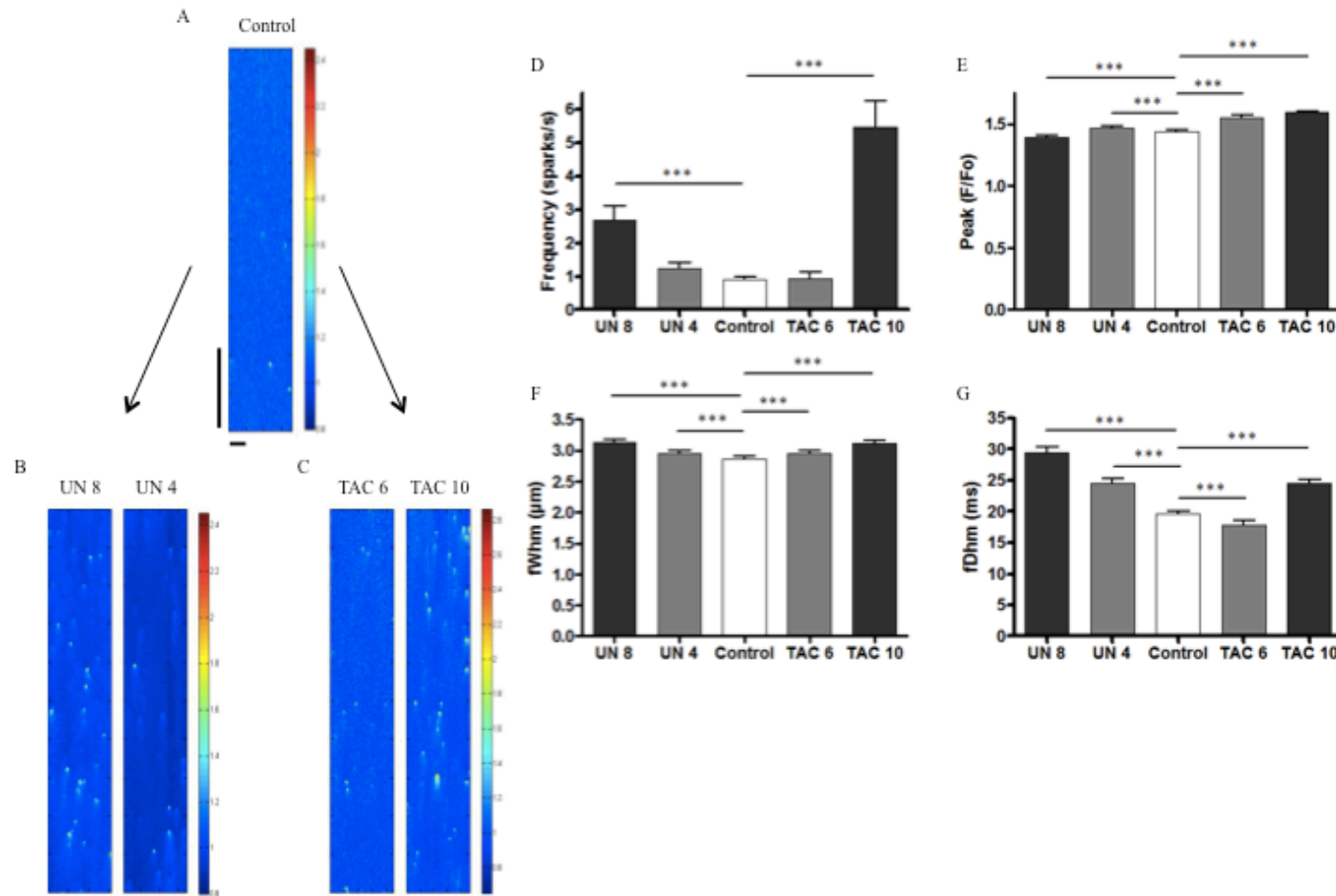


FIGURE C6-3: Ca^{2+} SPARK FREQUENCY AND MORPHOLOGY WERE AFFECTED BY DEGREE AND DURATION OF MECHANICAL LOAD VARIATION

(A) Representative traces of quiescent cardiomyocytes taken in line-scan mode to measure Ca^{2+} spark features in control, (B) unloaded and (C) overloaded cardiomyocytes. Graphs show (D) Ca^{2+} spark frequency (UN 8 n=75, UN 4 n=73, Control n=173, TAC 6 n=50, TAC 10 n=28), (E) peak, (F) width and (G) duration (UN 8 n=359, UN 4 n=242, Control n=447, TAC 6 n=152, TAC 10 n=513). The vertical scale bar represents 768ms and the horizontal scale bar represents 100 pixels. The colour chart is a normalised scale of fluorescence intensity.

6.3.4 T-Tubule Structure Is Sensitive To Degree And Duration Of Mechanical Load Variation

Neither 4 nor 8 weeks UN altered the t-tubule density, as was found in Chapter 4 (Figure C6-4). 8 but not 4 weeks UN reduced the t-tubule regularity, measured as the peak of the Fourier transform. At 6 weeks of mechanical overload, neither t-tubule density nor regularity were affected (Figure C6-4). However, 10 weeks of mechanical overload depressed both t-tubule density and regularity. Interestingly, all the data points of the experimental groups lie within the spread of the control data. The significant differences in the mean are the result of a different distribution of cell volumes within the same range. These data show that t-tubule structure is progressively disrupted by either chronic unloading or overloading of the myocardium.

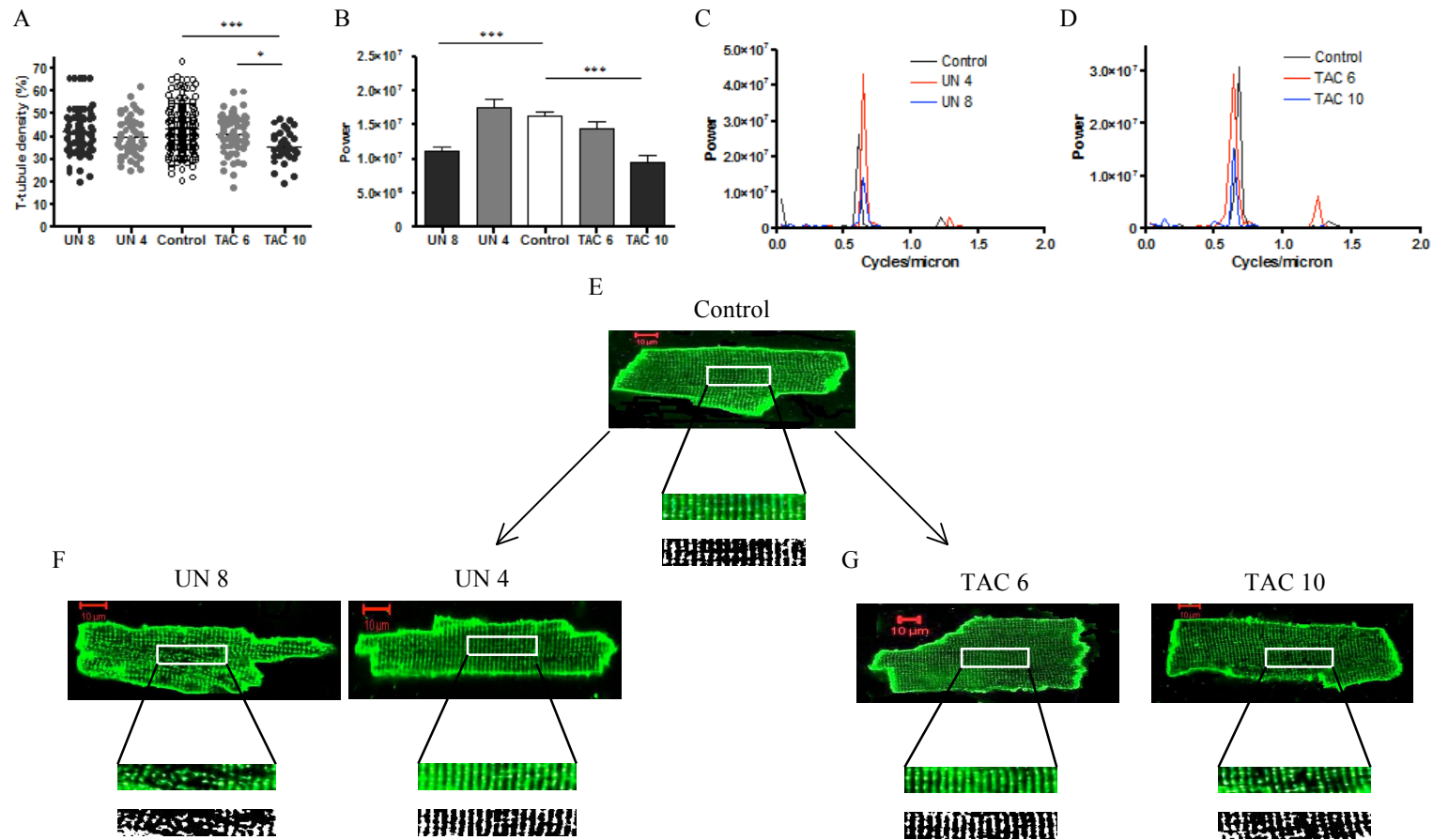


FIGURE C6-4: T-TUBULE STRUCTURE IS SENSITIVE TO DEGREE AND DURATION OF MECHANICAL LOAD VARIATION

(A) T-tubule density was measured in cardiomyocytes from hearts undergoing chronic load variation (UN 8 n=80, UN 4 n=50, Control n=157, TAC 6 n=61, TAC 10 n=31). (B) T-tubule regularity was recorded using Fourier transforms of (C) unloaded and (D) overloaded cardiomyocytes (UN 8 n=82, UN 4 n=53, Control n=151, TAC 6 n=42, TAC 10 n=33). (E) Representative images of Di-8-ANEPPS stained LV cardiomyocytes from control, (F) unloaded and (G) overloaded hearts. Inset shows binary images of central portions of the cardiomyocytes.

6.3.5 Severe But Not Moderate Mechanical Unloading At 4 Weeks Is Associated With Pathological Remodelling Of Local CICR, T-Tubule And Cell Structure

To examine the impact of moderate versus severe mechanical unloading, the results obtained after 4 weeks UN (as used throughout above) were compared with the results obtained using a model of severe unloading (S-UN), previously used in Chapter 4 and 5. S-UN was associated with significantly smaller cell volume than UN (Table C6-1). Neither severe nor moderate unloading affected the t-tubule density with respect to control, although UN was surprisingly associated with lower t-tubule density compared to S-UN. While 4 weeks of UN did not alter t-tubule regularity, S-UN was associated with significant loss of regularity of the t-tubule system (Table C6-1).

All values are ratio of control	UN 4	S UN 4	p value
Cell Volume	0.63 ± 0.04 (n=59)	0.43 ± 0.02 (n=90)	***
T-tubule Density	0.89 ± 0.03 (n=50)	1.03 ± 0.02 (n=90)	***
T-tubule Regularity	1.10 ± 0.08 (n=53)	0.13 ± 0.01 (n=49)	***
z-groove index	0.93 ± 0.15 (n=6)	0.47 ± 0.03 (n=17)	0.064
Ca²⁺ Transient TTP	1.12 ± 0.05 (n=46)	1.35 ± 0.08 (n=42)	*
Ca²⁺ Transient Amplitude	0.99 ± 0.04 (n=46)	0.71 ± 0.04 (n=42)	***
Ca²⁺ Transient T50	1.24 ± 0.03 (n=46)	1.66 ± 0.12 (n=42)	**
Ca²⁺ Transient T90	1.24 ± 0.02 (n=46)	1.36 ± 0.07 (n=42)	0.997
Ca²⁺ Transient VTTP	1.15 ± 0.07 (n=46)	1.80 ± 0.16 (n=42)	**
ICa,L Peak Current	0.77 ± 0.06 (n=26)	1.04 ± 0.04 (n=15)	**
Spark Frequency	1.18 ± 0.17 (n=73)	3.85 ± 0.65 (n=87)	***
Spark Peak	1.12 ± 0.02 (n=297)	0.96 ± 0.01 (n=459)	***
Spark Width	1.03 ± 0.02 (n=242)	1.23 ± 0.02 (n=410)	***
Spark Duration	1.23 ± 0.04 (n=242)	1.74 ± 0.06 (n=410)	***

TABLE C6-1: COMPARISON OF SEVERE UNLOADING (HAHT) VERSUS MODERATE UNLOADING (HAHLT)

Values were obtained by dividing each value from the experimental group in question by the mean control value. This represents the effect of either S-UN or UN compared to its respective control. These values were then compared using a non-parametric t-test. TTP – time to peak; T50 – time to 50% decline; T90 – time to 90% decline. VTTP – variance of the time to peak. In the case of ICa,L, the peak current density was used.

S-UN increased the variance, prolonged the mean of the time to peak and time to 50% decline and reduced the amplitude of the Ca^{2+} transient compared to S-UN (Table C6-1). The ICa_L was unaffected by either form of mechanical unloading compared to control, but UN was associated with lower peak ICa_L compared with S-UN. Given that with respect to their individual controls, there is no change to ICa_L , it appears that ICa_L is not affected by mechanical unloading. The Ca^{2+} spark frequency, width and duration were higher in S-UN compared to UN (Table C6-1). Ca^{2+} spark peak amplitude was lower in S-UN.

To assess the impact of different degrees of unloading on the cell surface, SICM was used. S-UN appeared to induce some changes to the cell surface but this effect was not significantly different to the effect of UN (Table C6-1). In summary, these experiments show that the effects on CICR and t-tubules are graded by the severity of mechanical unloading.

6. 4 Discussion

These results show that severe chronic increases or decreases in load are associated with significant changes in local CICR and t-tubule structure of normal LV cardiomyocytes. There were limited effects on these parameters by changes in load that are less severe or maintained for a shorter time period.

6.4.1 Effect Of The Degree Of Mechanical Unloading

The differential impact of the two models for severe and moderate unloading has been corroborated in previous studies (Wang *et al.*, 2009). This Chapter shows that reduction of cell size obtained with moderate/short periods of unloading is accompanied by preserved cell function, supporting the notion of *physiological hypotrophy*, similar, but opposite in direction, to physiological hypertrophy (Figure C6- 5). This is distinct from *pathological atrophy*, which is observed after prolonged and severe unloading and is associated with dysfunction. Mechanical unloading using LVADs can produce major reverse remodelling and promote cardiac recovery (as reviewed in the Introduction) but prolonged unloading-induced atrophy may be a major impediment. One possible approach to prevent atrophic remodelling of the LV is the use of moderate mechanical unloading (Wang *et al.*, 2008). The data presented in this study supports the concept that pathological atrophic remodelling is delayed with the use of moderate mechanical unloading. CICR remodelling is a central defect in failing cardiomyocytes and reversal of these changes is specifically associated with cardiac recovery (Terracciano *et al.*, 2004). However, prolonged mechanical unloading can impair CICR, as documented in Table C6-1.

6.4.2 Alterations In Cell Volume And Myocardial Hypertrophy

Both mechanical overload and unloading resulted in changes to the cell volume and LV weight. LV weight and cardiomyocyte volume did not necessarily change together, with a significant reduction in cell volume but not LV weight at 4 weeks of mechanical unloading, and a significant increase in LV weight but not cell volume at 6 weeks of mechanical unloading (Figure C6-1). The mechanisms of this are unclear. The measurement of LV weight occurs after enzymatic digestion on the Langendorff perfusion system, but while the LV is still relatively intact, and prior to dissection of the tissue into smaller pieces and isolation of single cardiomyocytes. As LV weighing occurs at this early stage it is possibly less vulnerable to confounding factors. The spread of the data might also play a role in preventing statistical significance. A greater number of experimental replicates could help to overcome the problem of spread of the data.

6.4.3 Load Variation And Local CICR

The Ca^{2+} transient undergoes pathological remodelling in mechanical unloading, and was depressed in amplitude and delayed in its time course at 8 weeks of mechanical unloading. Shorter term unloading did not result in such changes. Severe mechanical unloading resulted in significantly more Ca^{2+} transient remodelling than moderate unloading, indicating a graded effect of degree of unloading. Mechanical overload resulted in an augmented Ca^{2+} transient amplitude and faster decline at 6 and 10 weeks. These changes are likely to be a part of the physiological compensation to mechanical overload, and it is known that such physiological responses can be maintained up to 24 weeks after the institution of chronic mechanical overload (Del Monte *et al.*, 2002). HF and the maladaptive response are heralded by abnormal cellular structure and function. The variance of the time to peak of the Ca^{2+} transient

was increased after 8 weeks of mechanical unloading, indicating greater CICR dyssynchrony, related to the disruption of the t-tubule structure. Interestingly, the variance of the time to peak of the Ca^{2+} transient was reduced (better synchrony) at 6 weeks of mechanical overload, possibly indicating enhanced cellular activation due to the augmented Ca^{2+} transient. This effect was lost at 10 weeks of mechanical overload, which accompanied t-tubule loss and disorganisation. While shorter-term mechanical overload is within a physiological range and associated with preserved t-tubule structure, as in physiological hypertrophy of exercise (Stolen *et al.*, 2009), prolonged overload likely represents the beginning of a maladaptive response. The enhanced SR Ca^{2+} content observed at 10 weeks mechanical overload could account for the fact that, despite the loss of the t-tubule network, relatively normal Ca^{2+} transients were observed. The increased SR Ca^{2+} content could mitigate the reduced LTCC-RyR coupling, allowing an early stage of compensated T-tubule dysfunction. ICa_L was unchanged compared to control in both moderate and severe unloading.

Increased Ca^{2+} spark frequency, an indication of SR Ca^{2+} leak which is observed in HF (Venetucci *et al.*, 2008), occurs at the extremes of both mechanical overload and mechanical unloading. The enhanced SR Ca^{2+} content could also contribute to the increased Ca^{2+} spark frequency in the 10 week TAC group. Mechanical unloading, whether moderate or severe, of a normal or failing heart (Chapter 5), uniformly increased Ca^{2+} spark duration. The mechanisms mediating this are unclear, but may include altered phosphorylation of RyR clusters (Bers, 2006b). Changes to the width of the Ca^{2+} sparks may be related to altered Ca^{2+} diffusion and buffering properties of these cells. The mechanisms mediating altered Ca^{2+} spark duration during unloading were discussed in previous Chapters.

This Chapter was focused on examining the impact of chronic mechanical load variation on local CICR and its structural substrate, the t-tubule network. This system exists in a wider system, of excitation-contraction coupling elements including the NCX, the AP, SERCA2a (Louch *et al.*, 2010c). The assessment of these elements is an important point for future studies.

6.4.4 T-Tubule System As A Load-Sensitive Regulator Of CICR

Mechanical unloading appears to induce subtle disorder of the t-tubules, without reduction in their density. These changes are dependent on both the degree and duration of unloading (Figure C6-5). Mechanical overload appears to impair their density and regularity.

While there were multiple cellular examples of physiological adaptation in this study, including augmented Ca^{2+} transient amplitude, and the novel finding of enhanced variance of the time to peak, there was no evidence of enhanced t-tubule density. Kemi *et al* used exercise training to examine the effect of physiologic hypertrophy alone and as a possible therapy for post-myocardial infarction pathological hypertrophy (Kemi *et al.*, 2011). They found that the induction of physiological hypertrophy alone did not influence the t-tubule density, in agreement with the present findings. Interestingly, they showed that the induction of physiological hypertrophy could reverse the detubulating effect of pathological, post-infarct hypertrophic remodelling. This indicates that it is not only the degree of hypertrophy (by measures of heart weight or cell volume) but also its quality and timecourse, which is presumably signalled by separate pathways (Heineke and Molkentin, 2006). The pace of load variation may also be important since the gradual, intermittent

increase in cardiac load induced by exercise is quite different to the sudden increase in cardiac load induced by myocardial infarction or TAC.

The mechanisms mediating the load sensitivity of the t-tubule system are not clear, but a number of candidates are emerging including BIN-1, Tcap, JPH2 and others (for a detailed review, see Introduction). In particular, JPH2 expression appears to decrease as TAC-induced hypertrophy progresses, possibly mediating t-tubule disruption (Wei *et al.*, 2010).

6.4.5 Limitations And Questions For The Future

One limitation of this study is that not all the parameters were measured for every group. SR Ca^{2+} content measurements were only performed for the Sham and 10 week TAC groups to address the specific question of the aetiology of preserved Ca^{2+} transient amplitude in the light of t-tubule disruption. It was only possible to use SICM to examine the 4 week moderate unloading cells, and the effect of longer term 8 week moderate unloading has not been studied. The nature of the sensitivity to the degree of load was addressed for mechanical unloading, using models of moderate and severe unloading. These data suggest that more severe unloading brings about rapid cellular remodelling. Future studies should examine whether the same is true of mechanical overload, using different band tightness as well as using different durations of load increase. In this regard, it is noteworthy that most studies describe the degree of stenosis in a way which is not meaningful. The gauge of a cannula or needle is not a guarantee of fixed external diameter, but internal diameter. It is preferable to use the external diameter, and ideal to provide a pressure gradient, but

this was not possible in this study. Also, it is important to control the size of the aorta, which can be attempted by using animals within a small body weight range.

6.4.6 Clinical Relevance

Partial mechanical unloading is being suggested as a means to prevent mechanical unloading-induced atrophic remodelling in patients and these results and those of others (e.g. (Wang *et al.*, 2008)) support the concept that lower grade unloading delays or prevents adverse remodelling during treatment with LVADs.

6.4.7 Summary

Mechanical overloading and unloading result in graded changes to the t-tubule network and local CICR. This supports the notion that the t-tubule system is load-sensitive in both degree and duration and that there is a range of physiological loading conditions that allow normal t-tubule structure. Importantly, this study supports the notion of a physiological reduction in load that can be associated with normal cardiomyocyte structure and function.

Chapter 7

Telethonin Regulates T-Tubule Structure And Function In The Mammalian Heart

In this Chapter, the role of Tcap in regulating t-tubule structure and function during physiological conditions and during chronic mechanical overload (TAC) was studied. To do this, Tcap KO mice were used. Cellular Ca^{2+} transients, Ca^{2+} sparks, cell surface and t-tubule structure were studied.

These experiments assessed the following hypothesis: Tcap is a regulator of t-tubule structure, which is especially important during mechanical overload.

7.1 Introduction

T-tubule dysfunction is a major feature of cardiac diseases, and occurs early in overload-induced HF (Wei *et al.*, 2010). A major biophysical regulator of t-tubule structure appears to be chronic mechanical load, with both increases and decreases in mechanical load altering the t-tubule structure, as investigated in Chapters 4-6. Treatment of HF-induced overload with mechanical unloading can reverse t-tubule dysfunction (Chapter 5), indicating that normalisation of mechanical overload results in normalisation of the t-tubule structure and function. Therefore, the t-tubule system is tightly regulated by load variation in both health and disease.

Tcap is a 19KDa protein of the sarcomeric z-disk, which is involved in a class of muscular dystrophies (Valle *et al.*, 1997, Markert *et al.*, 2010). Recent work has suggested that one mechanism by which Tcap mutations result in such muscular dystrophies is by disruption to the t-tubule system (Zhang *et al.*, 2009). Tcap KO has been studied previously (Markert *et al.*, 2010, Knoll *et al.*, 2011). These studies show that while Tcap is not essential for skeletal or cardiac muscle development, contractile and stretch-sensing defects occur in Tcap KO. In the heart, Tcap KOs show no gross cardiac disease spontaneously, but its signalling with p53 results in profound apoptosis after the institution of mechanical overload, with contractile failure (Knoll *et al.*, 2011).

As has been discussed previously, a number of molecules are likely to be involved in regulating t-tubule structure and function (Table I-4). Tcap is a candidate load-dependent regulator of t-tubule structure because (i) it is a part of the stretch-sensitive complex of the cardiomyocyte z-disc (Hayashi *et al.*, 2004, Knoll *et al.*, 2002); (ii) it

links this stretch-sensitive complex with proteins localised in the t-tubule membrane (Furukawa *et al.*, 2001); (iii) it is stretch-sensitive in expression, and is required for normal t-tubule development in zebrafish skeletal muscle (Zhang *et al.*, 2009) and (iv) is mutated in a manner which increases stretch-sensitivity in HOCM and reduces stretch-sensitivity in DCM (Hayashi *et al.*, 2004), conditions where t-tubule abnormalities also exist (Lyon *et al.*, 2009). Recent evidence shows that in Tcap KOs cardiac overload results in pronounced apoptosis (Knoll *et al.*, 2011).

Based on these studies, it is timely to test the hypothesis that Tcap is a load-dependent regulator of t-tubule structure in the heart. This was done using a Tcap KO mouse, which was studied at 3 and 8 months, and following 4 weeks of TAC performed in 3 month old mice. Single cardiomyocytes were then isolated and assessed in terms of local CICR, t-tubule and cell structure.

7.2 Materials and Methods

All procedures were performed according to the guidance of the UK Home Office following ethical review.

7.2.1 Tcap Knock-Out Mice

Tcap KO animals were generated on a C57BL/6 background by replacing the coding regions of exon 1 and 2 by a lacz-neomycin cassette to monitor endogenous gene expression using homologous recombination as described previously (Knoll *et al.*, 2011). These animals were provided by Professor Ralph Knoll, Imperial College London. Four animals were used in each group.

The whole heart phenotype of this specific model has been described previously (Knoll *et al.*, 2011). Using echocardiography in 3 to 4 month old KO mice, Knoll *et al.* were unable to identify any defect in contractility or heart dimensions under spontaneous conditions. In addition, electrocardiogram analyses revealed no abnormalities. (Knoll *et al.*, 2011)

In this Chapter, to investigate the status of the t-tubule system and local CICR at a young and mature stage, animals were studied at 3 months of age (3mKO) and at 8 months of age (8mKO). Age- and sex-matched C57BL/6 non-littermate wild-type (WT) controls were used.

7.2.2 TAC

To investigate the impact of chronic mechanical overload, TAC was performed in animals at 8-12 weeks of age. A small portion of the second rib was excised to

provide access to the transverse thoracic aorta. A band of 7-0 Prolene was placed under the aorta and tied around a blunted 26-gauge needle, which was later removed. The animals were studied 4 weeks after TAC. Sham-operated matched animals were used as controls. These operations were performed by Dr Mutsuo Harada at South Kensington Campus, Imperial College London, due to an infection which prohibited their entrance to the bioscience unit in Harefield.

7.2.3 Cell Isolation

LV cardiomyocytes were isolated as described previously in the Methods. All recordings were performed at 37°C with cells superfused with NT unless otherwise indicated.

Following cell isolation, the following experiments were performed:

- Ca²⁺ transient and Ca²⁺ spark recordings
- T-tubule imaging using Di-8-ANEPPS
- Cell surface imaging using SICM (largely by Dr Anamika Singh)
- ICa_L recordings (Dr Urszula Siedlecka)
- AP recordings (Dr Christopher Rao)

7.2.4 Statistical Analysis

Statistical analysis was performed using non-parametric one-way ANOVA (Kruskal-Wallis test). Dunn's post-hoc test was used to test for differences between groups. The analysis was performed using Prism4 software (GraphPad software Inc., San Diego, CA, USA). P<0.05 was taken as significant.

7.3 Results

7.3.1 Lack Of Tcap Caused Progressive Ca²⁺ Handling Defects

In isolated LV cardiomyocytes, the whole cell Ca²⁺ transient was assessed during aging. 3 month old mice were studied, corresponding to young mice that were previously described (Knoll *et al.*, 2011), as well as 8 months of age.

In 3mKO, the Ca²⁺ transients had a normal time to peak, amplitude, and time to 50 and 90% decline, as previously shown by Knoll *et al* (Knoll *et al.*, 2011). Only the variance of the time to peak, a measure of the synchrony of Ca²⁺ release across the cell, was increased (Figure C7-1). This indicated a reduced Ca²⁺ transient synchrony, which may be due to altered AP morphology or t-tubule disruption.

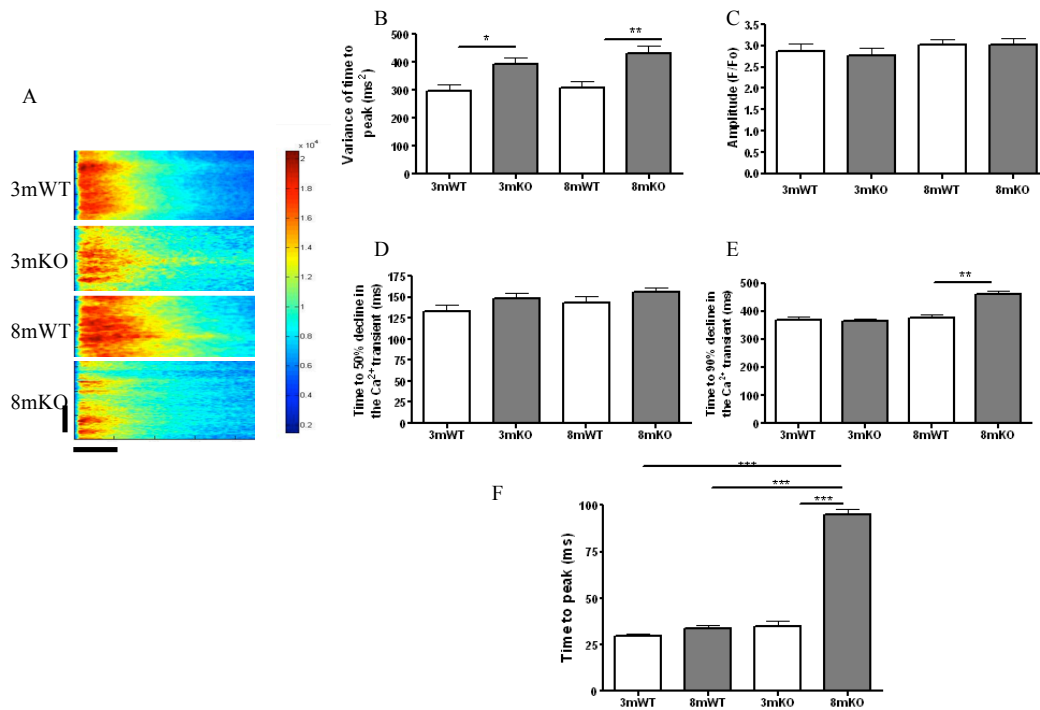


FIGURE C7-1: TCAP KO WAS ASSOCIATED WITH PROGRESSIVE CA²⁺ TRANSIENT DEFECTS

(A) The figure shows line scans of stimulated LV cardiomyocytes from 3 month old or 8 month old WT and KO hearts. (B) Variance of the time to peak of the Ca²⁺ transient, (C) amplitude of the Ca²⁺ transient; (D) time to 50% decline of the Ca²⁺ transient, (E) time to 90% decline of the Ca²⁺ transient, (F) time to peak of the Ca²⁺ transient. 3mWT n=40, 3mKO n=48, 8mWT n=48, 8mKO n=43. The vertical scale bar represents 100 pixels and the horizontal scale bar represents 100ms. The colour chart is a normalised scale of fluorescence intensity.

In 8mKO, the time to peak and time to 90% decline were also prolonged, indicating progression of Ca²⁺ handling abnormalities. It is important to note that the functional status of 8mKO animals at the whole heart level has not been described. It was not possible to assess these for regulatory reasons concerning infection with a benign

7.3.2 Tcap KO Was Associated With Progressive Ca²⁺ Spark And LTCC Defects

Ca²⁺ sparks are the functional constituents of the Ca²⁺ transients. In 3mKO and 8mKO, there was an elevated Ca²⁺ spark frequency, with a small but significant reduction in Ca²⁺ spark peak amplitude (Figure C7-2). This indicates impaired SR Ca²⁺ release function, which could be due to altered I_{Ca,L}, dyadic disruption or primary changes to the RyR.

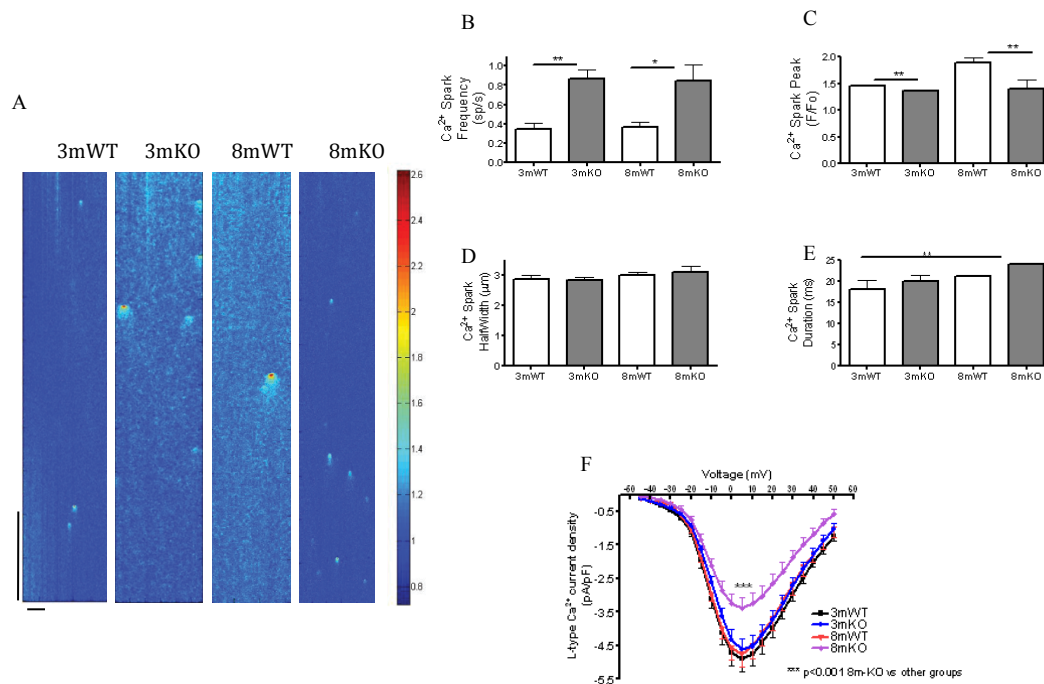


FIGURE C7-2: Tcap KO WAS ASSOCIATED WITH PROGRESSIVE CA²⁺ SPARK AND LTCC DEFECTS

(A) Line scans of quiescent LV cardiomyocytes from 3 month or 8 month WT and KO hearts. Ca²⁺ spark (B) frequency, (C) peak, (D) width and (E) duration. 3mWT n=40, 3mKO n=48, 8mWT n=48, 8mKO n=43. (F) The I_{Ca,L} density is shown. 3mWT n=24, 3mKO n=25, 8mWT n=16, 8mKO n=20. The vertical scale bar represents 768ms and the horizontal scale bar represents 100 pixels. The colour chart is a normalised scale of fluorescence intensity.

There was no difference in $I_{Ca,L}$ density in 3mKO cardiomyocytes; however, cardiomyocytes from 8mKO had a significant reduction in $I_{Ca,L}$ density (Figure C7-2). The shape of the current-voltage curve was not affected at any timepoint.

7.3.3 Loss Of Tcap Did Not Influence The Action Potential

The AP was unchanged in amplitude or time to 50 and 90% depolarisation (Figure C7-3). These results indicate that at 3 months of age, the triggers for SR Ca^{2+} were unaffected. The results at 8 months need to be interpreted in light of the loss of t-tubule density, as the LTCC is concentrated in the t-tubule membrane (Gu *et al.*, 2002).

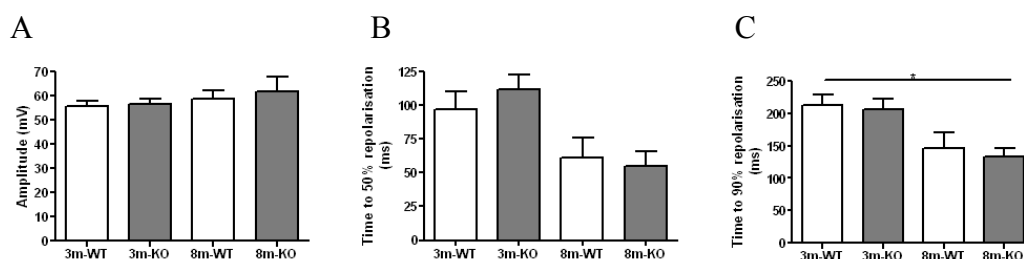


FIGURE C7-3: THE ACTION POTENTIAL WAS UNAFFECTED IN TCAP KO

The figure shows the (A) amplitude and (B) time to 50 and (C) 90% repolarisation in the AP at 1 Hz.

Similar recordings were made at 3 and 5Hz. 3mWT n=25, 3mKO n=26, 8mWT n=9, 8mKO n=10.

7.3.4 The T-Tubule System Was Disrupted In Tcap KO

The isolated loss of Ca^{2+} transient synchronicity in cardiomyocytes from 3mKO could be due to pathological changes to the t-tubule system. In 3mKO, there was a normal density of the t-tubules, but the regularity of the t-tubules, measured by the power of the dominant peak of the Fourier transform, was significantly depressed (Figure C7-4).

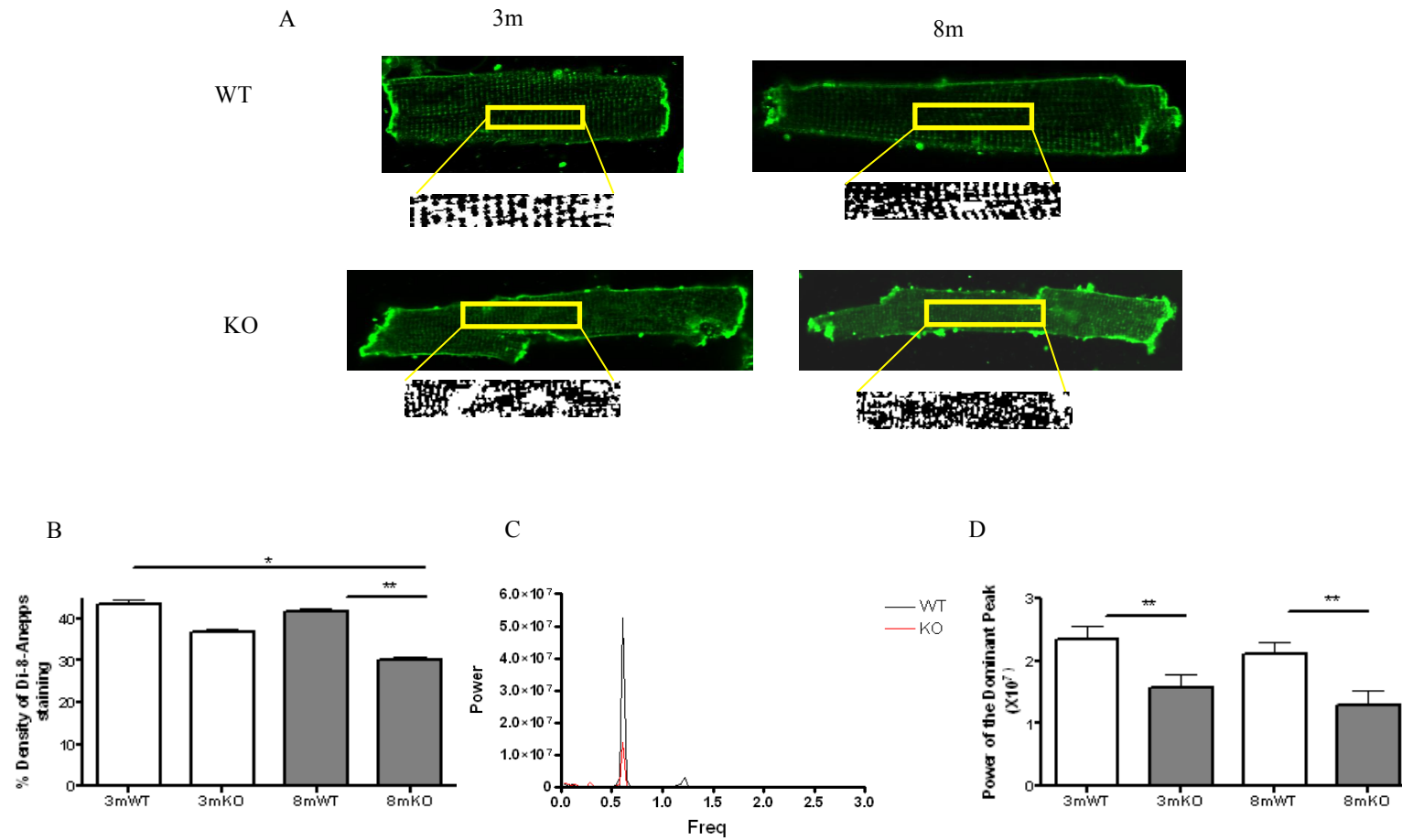


FIGURE C7-4: T-TUBULE STRUCTURE WAS DISRUPTED IN TCAP KO

(A) Single Di-8-ANEPPS stained cardiomyocytes from which t-tubule density (B) and regularity (C & D) are calculated. This provides a power-frequency curve, the peak of which is taken as an index of t-tubule regularity. 3mWT n=30, 3mKO n=32, 8mWT n=45, 8mKO n=44.

In 8mKO, the t-tubule abnormalities progressed, with loss of both t-tubule density and regularity (Figure C7-4).

7.3.5 The Cell Surface Was Initially Unaffected by Tcap KO

To study the structure of the cell surface, SICM was used. 3mKO had a normal cell surface structure with a typical pattern of fine membrane striations (z-grooves) in which orderly t-tubules reside (Figure C7-5). However, in 8mKO there was loss of the fine membrane architecture with a reduced z-groove index (Figure C7-5).

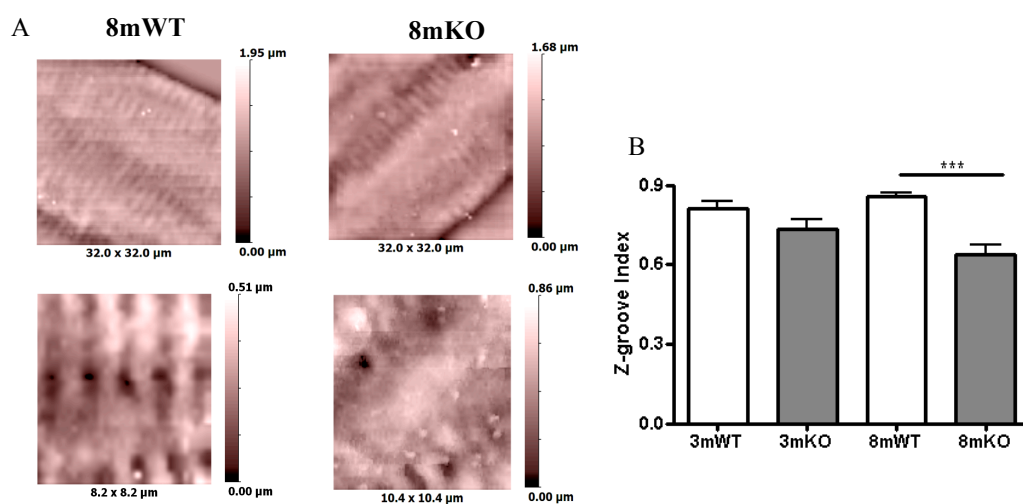


FIGURE C7-5: CELL SURFACE STRUCTURE WAS DISRUPTED IN TCAP KO

(A) Low (top images) and high (bottom images) power views of the cell surface of 8m WT and KO cells are shown. (B) The z-groove index is reduced in 8mKO, indicating loss of cell surface organisation. 3mWT n=7, 3mKO n=16, 8mWT n=30, 8mKO n=20.

7.3.6 Mechanical Overload Caused Marked Ca^{2+} Transient Abnormalities In Tcap KO

After chronic mechanical overload induced by TAC for 4 weeks in 3 month old mice, both WT and KO cells showed prolongation of the time to peak (WT increased by 16%, KO increased by 19%) of the Ca^{2+} transient (Figure C7-6). Both WT and KO

showed an increase in the variance of the time to peak of the Ca^{2+} transient than WT (WT increased by 34%, KO by 25%, with respect to control), and additional prolongation of the time to 50% (WT increased by 11%, KO increased by 40%) and 90% (WT increased by 5%, KO increased by 15%) decline of the Ca^{2+} transient.

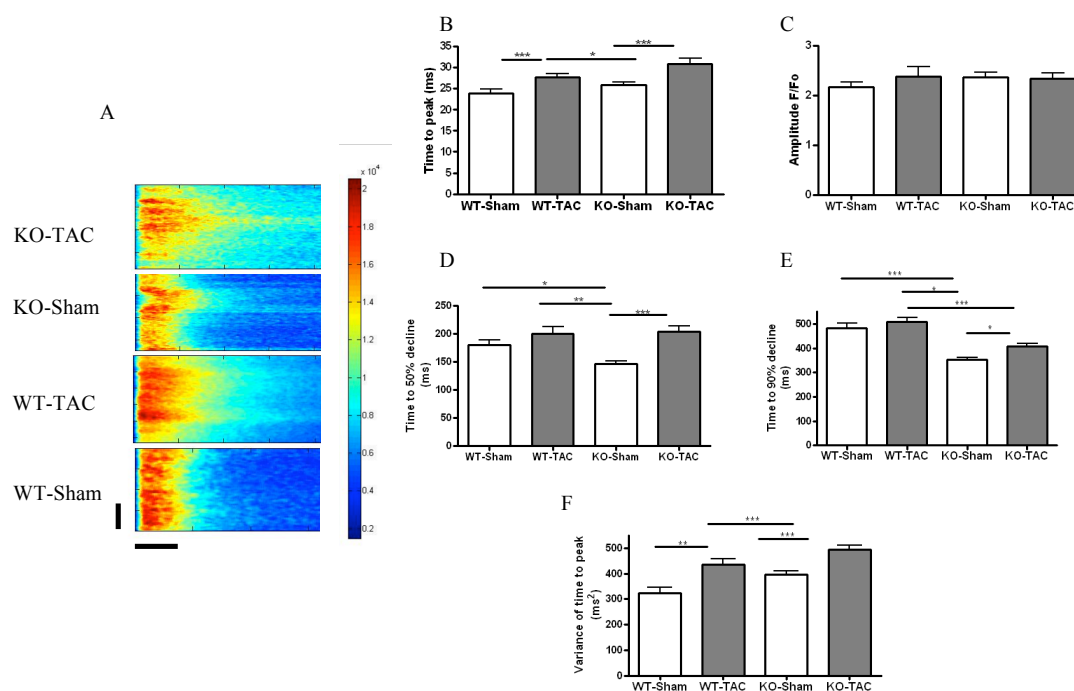


FIGURE C7-6: MECHANICAL OVERLOAD CAUSED SEVERE Ca^{2+} TRANSIENT DEFECTS IN TCAP KO

(A) Line scans of stimulated LV cardiomyocytes from Sham or TAC, WT and KO hearts. (B) Time to peak of the Ca^{2+} transient, (C) amplitude of the Ca^{2+} transient, (D) time to 50% decline of the Ca^{2+} transient, (E) time to 90% decline of the Ca^{2+} transient, (F) variance of the time to peak of the Ca^{2+} transient. WT-Sham n=34, WT-TAC n=32, KO-Sham n=31, KO-TAC n=34. The vertical scale bar represents 100 pixels and the horizontal scale bar represents 100ms. The colour chart is a normalised scale of fluorescence intensity.

7.3.7 Mechanical Overload Caused Marked Increases In Ca^{2+} Spark Frequency In Tcap KO

To understand the mechanisms involved in the Ca^{2+} transient abnormalities in response to TAC, Ca^{2+} spark properties were studied. KO, but not WT, cells showed increased Ca^{2+} spark frequency (Figure C7-7). There was also a prolongation of the Ca^{2+} spark duration in both WT and KO cells after TAC. The Ca^{2+} spark duration was increased in the KO-Sham group, compared to the WT-Sham group. This was not observed in the study using 3 and 8 month old animals above.

There was no change to ICa_L after mechanical overload in KO or WT (Figure C7-7). This was different to the finding in the ageing study above (Figure C7-2), and will be discussed below.

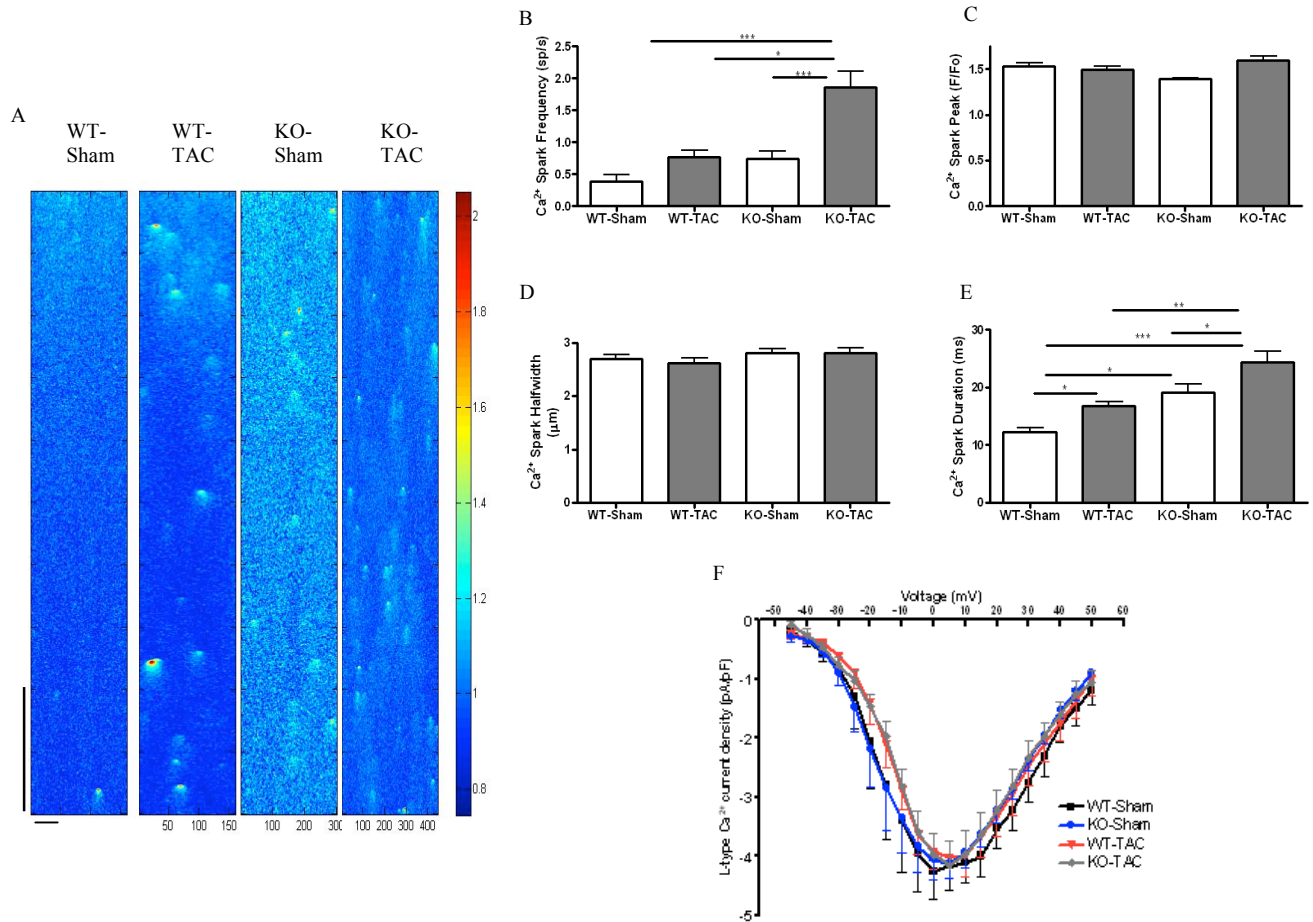


FIGURE C7-7: MECHANICAL OVERLOAD ALTERED Ca^{2+} SPARK FREQUENCY IN TCAP KO BUT NOT WT AND DID NOT AFFECT LTCC

(A) Line scans of quiescent ventricular cardiomyocytes from 3 month or 8 month WT and KO hearts. Ca^{2+} spark (B) frequency, (C) peak, (D) width and (E) duration. WT-Sham $n = 34$, WT-TAC $n = 32$, KO-Sham $n = 31$, KO-TAC $n = 34$. (F) ICa_L density is shown in lower graph. WT-Sham $n = 11$, WT-TAC $n = 16$, KO-Sham $n = 13$, KO-TAC $n = 14$. The vertical scale bar represents 768ms and the horizontal scale bar represents 100 pixels. The colour chart is a normalised scale of fluorescence intensity.

7.3.8 The Action Potential Was Unaffected by Loss of Tcap Following TAC

The AP was also unaffected in amplitude or time to 50% or 90% depolarisation at all stimulation frequencies studied (1Hz (shown) 3Hz and 5Hz) (Figure C7-8).

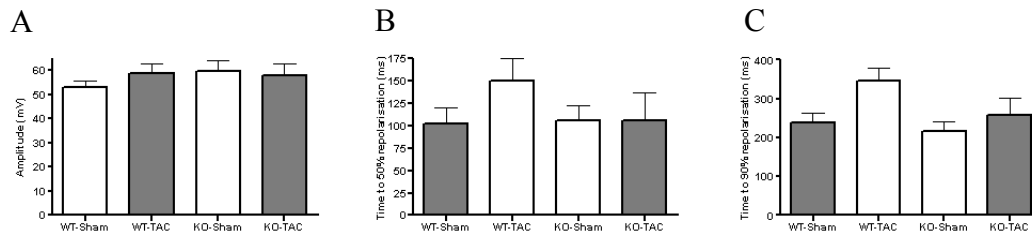


FIGURE C7-8: THE ACTION POTENTIAL WAS UNAFFECTED BY OVERLOAD IN WT AND KO CELLS

The figure shows the (A) amplitude and (B) time to 50 and (C) 90% repolarisation in the AP at 1Hz. Similar recordings were made at 3 and 5Hz. WT-Sham n=16, WT-TAC n=13, KO-Sham n=24, KO-TAC n=23.

7.3.9 Pronounced T-Tubule Loss And Disorder Following Mechanical Overload In Tcap KO

It is known that chronic mechanical overload induces loss and disorder in the t-tubule system, and this was confirmed by comparing Sham and TAC WT cells (Figure C7-9) Tcap KO showed a profound loss of t-tubule density and regularity, which was greater than the loss which occurred in WT cells (density was reduced by 27% in WT and by 30% in KO).

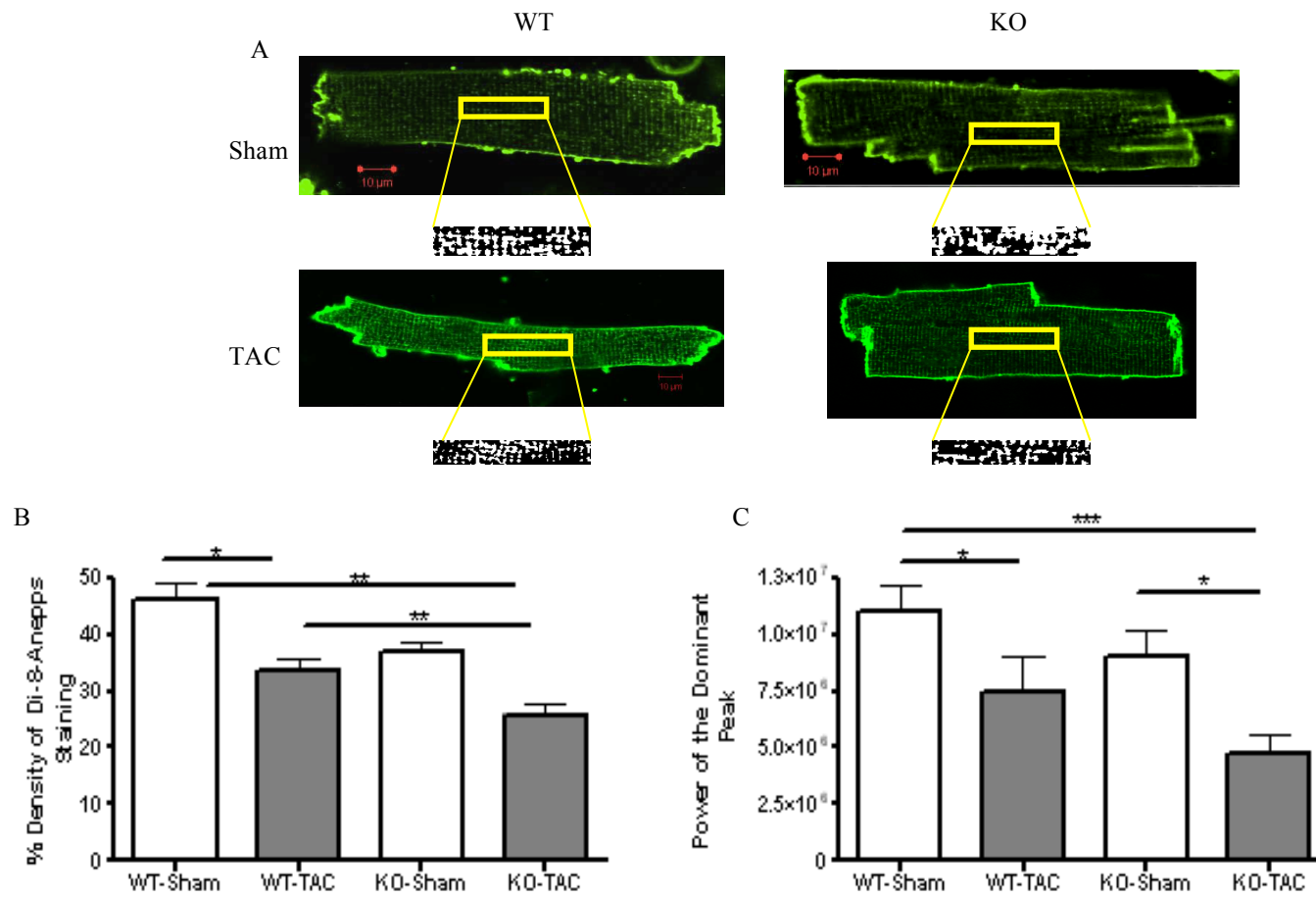


FIGURE C7-9: PROFOUND T-TUBULE LOSS OCCURRED AFTER MECHANICAL OVERLOAD IN TCAP KO

(A) Single Di-8-ANEPPS stained cardiomyocytes from which t-tubule density (B) and regularity (C) are calculated. WT-Sham n= 31, WT-TAC n= 28, KO-Sham n= 30, KO-TAC n= 34.

7.3.10 The Cell Surface Was Disrupted By Mechanical Overload in Tcap KOs

The cell surface was examined using SICM and found that TAC induced pathological remodelling of the cell membrane surface with loss of the z-grooves (Figure C7-10).

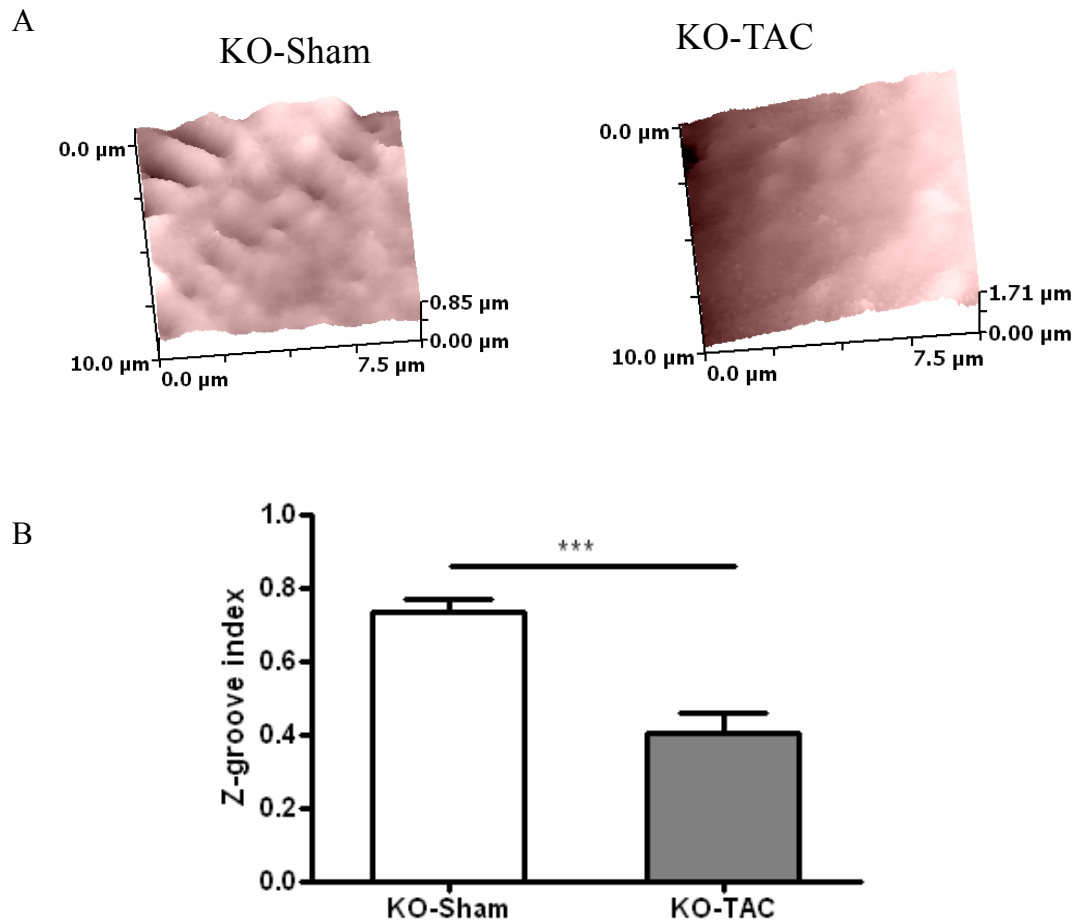


FIGURE C7-10: CELL SURFACE STRUCTURE WAS DISRUPTED IN TCAP KO FOLLOWING MYOCARDIAL OVERLOAD

(A) The cell surface of Tcap KO Sham and TAC cardiomyocytes is shown, from which the z-groove is calculated (B). KO-Sham n=16, KO-TAC n=14.

7.4 Discussion

This study shows that loss of Tcap from cardiomyocytes is associated initially with an isolated t-tubule defect, with minor Ca^{2+} handling changes and normal heart and cellular function, suggesting that this effect is primary to the t-tubule. This defect becomes worse with ageing and is associated with significant changes in Ca^{2+} cycling. During mechanical overload, there is pronounced t-tubule loss and remodelling indicating that Tcap is necessary to maintain t-tubule structure and function, especially after mechanical overload.

7.4.1 T-Tubule Regulation

The t-tubules are regulated by a number of molecules, each acting at different levels, as discussed in the Introduction (Table I-4). A major biophysical regulator of the t-tubule system is mechanical load variation, as both increased and reduced mechanical load can disrupt t-tubule structure when prolonged (as shown in Chapters 4-6). Zhang *et al* identified Tcap as a load-dependent promoter of t-tubule formation in response to stretch in zebrafish skeletal muscle (Zhang *et al.*, 2009). It was recently reported that during HF Tcap expression declines, then recovers after SERCA2a gene therapy-induced reverse remodelling, correlating with observed loss and reappearance of the t-tubules (Lyon *et al.*, 2012). In this present study, Tcap is identified as a molecular mechanism governing t-tubule structure and function in the mammalian heart. Importantly, lack of Tcap results initially in isolated abnormal t-tubule structure, with few Ca^{2+} handling changes (discussed below). This is suggestive of a direct t-tubule regulatory role by Tcap. In the absence of Tcap, mechanical overload results in a more pronounced t-tubule dysfunction, with major implications for CICR. Taken

together, these data suggest that Tcap is involved in the regulation of t-tubule structure, especially after mechanical overload.

7.4.2 Electrophysiological Abnormalities

The AP morphology was unaffected by either lack of Tcap or mechanical overload.

The AP was unaffected despite a reduced $I_{Ca,L}$. This may be due to the enhancement of other currents such as the NCX current. Further, the changes in $I_{Ca,L}$ may have been too small to influence the AP.

The effect on $I_{Ca,L}$ reported in this Chapter is complex. In 8mKO, where t-tubule density was depressed, the $I_{Ca,L}$ density was also significantly depressed. Such $I_{Ca,L}$ depression could result in smaller increases in the dyadic $[Ca^{2+}]$, resulting in slower Ca^{2+} release from the SR and therefore less activation of the contractile apparatus. Indeed, the time to peak and time to 90% decline of the Ca^{2+} transient were prolonged in 8mKO. During conditions of overload in both WT and KO cells, where t-tubule density was also depressed, $I_{Ca,L}$ was unaffected. There are two possible explanations for this discrepancy. First, $I_{Ca,L}$ density is a normalised measure to cell capacitance, which depends on the membrane area. Therefore, if the membrane area were to decrease to the same extent as loss of LTCCs, the overall $I_{Ca,L}$ density measure would be the same. Secondly, there could be a compensatory increase in LTCCs to counter the loss through detubulation, (whether driven by proteins linked to the t-tubule biogenesis such as BIN1, or independent) which results in a relatively normal $I_{Ca,L}$:membrane area measurement. Whether these or other mechanisms explain this finding is an important question for future studies. Lastly, most studies do not report depression of $I_{Ca,L}$ after models of overload and HF (Bers, 2006a), and it

could be that the t-tubule loss induced by ageing is distinct (driven by distinct molecular pathways) from that driven by overload-induced HF; this also needs to be addressed by future studies.

In 3mKO, as previously reported (Knoll *et al.*, 2011), the amplitude and duration of the whole cell Ca^{2+} transient were not different from control cardiomyocytes. However, using confocal microscopy, it was possible to perform an additional examination of CICR synchronicity, and it was found that this was significantly disrupted in 3mKO. The points of delayed Ca^{2+} release spatially colocalise to defects in the t-tubule system, indicating that these areas of dysynchrony are caused by delayed RyR activation due to LTCC-RyR uncoupling (Louch *et al.*, 2004, Meethal *et al.*, 2007). T-tubule morphology is not the only regulator of CICR synchrony, but given that Ca^{2+} handling appears intact at this stage, t-tubule remodelling is likely the cause of this loss of synchrony. In 8mKO, in addition to loss of synchronous CICR, there was also prolongation of the time to peak and time to 90% decline of the Ca^{2+} transient. This was accompanied by loss of t-tubule density, which would further uncouple CICR.

After mechanical overload, Ca^{2+} transient remodelling occurs with a prolongation of the time to peak, loss of Ca^{2+} release synchrony and prolongation of time to 50% and 90% decline in the Ca^{2+} transient. In cardiomyocytes lacking Tcap, mechanical overload-induced additional prolongation of the Ca^{2+} transient occurred. One explanation for this effect is that pronounced t-tubule defects in this group resulted in altered interaction between ion channels responsible for efficient CICR, resulting in Ca^{2+} transient remodelling. Interestingly, despite longer Ca^{2+} spark duration after

TAC, the Ca^{2+} transient decline was faster in KO compared to WT Shams. The reasons for this are unknown and could be related to Tcap interactions with other receptors and ion channels which were not studied, including NCX.

SR Ca^{2+} release function was assayed by measuring Ca^{2+} sparks. In 3mKO, Ca^{2+} spark frequency was elevated. The mechanisms governing Ca^{2+} spark properties are complex, but this could be due to uncoupling of LTCC and RyR, which can induce destabilisation of the RyR. Increased Ca^{2+} spark frequency occurs in HF where the t-tubules are abnormal or missing (Meethal *et al.*, 2007) and this increased Ca^{2+} spark frequency is spatially localised to gaps in the t-tubule system. Ca^{2+} spark frequency was elevated in KOs but not WT after TAC, possibly due to the pronounced t-tubule loss, resulting in LTCC-RyR uncoupling. The Ca^{2+} spark duration was prolonged in both WT and KO after TAC. One mechanism which could mediate this is displacement or loss of NCX, which is concentrated in the t-tubules. Loss of NCX could delay the extrusion of Ca^{2+} , resulting in prolonged Ca^{2+} sparks (Biesmans *et al.*, 2011). KO Sham cells showed longer Ca^{2+} spark duration compared to WT Sham. Ca^{2+} spark duration is a complex parameter and depends on factors promoting Ca^{2+} leak, altering Ca^{2+} diffusion properties throughout the cytoplasm and Ca^{2+} extrusion from the cytoplasm (Cheng *et al.*, 1999).

7.4.3 Changes To The Cell Surface

In 3mKO, the cell surface was not altered but in 8mKO, cell surface remodelling occurred. After mechanical overload, the cell surface was also disrupted. The implications for CICR of z-groove loss is not clear, although in all conditions studied so far, where z-groove remodelling has occurred, there have also been CICR defects (Lyon *et al.*, 2009). Importantly, the intact cell surface structure of 3mKO indicates

that these cells were not in a state of general pathological remodelling and that their gross structural features were intact. Together with previous reports (Zhang *et al.*, 2009), this supports the notion that the t-tubule changes in these cells were an isolated, primary defect.

7.4.4 Molecular Mechanism Mediating Tcap-T-Tubule Interaction

In the heart, Tcap interacts with a number of proteins to form a stretch-sensing complex (Knoll *et al.*, 2002), Figure C7-11. Importantly, Tcap also binds minK, the regulatory β -subunit of the delayed rectifier K^+ current channel that resides in the t-tubules (Furukawa *et al.*, 2001), providing a possible molecular mechanism for the findings observed in this study. This suggests a role for Tcap in coupling stretch-sensation with the t-tubule membrane. Zhang *et al* showed in zebrafish that t-tubule development, which corresponds to body twitch initiation, is dependent on normal Tcap expression (Zhang *et al.*, 2009). Reduction of body movement by anaesthesia delayed t-tubule development. The authors concluded that Tcap could be regulating the t-tubule system in response to changes in stretch. One way in which Tcap could perform mechanosensation functions might be using the integrin-linked kinase system, which should be tested in future studies (Knoll *et al.*, 2007).

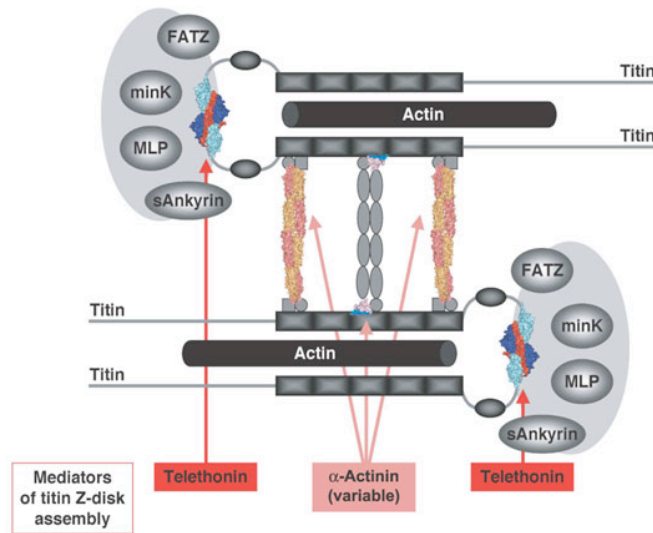


FIGURE C7-11: TELETHONIN BINDS WITH A NUMBER OF MOLECULES IN THE Z-DISK

The binding partners and arrangement of Tcap (Telethonin) in the z-disk is shown. This includes minK, present in the t-tubule membrane of the ventricular muscle cells. Figure from (Zou et al., 2006).

Given the large body of evidence showing that the t-tubule system is load-sensitive in both myocardial overloading and unloading (Chapters 4-6), it was hypothesised that Tcap would regulate the t-tubule system of mammalian ventricular cardiomyocytes in response to load. In this study, it was shown that cardiomyocytes lacking Tcap have deranged t-tubule structure with limited other defects to the CICR system, suggesting Tcap has a primary role in regulating the t-tubule structure. When subjected to mechanical overload, the t-tubule system undergoes even more profound remodelling, indicating a failure of load-dependent regulation. This supports the hypothesis that Tcap is a critical, load-dependent regulator of t-tubule structure. Further studies are required to elucidate the precise mechanisms mediating the biochemical interactions between Tcap and the t-tubule membrane.

Chapter 8

General Discussion

“Truth in science can be defined as the working hypothesis best suited to open the way to the next better one.”

Konrad Lorenz (1903-89), Austrian founder of Ethology [Nobel prize for Medicine, 1973]

In this Chapter, the evidence presented in respect of the general hypothesis that mechanical load affects t-tubule structure and function in LV cardiomyocytes in a graded and reversible manner via specific molecular mechanisms is synthesised and evaluated.

8.1 Summary

8.1.1 Whole Heart

The studies in this thesis were aimed at evaluating t-tubule structure and local CICR during various loading conditions. The evaluation of the whole heart was limited to the validation of the animal models through the measurement of heart weight and echocardiography.

Heart weights increased in proportion to the degree of mechanical overload, secondary to CAL or TAC (Chapter 3, 5 and 6). Likewise, heart weights were reduced following mechanical unloading (Chapter 3 and 6).

Echocardiographic assessment was employed for functional analysis of CAL and TAC, and for measurement of wall and cavity dimensions in the case of mechanical unloading. HF was assessed as an EF of below 40%, representing a class of functionally very impaired patients, who would be candidates for mechanical circulatory support (Stewart and Givertz, 2012), although the CAL model does not result in any detectable functional symptoms or signs of HF. CAL was accompanied by dilation of the LV cavity (Chapter 3). The contractility of the TAC model was enhanced, indicating compensated hypertrophy (Chapter 3). Mechanical unloading was associated with thinning of the LV wall and a smaller LV cavity. No whole heart functional assessment of the mechanically unloaded heart was performed.

These results indicate that mechanical load variation results in myocardial hypertrophy or atrophy. Overload resulted in a compensated hypertrophy and CAL caused clinically-relevant HF.

8.1.2 Whole Cell

A summary of cellular findings and what was not studied, for each group of experiments is given in Figures C8-1 to C8-5.

Cell volume generally mirrored the changes in whole heart weight, with hypertrophy in response to CAL and TAC and reductions in response to mechanical unloading. The cellular hypertrophy after CAL was associated with cell volumes in the order of $6 \times 10^4 \mu\text{m}^3$, while 10 weeks after TAC the cell volume reached approximately $5 \times 10^4 \mu\text{m}^3$. Cells from the TAC 10 week group showed some preserved functional properties (e.g. Ca^{2+} transient amplitude) and some signs of deterioration (e.g. raised Ca^{2+} spark frequency and altered t-tubule structure). This may represent a transitional period between compensated hypertrophy and HF.

Mechanical unloading results in reductions in cell volume (Chapter 4, 5 and 6). It was shown that while severe mechanical unloading, induced by heart-only transplantation resulted in major diminution of cell volume, moderate mechanical unloading resulted in graded reductions in cell volume. The regression of hypertrophy (Chapter 5) was associated with enhanced cellular function. However, very low cell volumes (Chapter 4) were associated with deterioration in function.

This thesis reveals that the relationship between cell volume and function is complex, with very high or low volumes associated with dysfunction and a physiologic intermediate range.

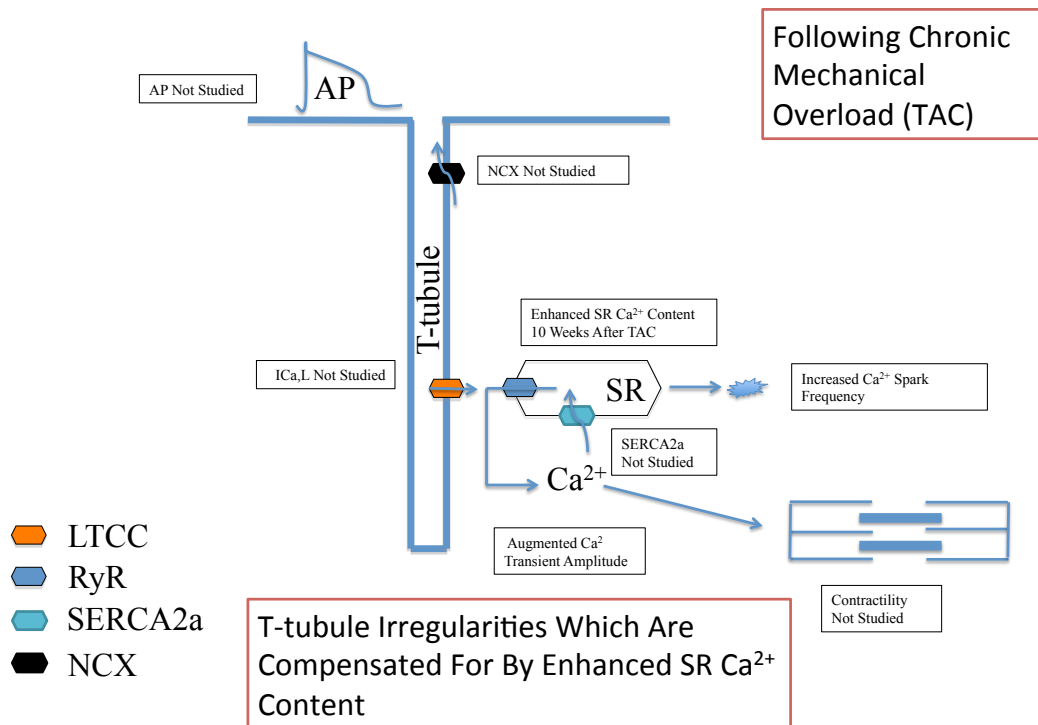


FIGURE C8-1: SUMMARY OF CHANGES REPORTED AFTER MECHANICAL OVERLOAD

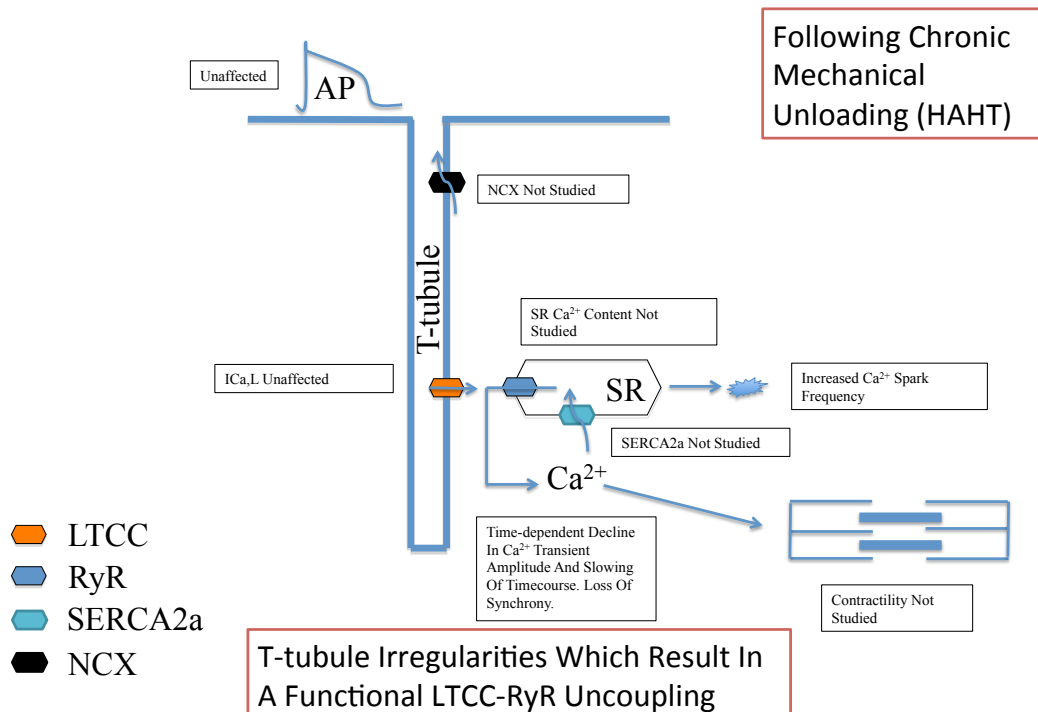


FIGURE C8-2: SUMMARY OF CHANGES REPORTED AFTER MECHANICAL UNLOADING (HAHT)

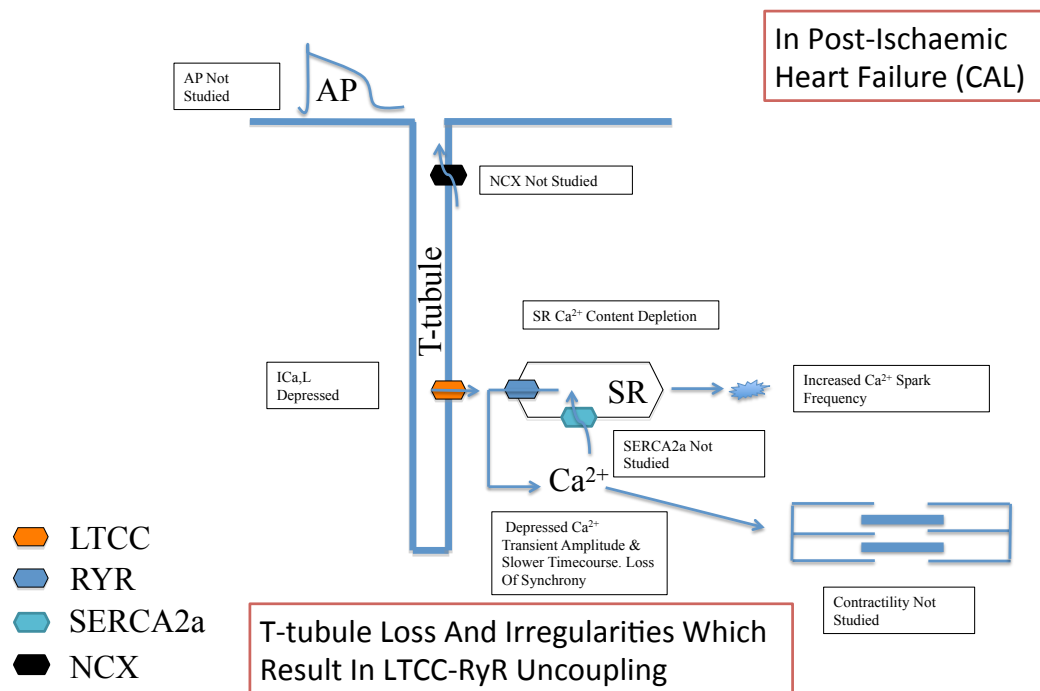


FIGURE C8-3: SUMMARY OF CHANGES REPORTED IN HEART FAILURE

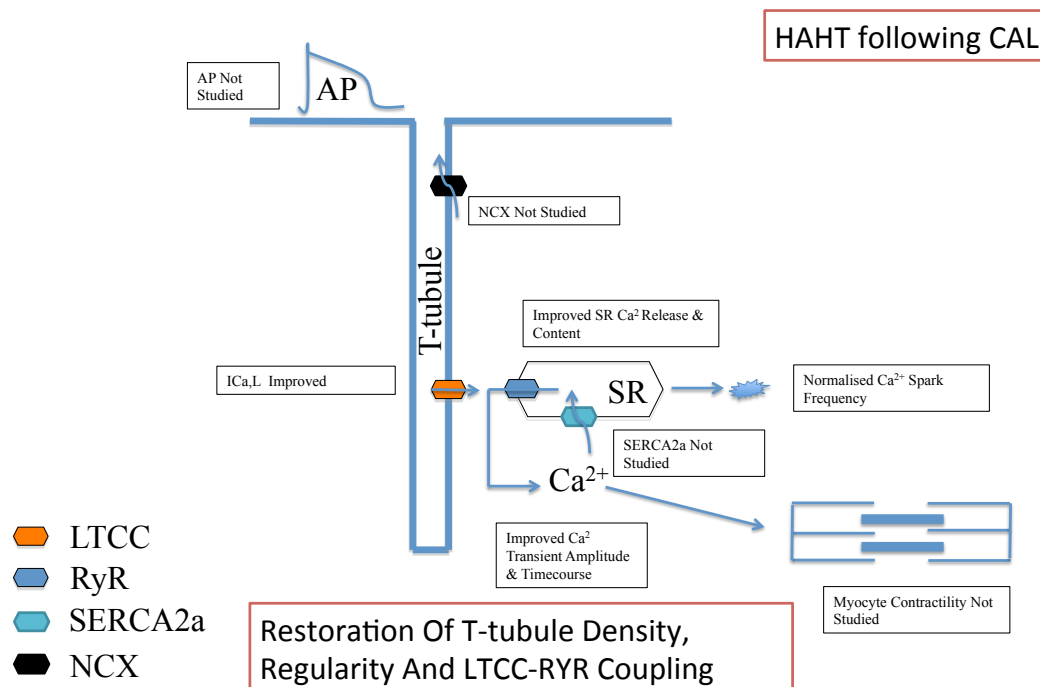


FIGURE C8-4: SUMMARY OF CHANGES REPORTED AFTER MECHANICAL UNLOADING OF THE FAILING HEART

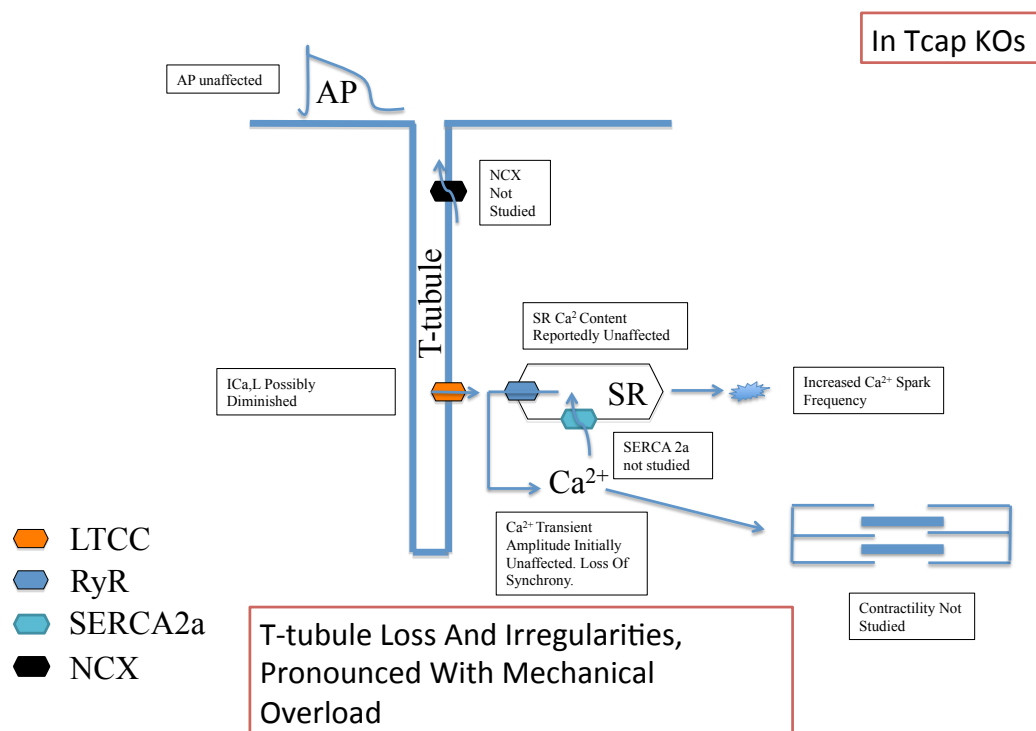


FIGURE C8-5: SUMMARY OF CHANGES REPORTED IN TCAP KO

8.1.3 The Cell Surface

The cell surface is a highly sophisticated structure which interacts with the extracellular matrix and the milieu of extracellular signaling molecules (Louch *et al.*, 2010c). It is a communication hub, sensing external stressors, and containing microdomains of β -adrenergic and other cell signalling centres (Nikolaev *et al.*, 2010), which shift during disease.

Using SICM, the cell surface was shown to be modulated by mechanical unloading in Chapters 4 and 6. Its disruption during HF was confirmed in Chapter 5, which showed for the first time that these changes were reversible. Chapter 6 showed that the degree of mechanical unloading might influence the cell surface, but more evidence is needed to reach a conclusion.

The method used to characterise the cell surface provides a general picture of the cell surface microarchitecture (Gorelik *et al.*, 2006). It is now routinely combined with assessment of the functional ion channel constituents of these membrane fractions (Gu *et al.*, 2002) and assessment of the β -adrenergic response of these fractions (Nikolaev *et al.*, 2010). These additional features will provide a much more detailed understanding of the dynamic regulation of the cell surface in response to various physiological and pathological stimuli.

This thesis supports the notion that mechanical load variation, in particular mechanical unloading, may impact the cell surface and highlights it as an important target for new studies. In particular, the relationship between the structural regulation of the membrane and the t-tubules needs to be investigated.

8.1.4 The T-Tubule System Or Network

This thesis was focused on understanding the response of the t-tubule system to altered physiological and pathological loading conditions. T-tubule structure is a major determinant of cellular Ca^{2+} handling, as has been demonstrated by many studies and perhaps most powerfully by a series of seminal papers using the detubulating agent formamide (Brette *et al.*, 2002, Chase *et al.*, 2010, Chase and Orchard, 2011, Despa *et al.*, 2003, Fowler *et al.*, 2007, Kawai *et al.*, 1999, Komukai *et al.*, 2002, Yang *et al.*, 2002). This thesis has confirmed the tight relationship between t-tubule structure and subcellular Ca^{2+} handling.

This thesis used advanced surgical models to test the relationship between chronic mechanical load and the t-tubule system. Chapter 4 showed that prolonged

mechanical unloading could alter the t-tubule structure. Chapter 5 furthered the concept of mechanical load as a regulator of the t-tubules by showing that normalisation of mechanical load restored the t-tubule structure. What is more, the study reported in Chapter 5 was the first demonstration of recovery of loss of t-tubule density after a pathological insult. This shows that not only are t-tubule structural properties regulated by mechanical load, but that t-tubule formation is also regulated by mechanical load, and the phenomenon is reversible. Chapter 6 refined these findings, showing that graded, slower decreases in mechanical load can be consistent with normal t-tubule structure, as can graded increases in mechanical load. The molecular mechanisms mediating the load-dependent regulation of the t-tubule structure are certainly multiple. Tcap became a target of interest due to its load-sensitivity and apparent role in load-dependent t-tubule regulation (Zhang *et al.*, 2009).

Chapter 7 showed that Tcap is essential for normal t-tubule structure at rest. What is remarkable is that there is no cardiac dysfunction at the earliest time point studied, and also no stigmata of abnormal cellular Ca^{2+} handling, indicating, as far as possible, that the defect is primary to the t-tubule. However, it is pertinent that overload-induced t-tubule dysfunction occurs prior to the advent of overt myocardial failure in rats (Wei *et al.*, 2010). As such, it cannot at this stage be ruled out that Tcap acts on the t-tubule membrane via another, as yet undiscovered, mechanism. Such a mechanism x could not be found after rigorous biochemical analysis (Knoll *et al.*, 2011). This investigator therefore submits, with some reservations, that Tcap has a primary role in t-tubule regulation. The load-dependence of Tcap expression, and the profound t-tubule disruption observed when the heart is challenged in its absence,

supports the notion that Tcap is particularly important during load-variation (although it is important to note that the percentage reduction of t-tubule density following TAC in WT and KO is approximately the same [27% and 30%, respectively]). Several questions remain unanswered, and will be outlined below.

8.1.5 Local CICR

Local CICR is profoundly dependent on t-tubule structural integrity. That is not to say that a physiological stimulus, such as mechanical load variation – which alters the t-tubule structure – cannot independently influence the determinants of local CICR. This is a problem when interpreting the influence of mechanical load variation on a system whose function is determined by multiple elements.

The whole cell Ca^{2+} transient was dyssynchronous when the t-tubules were also impaired (Chapter 4, 5, 6, 7). Other investigators used double-staining experiments (with membrane-staining and Ca^{2+} sensitive dyes) to colocalise the gaps in the t-tubule system with inhomogeneous Ca^{2+} transient (Louch *et al.*, 2004, Louch *et al.*, 2006, Louch *et al.*, 2010c). The finding that synchrony improved following restoration of normal t-tubule structure in Chapter 5 was suggestive of a direct relationship. However, other factors, especially the AP, play a role in regulating Ca^{2+} release synchrony (Louch *et al.*, 2010a) and should be investigated in future studies.

The Ca^{2+} transient amplitude was depressed in HF, and recovered following mechanical unloading (Chapter 5). Prolonged mechanical unloading of the normal heart depressed the Ca^{2+} transient amplitude (Chapter 4). Moderate unloading achieved the same effect, over twice the duration (Chapter 6). Mechanical overload

enhanced the Ca^{2+} transient amplitude, as expected for a compensated hypertrophy picture (Chapter 6). In some cases, these results could be explained by alterations to the SR Ca^{2+} content.

The timecourse of the Ca^{2+} transient was also affected by mechanical unloading alone, which induced prolongation of the time to peak and time to 50% and 90% decline. This may be related to the loss of the normal t-tubule architecture, which separates the normally close interaction with LTCC and the RyR (Chapter 4). The t-tubules also contain Ca^{2+} extrusion pathways, including NCX, which help to determine the transient. HF-induced prolongation of the time to peak and decay parameters of the transient was reversed following mechanical unloading, at least partly driven by the reappearance of the t-tubule network (Chapter 5). The physiologic hypertrophy induced by mechanical overload did not influence the time to peak, but caused reduction of the time to 50% and 90% decline (Chapter 6). Moderate mechanical unloading induced a slowing of the Ca^{2+} transient, over a longer duration of unloading than severe mechanical unloading. Together, these results imply that all the features of the Ca^{2+} transient can be regulated by mechanical load. There is thus substantial evidence which ties these changes to alterations in t-tubule structure. Further experiments which simultaneously map the changes in Ca^{2+} transients and t-tubule structure in the same cells will be helpful in understanding this link in greater detail.

The Ca^{2+} spark properties are regulated by a number of factors and have been discussed in the preceding chapters. The cardinal sign of altered SR Ca^{2+} release function, in particular enhanced RyR leak, is Ca^{2+} spark frequency. Prolonged

mechanical unloading increased the Ca^{2+} spark frequency, in line with altered t-tubule structure which may uncouple the RyR and LTCC. This was also found in the failing heart, where t-tubule density was reduced. In support of the notion that disruption of the t-tubule membrane can result in increased Ca^{2+} spark frequency, when the t-tubule density was enhanced following mechanical unloading, the Ca^{2+} spark frequency was reduced (Chapter 5). Chapter 6 showed that Ca^{2+} spark frequency was raised at either end of prolonged mechanical unloading and mechanical overload. These conditions were accompanied with t-tubule aberrations. In addition, Ca^{2+} spark frequency was increased in young Tcap KOs, where there appeared to be isolated t-tubule irregularity. These results may present a challenge to the current paradigm of the functional consequences of LTCC-RyR interaction. It would be expected that LTCC-RyR uncoupling would reduce the Ca^{2+} spark frequency. Evidence against this was summarised in the preceding Chapters.

There was no evidence that the $\text{I}_{\text{Ca,L}}$ or AP were affected by mechanical unloading. HF depressed the $\text{I}_{\text{Ca,L}}$ and mechanical unloading restored this.

8.2 What Remains To Be Discovered?

This thesis has served to highlight the dynamism of the t-tubule network's structure and function in relation to various physiologic and pathologic stimuli. It has shed light on the details of this interaction, but raised a number of questions of importance.

First, to what extent precisely can the corollaries of mechanical unloading (altered phosphorylation, metabolic changes and so on) explain the changes which have been ascribed to the t-tubule network?

In particular, denervation may have considerable consequences on the transplanted heart. The main consequence of denervation appears to be abolition of the fine extrinsic neuroendocrine regulation (Kent and Cooper, 1974), necessary for the feedforward anticipatory changes which contribute to the response to increased cardiac workload. This leaves intrinsic mechanisms to cope, mainly Starling's law. This is augmented by catecholamine surges which stimulate a number of subcellular phosphorylation pathways to augment excitation-contraction coupling. In the absence of vagal tone, turning off of these responses is mediated by the termination of the catecholamine surge and reduced cardiac workload. The importance of these effects in an unloaded heart is uncertain.

Second, what mechanisms govern the impact of Tcap's loss on the t-tubule network's structure?

Third, it is necessary to understand the functional consequences of LTCC and RyR interactions to a more sophisticated extent than this thesis has achieved.

Finally, it is necessary to use the substantial data which has amassed in the last fifteen years to tackle the major, but neglected, question of what the purpose of (or survival advantage of) t-tubule restructuring, loss and reappearance is. It may be a bystander effect, simply a consequence of a ruined dyadic neighbourhood (Louch *et al.*, 2010c). But might it be of physiological benefit to impair contractility in an energy-deplete system? There are two approaches that could help to answer this major question.

First, it is relevant to compare physiological with pathological hypertrophy (Kemi *et al.*, 2011). To the extent that this has been possible, it appears that physiological hypertrophy is not associated with t-tubule density changes, while this parameter is reduced in pathological hypertrophy. What do these terms mean, however, if the cell sizes compared (e.g. (Kemi *et al.*, 2011)) are so wildly different? Is it possible to have a physiological hypertrophy of the order of magnitude reported for pathological conditions? The same is true of mechanical unloading and atrophic hearts, where the cell volumes compatible with normal function are significantly larger than those that are damaged. This is a major problem which needs to be addressed.

Second, the perhaps more fruitful avenue is to address the molecular mechanisms governing these changes. By understanding the molecular pathways governing the change in t-tubule density, perhaps it will be possible to better understand its aetiology. Is it perhaps a consequence of reversion to a foetal gene programme? Is it part of a wider energy-conserving programme? These approaches may allow us to understand the purpose and first-cause of the reported changes in t-tubule structure and function.

8.3 Clinical Implications

The phenomenon of significant myocardial mechanical unloading is essentially confined to the relatively rare setting of LVADs. That is not to understate the significance of understanding the physiological mechanisms mediating the response to myocardial unloading. It represents one half of a cycle which is understudied. It is impossible to understand how the heart responds to load variation without studying mechanical unloading. Nonetheless, the prime motivation of this work is to

understand the spectrum of effects of mechanical unloading because of the advent of the LVAD. In that regard, this thesis has contributed original knowledge to three major clinical questions.

First, what mechanisms explain the regression of the improvement to myocardial function in the setting of prolonged mechanical unloading? This thesis suggests that subcellular Ca^{2+} handling defects, likely the result of structural dysregulation of the t-tubules, may play a role in so-called atrophic remodelling.

Second, what mechanisms explain the improved function following mechanical unloading of the failing heart? This thesis suggests that one mechanism (of many) is restitution of the normal tight LTCC-RyR coupling mediated by the t-tubule.

Third, can a shorter duration or less severe degree of mechanical unloading influence the enhancement of bridge to recovery? This thesis suggests that the heart is sensitive to the degree and duration of mechanical unloading; in particular, that mechanical unloading of a lesser degree can preserve the structure and function of the t-tubule system.

8.4 General Conclusion

Chronic mechanical load variation influences t-tubule structure and function in a time- and degree-dependent manner. The degradation of the t-tubule system is reversible by reducing mechanical load. Tcap is a regulator of t-tubule structure, which is especially important during mechanical overload.

These results support a general model of t-tubule regulation by mechanical load variation, where a physiological range exists, beyond which t-tubule abnormalities occur and local CICR is disturbed. This thesis has highlighted a potentially critical molecule involved in the load-dependent regulation of t-tubule structure, Tcap. Further work will reveal the details of the specific pathways which link the physiological processes described here with their molecular regulators.

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