THE ROLE OF THE L-TYPE CALCIUM CHANNEL AND ITS CARBOXYL-TERMINUS

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ABSTRACT OF DISSERTATION

Miranda Jean Byse

The Graduate School
University of Kentucky
2010
ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
Miranda Jean Byse
Lexington, KY

Director: Dr. Jonathan Satin, Professor of Physiology
Lexington, KY

2010

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THE ROLE OF THE L-TYPE CALCIUM CHANNEL AND ITS CARBOXYL-TERMINUS IN THE DEVELOPING AND ADULT HEART

In the heart, the primary role of the L-type calcium channel (LTCC) Ca\textsubscript{v}1.2 is to conduct calcium into cardiomyocytes and initiate contraction. However, part of the Ca\textsubscript{v}1.2 channel itself, the cleaved carboxyl-terminus (CCt) can also localize to the nucleus and regulate gene transcription. Therefore, the goal of this dissertation project was to determine the role and regulation of CCt in the embryonic and adult heart. The global hypothesis of my dissertation project is that CCt localizes to the nucleus in embryonic and adult cardiomyocytes via a calcium-mediated mechanism and regulates transcription. A model of pharmacological LTCC block-induced perturbation of murine embryonic heart development was first utilized to study the role of CCt. Pharmacological block at embryonic day 10 perturbed cardiogenesis and increased Ca\textsubscript{v}1.2 expression. This result was not mimicked by removal of extracellular calcium or inhibition of calcium release from the sarcoplasmic reticulum. Co-currently, pharmacological block decreased CCt nuclear localization in embryonic cardiomyocytes. At the transcriptional level, CCt suppressed the Ca\textsubscript{v}1.2 promoter. This indicated that the observed upregulation of Ca\textsubscript{v}1.2 induced by pharmacological block may be caused by nuclear localization of the transcriptional repressor, CCt. Therefore, the conclusion was made that pharmacological LTCC block perturbed embryonic cardiogenesis by decreasing nuclear localization of the transcription factor CCt; implying a role for CCt in embryonic heart development. Next, CCt regulation was studied in the adult heart. Similar to the embryonic heart, pharmacological LTCC block decreased nuclear localization of CCt. Inhibition of the calcium activated phosphatase calcineurin also decreased CCt nuclear localization. To determine a role for CCt in the adult heart, CCt nuclear localization was measured in response to hypertrophic stimuli. Serum-induced cardiomyocyte hypertrophy significantly increased nuclear localization of CCt. In conclusion, this dissertation supports the hypothesis that CCt localizes to the nucleus in embryonic and adult cardiomyocytes, and that this regulation is mediated by calcium entry into the cardiomyocyte. Furthermore, data from this dissertation suggests that CCt nuclear localization may play an important role in embryonic heart development and adult cardiac hypertrophy.
Miranda Byse

[Signature]

06/04/2010
THE ROLE OF THE L-TYPE CALCIUM CHANNEL AND ITS CARBOXYL-TERMINUS IN THE DEVELOPING AND ADULT HEART

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06/04/2010
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Copyright © Miranda Jean Byse 2010
This dissertation is dedicated to my loving parents, Brian and Linda Byse.
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Chapter 1: Background

1.1 The Calcium Channels and their Expression in the Murine Heart

In the murine heart there are two types of voltage-gated calcium channels present, the L-type calcium channels and the T-type calcium channels. Both L-type and T-type calcium channels conduct calcium in response to changes in membrane potential; however, compared to T-type calcium channels, L-type calcium channels activate at more positive membrane potentials, inactivate more slowly, and are inactivated by intracellular calcium [1-3]. The L-type and T-type calcium channels each have two subtypes that are expressed in the heart at varying levels throughout development and into adulthood. The two L-type calcium channel subtypes are Ca$_{v}$1.2 and Ca$_{v}$1.3 (encoded by the $\alpha_{1C}$ and $\alpha_{1D}$ gene, respectively), and the two T-type calcium channel subtypes are Ca$_{v}$3.1 and Ca$_{V}$3.2 (encoded by the $\alpha_{1G}$ and $\alpha_{1H}$ gene, respectively). The exact expression pattern of calcium channels prior to embryonic day (E) 9.5 of murine heart development is unclear. However, at E9.5 it is known that in the ventricle, the L-type calcium channel Ca$_{v}$1.3 is expressed at higher levels than Ca$_{v}$1.2. The only T-type calcium channel expressed is Ca$_{V}$3.2 [4-6]. By E18, which is close to parturition, Ca$_{v}$1.2 expression is greater than Ca$_{v}$1.3 expression, Ca$_{V}$3.2 expression has decreased, and Ca$_{v}$3.1 is expressed [7-8]. From neonatal onward, the L-type calcium channel, Ca$_{v}$1.2, is the predominantly expressed calcium channel subtype in the atrial and ventricular cardiomyocytes, and is the major contributor of calcium for excitation-contraction coupling [1, 9-11]. The L-type calcium channel, Ca$_{v}$1.3, is expressed primarily in the sinoatrial node, atrioventricular node, and atrial cardiomyocytes [12-13]. The T-type calcium channel, Ca$_{v}$3.1, is expressed primarily in the sinoatrial node, atrioventricular node, and the purkinje fibers of the adult murine heart [1-2, 14]. The T-type calcium channel, Ca$_{v}$3.2, is not known to be present in the adult murine heart [5].
1.2 The Structure and Regulation of CaV1.2 and its Carboxyl-terminus

The L-type calcium channel, CaV1.2, exists as a heteromultimer (Figure 1.1). The main pore-forming region of CaV1.2 is the α1C subunit (encoded by the α1C gene). The α1C subunit is composed of four homologous motifs (I-IV) containing six trans-membrane segments each. The α1C subunit contains the selectivity filter for calcium, the voltage sensor, and the gating machinery that controls whether the channel is open or closed [3, 11, 15]. Associated with the α1C subunit are two auxiliary subunits, a cytosolic β-subunit (CaVβ2) and covalently-linked α2-δ subunit (reviewed by [11, 16]). The CaVβ2 subunit regulates channel kinetics and increases current density by increasing trafficking of α1C to the plasma membrane [3, 17-24]. In contrast to CaVβ2, the α2-δ subunit has little effect on channel current density, but can affect channel voltage dependence and kinetics [3, 25-27].

In addition to being regulated by CaVβ2 and the α2-δ auxillary subunits, the α1C subunit is autoregulated by its carboxyl-terminus, which spans from amino acids 1507-2171 based on the rabbit sequence [28]. In the heart, the CaV1.2 carboxyl-terminus is post-transcriptionally cleaved, purportedly by calpain, producing a ~37kDa protein [29-32]. CaV1.2 is cleaved by calpain at a consensus site that is shared by two other L-type calcium channel family members, CaV1.3 and CaV1.1. In CaV1.2, this site is located between amino acids 1800 and 1801 of the rabbit sequence [33]. However, the same lab mistakenly published a paper in 2006 stating that CaV1.2 is cleaved at amino acid 1821 [34]. Unfortunately, this mistake has been perpetuated in the literature by myself and others [29, 35]. It wasn’t until just a few months ago that the error was recognized. In this dissertation work I extensively utilize a vector that over-expresses rabbit CaV1.2 from 1821-2171. I will refer to this piece as the cleaved carboxyl-terminus (CCt), as I mistakenly believed at the time of the experiments that amino acids 1821-2171 comprised CCt. Please note that CCt actually spans from amino acid 1801-2171 of CaV1.2.
The proximal portion of the carboxyl-terminus upstream from the cleavage site contains the EF-hand [36-37], A [38-39], C [38, 40], and IQ [39-41] motifs spanning from amino acids 1526-1664 (Figure 1.1). This domain binds calmodulin, which is involved in calcium-dependent facilitation (CDF) and calcium-dependent inactivation (CDI) of the Ca\textsubscript{V}1.2 channel [42-44]. The distal portion of the carboxyl-terminus, CCT, re-associates with and acts as an inhibitor of Ca\textsubscript{V}1.2 [28, 34]. (Of note, recently published work shows that over-expression of amino acids 1801-2171 of Ca\textsubscript{V}1.2 has the same functional, inhibitory effect on Ca\textsubscript{V}1.2 channel as over-expression of amino acids 1821-2171 of Ca\textsubscript{V}1.2 [45].) However, CCT also contains Serine 1928, which can be phosphorylated by Protein Kinase A (PKA) or Protein Kinase C (PKC) [46-50]. Calmodulin kinase II (CaMKII) can also phosphorylate the carboxyl-terminus upstream of the cleavage site at amino acids 1512 and 1570 [51]. In addition to re-associating with Ca\textsubscript{V}1.2, CCT is also capable of localizing to the nucleus of neurons and acting as a transcriptional regulator [35]. In neurons, localization of CCT is regulated by calcium [35]. Depolarization of neurons, which increases Ca\textsubscript{V}1.2 activity, decreases nuclear localization of CCT. Application of the pharmacological L-type calcium channel blocker nimodipine reverses the effect of depolarization, causing an increase in nuclear localization of CCT. In addition to being regulated by calcium, levels of nuclear CCT are also regulated developmentally; nuclear CCT levels in brain tissue increase from E18 to adulthood. Although Ca\textsubscript{V}1.2 is cleaved in both the brain and in the heart [30, 32], it has not yet been established whether or not the cleaved portion of Ca\textsubscript{V}1.2 (CCT) can also localize to the nucleus in cardiomyocytes. Therefore, a central goal of this dissertation will be to determine if CCT localizes to and has a functional role in the nucleus of cardiomyocytes.

1.3 The Roles of Ca\textsubscript{V}1.2 in the Heart

The primary function of Ca\textsubscript{V}1.2 in the heart is to provide calcium current (I\textsubscript{Ca,L}) that triggers contraction through a process known as excitation-contraction coupling [1, 11, 52]. Depolarization of the heart activates the voltage-gated Ca\textsubscript{V}1.2 channel, allowing calcium to enter (I\textsubscript{Ca,L}). This calcium activates ryanodine receptors (RYRs) on the sarcoplasmic reticulum (SR), a process...
known as calcium-induced calcium release. Calcium released from SR increases global cytosolic calcium concentrations ultimately leading to contraction. Relaxation occurs by removal of calcium from the cytosol via the sodium/calcium exchanger (NCX), which pumps calcium into the extracellular space, and the sarco-endplasmic reticulum calcium ATPase (SERCA) pump, which pumps calcium back into the SR.

CaV1.2 can also play a very different role in the heart as a regulator of signaling. The role of CaV1.2 in signaling has primarily been studied in the context of adult cardiac hypertrophy because an increase in CaV1.2 channel density is sufficient to induce a hypertrophic response [11, 53-54]. CaV1.2 has been shown to be involved in regulation of the developmentally expressed gene, atrial naturetic factor (ANF), and the transcription factor, NFAT, which are both involved in pathological hypertrophy [55-56]. Interestingly, signaling pathways involving CaV1.2 have only been implicated in pathological cardiac hypertrophy, not physiological hypertrophy [57-60]. This may in part be due to the differences in how pathological hypertrophy and physiological hypertrophy are stimulated. Pathological hypertrophy can be caused by, among other things, increased plasma noradrenaline and increased cardiac sympathetic activity [61-63]. Noradrenaline and sympathetic activity activate the β-adrenergic receptors, which will ultimately increase I_{Ca,L} and calcium signaling. Indeed, pathological cardiac hypertrophy is characterized by an increase in ANF, which has been shown to be regulated by CaV1.2 in cardiomyocytes. Pathological hypertrophy also exhibits an increase in the expression of fetal genes such as β-Myosin Heavy Chain (β-MHC) and α-skeletal actin and a decrease in α-MHC and SERCA [64-69]. In contrast to pathological hypertrophy, physiological cardiac hypertrophy is caused by exercise or pregnancy and is reversible [70-72]. Molecularly, physiological cardiac hypertrophy exhibits an increase in α-MHC [73-74] and β1-adrenergic receptor (β1-AR) [74], a decrease in cardiac troponin T type 2 (cTNT)[73] and α-skeletal actin[65], and no change in SERCA[65]. There is another potential CaV1.2 mediated pathway that could play a role in signaling in cardiac hypertrophy, CaV1.2 regulating nuclear localization of CCt, Therefore, in
addition to establishing whether or not CaV1.2 regulates nuclear localization of CCt in cardiomyocytes (Section 1.2), this dissertation will also explore CCt nuclear localization in cardiac hypertrophy.

1.4 Heart Maturation and the Importance of CaV1.2

In addition to playing an important role in contraction and signaling in the adult heart, CaV1.2 is essential for cardiac development. The process of heart maturation is complex [75-80]. Mouse cardiac organogenesis begins with the formation of a linear heart tube that first contracts around embryonic day (E) 8.5 [81]. Between E8.5 and E10, the heart tube folds due to accelerated ventricular growth and the endocardial cushions (EC) form between the sinus venosus (SV) and right ventricle (RV). At E10, there is a distinct SV and left ventricle (LV) divided by the endocardial cushion-filled atrioventricular canal (AVC) [82]. The LV and RV are separated by the interventricular foramen, externally marked by the interventricular sulcus (IVC) [82], and the RV continues into the outflow tract (OFT) (Figure 1.2). Between E10 and E12 the heart continues to fold until the SV and outflow tract (OFT) are both oriented cranially. At E13.5 the first mature components of the His-Purkinje system appear [83]. Between E12 and birth, the SV matures into the right atria and the coronary sinus, the OFT matures into the aorta and pulmonary artery, and septation occurs between the atria and ventricles [84]. At E16, the heart is at the fetal stage of maturation [85] and septation has completed, resulting in distinct left and right atria and ventricles. At this time the morphological development of the heart is completed, with some modifications, such as transverse-tubule formation [86], occurring after birth (E20).

The necessity of functional L-type calcium channels for development has been exemplified in the literature by three separate mouse models. The first model is in vitro pharmacological treatment of excised E7.5-E8.5 mouse embryos [87]. Treatment of excised embryos for two days with the L-type calcium channel antagonist nifedipine or verapamil induced abnormal development of the right ventricle without altering cardiac looping. Concurrently, L-type calcium channel
block altered the expression pattern of GATA4, a transcription factor that plays an essential role in ventricular development [88-89]. GATA4 mRNA was not expressed in the ventricles of L-type calcium channel block treated embryos. This finding suggested that L-type calcium channel block regulates GATA4 expression and that loss of GATA4 may explain the abnormal ventricular development that was observed. Additionally, L-type calcium channel block acutely decreased resting and peak intracellular calcium in the hearts of intact embryos. However, embryos treated for two days with L-type calcium channel block did not exhibit a significant change in heart rate compared to control.

The second mouse model providing evidence of the necessity of L-type calcium channels for development is a Ca\textsubscript{v}1.2 knockout mouse model [90]. Knockout of Ca\textsubscript{v}1.2 did not significantly alter embryonic development or heart rate up to E12.5, but caused embryonic death by E14.5. Whole-cell patch clamp studies indicated that 81% of cardiomyocyte from Ca\textsubscript{v}1.2 knockout mice exhibited currents consistent with L-type calcium channel kinetics that were L-type calcium channel block sensitive. These currents were most likely due to expression of another L-type calcium channel, Ca\textsubscript{v}1.3. In normal embryonic development, Ca\textsubscript{v}1.3 is expressed at E9.5 at levels higher than Ca\textsubscript{v}1.2, but by E18 Ca\textsubscript{v}1.2 levels are higher than Ca\textsubscript{v}1.3 [4-8]. The decrease in Ca\textsubscript{v}1.3 levels seen in development after E9.5 may explain why the Ca\textsubscript{v}1.2 knockout is unable to survive past E14.5. In line with this supposition, a follow-up study showed that Ca\textsubscript{v}1.2 knockout mice displayed a significant increase in Ca\textsubscript{v}1.3 compared to controls, suggesting that an upregulation in Ca\textsubscript{v}1.3 may allow for embryonic survival until E14.5.

The third mouse model providing evidence of the necessity of L-type calcium channels for heart development is a Ca\textsubscript{v}β2 knockout mouse model [91]. Knockout of Ca\textsubscript{v}β2 decreased L-type calcium channel current density by approximately 30% and decreased spontaneous heart rate by approximately 50%. Hearts in Ca\textsubscript{v}β2 embryos exhibited an abnormal progression of cardiac looping, narrowing between the right ventricle and outflow tract, and decreased
myocardial wall thickness. Embryonic lethality was observed after E10.5. Cardiac-specific disruption of Ca\(\textsubscript{V}\beta2\) caused embryonic lethality after E13.5, which may have been due to incomplete penetrability of the cardiac-specific promoter driving Ca\(\textsubscript{V}\beta2\) disruption. Similar to whole-animal Ca\(\textsubscript{V}\beta2\) knockout, cardiac-specific Ca\(\textsubscript{V}\beta2\) disruption decreased spontaneous heart rate and L-type calcium channel current density. Interestingly, treatment of pregnant mice with the calcium channel agonist (-)-BayK8644 increased the survival of cardiac-specific Ca\(\textsubscript{V}\beta2\) disrupted embryos by one day.

The L-type calcium channel also plays an essential role in heart development in humans. Timothy Syndrome is caused by a missense mutation in exon 8A of Ca\(\textsubscript{V}1.2\). This mutation alters channel kinetics, leading to electrophysiological and morphological heart defects ([92-95], reviewed by [96]).

The above models provide evidence that the L-type calcium channel, Ca\(\textsubscript{V}1.2\) is necessary for embryonic heart development. However, what has yet to be established is a mechanism for the exact role of Ca\(\textsubscript{V}1.2\) in development. One possibility is that, similar to the adult heart, calcium entering through Ca\(\textsubscript{V}1.2\) activates signaling pathways that ultimately regulate gene transcription factors. A second possibility is that Ca\(\textsubscript{V}1.2\) regulates nuclear localization of CCt, which acts as a transcription factor that regulates genes involved in development. These two postulates will be explored in this dissertation.

1.5 Overview of the Dissertation Project

The global hypothesis of my dissertation project is that CCt localizes to the nucleus in embryonic and adult cardiomyocytes via a calcium-mediated mechanism and regulates transcription. This finding would provide a novel function for CCt in the heart. Furthermore, it would potentially provide a mechanism to explain how Ca\(\textsubscript{V}1.2\) directly regulates embryonic heart development and adult pathological cardiac hypertrophy. This hypothesis will be tested by the following chapters and specific aims; Figure 1.3 provides a graphical representation of these aims.
Specific Aim 1: Establish a model for testing the effects of cardiac-specific pharmacological L-type calcium channel block on embryonic cardiogenesis.

Rationale: Previous work has shown that pharmacological L-type calcium channel block, when applied to the entire embryo, can perturb morphogenesis. However, it is unclear if pharmacological L-type calcium channel block mediates its effects by directly acting on the heart. Therefore, the effects of pharmacological L-type calcium channel block will be tested on excised embryonic hearts to determine if the effects are the same as those seen in previous whole-embryo studies.

Specific Aim 2: Determine if pharmacological L-type calcium channel block perturbs cardiogenesis by altering embryonic cardiomyocyte calcium handling.

Rationale: It is known that in the adult heart Ca\textsubscript{V}1.2 can directly regulate hypertrophic signaling pathways. However, it has also been shown that in the adult heart a general increase in intracellular calcium can induce hypertrophy. This raises the possibility that L-type calcium channel block perturbs embryonic cardiogenesis by a general alteration of calcium that is secondary to the L-type calcium channel block. Therefore, the effects of L-type calcium channel block on calcium handling properties of embryonic cardiomyocytes will be studied. Additionally, the effects of perturbing calcium independent of the L-type calcium channel will be studied.
Chapter 3: CCt Localizes to the Nucleus in Developing Cardiomyocytes and Regulates CaV1.2 Transcription

**Specific Aim 1:** Determine if CCt localizes to the nucleus of embryonic cardiomyocytes.

**Rationale:** CaV1.2 is cleaved in neurons and in cardiomyocytes. In neurons this cleaved portion, CCt, has been shown to localize to the nucleus in neurons. Therefore, it is possible that CCt also localizes to the nucleus in cardiomyocytes.

**Specific Aim 2:** Determine if pharmacological L-type calcium channel block alters nuclear localization of CCt.

**Rationale:** In neurons it was established that CCt nuclear localization is regulated by CaV1.2 activity. However, neurons and cardiomyocytes have very different functions and properties. Specifically, CaV1.2 is involved in excitation-contraction coupling in cardiomyocytes; therefore CCt nuclear localization may be differentially regulated in cardiomyocytes.

**Specific Aim 3:** Determine if CCt can regulate transcription in embryonic cardiomyocytes.

**Rationale:** CCt acts as a transcriptional regulator in neurons. Therefore, the ability of CCt to regulate transcription in cardiomyocytes will also be determined; this will assist in establishing a physiologically relevant role for CCt.

Chapter 4: CCt Nuclear Localization is Regulated in the Adult Heart

**Specific Aim 1:** Confirm that endogenous CCt localizes to the nucleus in an L-type calcium channel block dependent fashion in the adult heart, similar to what is seen in embryonic cardiomyocytes.
**Rationale:** Embryonic and adult hearts differ in their expression levels of CaV1.2 as well as their overall structure. Additionally, it has been shown that CCT nuclear localization levels alter throughout development and into adulthood. This suggests the possibility that CCT localization could alter and/or be differentially regulated in the adult heart.

**Specific Aim 2:** Determine the specific mechanism by which CCT nuclear localization is regulated.

**Rationale:** CaV1.2 channel activity regulates CCT nuclear localization. However, CCT is not known to contain a calcium-binding domain; instead, the calcium-binding EF hand domain is located upstream in the proximal carboxyl-terminus. This begs the question of how CaV1.2 signals CCT to localize to the nucleus. Calmodulin, bound to CaV1.2 and activated by calcium entry, is capable of activating CaMKII and CaN. It is possible that this kinase and phosphatase act on CCT to regulate its nuclear localization. Indeed, phosphorylation and dephosphorylation are well established regulators of nuclear transport. Additionally, CCT contains Serine 1928, which is a known site of phosphorylation by PKA and PKC. Therefore, the role of CaMKII, CaN, PKA, and PKC on CCT nuclear localization will be explored.

**Specific Aim 3:** Determine if CCT nuclear localization is altered in cardiac hypertrophy.

**Rationale:** Increasing calcium is sufficient to induce pathological cardiac hypertrophy in vitro. Additionally, CaV1.2 can directly induce hypertrophy in vitro. Clinical studies have shown that pharmacological L-type calcium channel block is beneficial in some cases of adult cardiac hypertrophy. This suggests that CaV1.2, or more specifically CCT, may be playing a role in pathological cardiac hypertrophy. To begin to test this supposition, nuclear localization of CCT will be compared in different models of pathological and physiological hypertrophy.
Figure 1.1 The Structure of CaV1.2.

The pore-forming region of the channel (α1C) with its four homologous motifs, each containing six trans-membrane motifs (S1-S6). The carboxyl-terminus of α1C contains EF hand, A, C and IQ motifs from amino acids 1526-1664 that are involved in regulation of channel kinetics. The carboxyl-terminus also contains a cleavage site at amino acid 1822 and a site for PKA and PKC phosphorylation at serine 1928 (S1928). In addition to the pore-forming unit there are accessory subunits, β and α2-δ that are involved in channel localization to the membrane and regulation of channel function. (Adapted from [11])
Figure 1.2 Representative Stages of Murine Heart Development.

Embryonic day (E) 8.5 shows the heart tube consisting of the outflow tract (OFT), immature right ventricle (RV) and left ventricle (LV), and the sinus venosus (SV), which ultimately become the atria. (Adapted from Zaffran et al., 2002). At E10 the heart is beginning to fold and there is a distinct separation between the SV and LV, the atrioventricular canal (AVC); the interventricular canal (IVC) has also begun to form between the LV and RV. Folding continues through E12 and by E16 there is a fully formed heart containing two distinct atria and ventricles.
Figure 1.3 Graphical Representation of the Dissertation Project.
The overall goal of the dissertation project is to determine if CCT is capable of localizing to the nucleus and regulating transcription in the embryonic and adult heart. Chapter 2 of the dissertation will determine if L-type calcium channel block perturbs embryonic cardiogenesis by altering calcium. Chapter 3 will determine if 1) CCT localizes to the nucleus in embryonic cardiomyocytes via a calcium-dependent mechanism and 2) if CCT regulates transcription and expression of Cav1.2. Chapter 4 will examine whether or not 1) CCT localizes to the nucleus in adult cardiomyocytes via a calcium dependent, 2) CCT nuclear localization is regulated by the adrenergic receptors, protein kinase A (PKA), protein kinase C (PKC), calmodulin kinase II (CaMKII), 3) CCT nuclear localization is regulated by calcineurin and the ERK1/2 pathway, and 4) CCT nuclear localization is regulated in cardiac hypertrophy.
Chapter 2: Pharmacological L-type Calcium Channel Block Perturbs Embryonic Cardiogenesis

2.1 Introduction

Previous work indicates that regulation of calcium is necessary for normal embryonic heart development. Pharmacological block of the L-type calcium channel in E7.5-E8.5 excised embryos causes abnormal ventricular development [87]. Additionally, Ca_\text{v}β2 knockout mice, which have decreased L-type calcium channel current density, exhibit morphological cardiac defects [91]. However, there are drawbacks to these two published models. Both models look at heart development in the intact embryo. While this is a developmentally relevant model, it raises the question of whether the perturbations performed in each model affect the heart directly. For example, treatment of the E7.5-E8.5 embryos with pharmacological L-type calcium channel block acutely decreased peak cytosolic calcium in the heart, consistent with L-type calcium channel block. However, treatment with the same dose of L-type calcium channel block over two days did not significantly alter heart rate. This suggests that the abnormal cardiogenesis observed may not have been due to blocking L-type calcium channels on the heart, but instead due to block of L-type calcium channels elsewhere in the embryo. A similar implication could be made with the Ca_\text{v}β2 knockout mice, since the deletion of Ca_\text{v}β2 was present throughout the embryo. Indeed cardiac-specific disruption of Ca_\text{v}β2, which was examined in the same paper as the Ca_\text{v}β2 knockout mouse, was not reported to exhibit perturbed cardiogenesis [91]. Further evidence that there is an unclear role for L-type calcium channel in heart development can be seen in Ca_\text{v}1.2 knockout mice. Knockout of the L-type calcium channel, Ca_\text{v}1.2, leads to embryonic lethality by E14.5, but there is no reported change in cardiac morphology [90]. However, there is a compensatory upregulation of the L-type calcium channel, Ca_\text{v}1.3, in these knockout mice, which may explain the lack of perturbed cardiogenesis [97]. Since there is an unclear role for the L-type calcium channel in normal heart development...
development, I wanted to test the hypothesis that specifically blocking the L-type calcium channel in the heart would perturb embryonic heart development. For these experiments I chose to use a model of explanted embryonic mouse hearts, which allows me to look at heart-specific effects of L-type calcium channel block. This model allows me to address my hypothesis without the perplexities that are present in the previously published model systems.

2.2 Materials and Methods

2.2.1 Whole-heart Explants and Cell Cultures

All cultures were maintained in Dulbecco’s Minimal Essential Media (DMEM) supplemented with 10% fetal bovine serum (FBS), 100μg/ml penicillin, 100μg/mL streptomycin, and 2μM L-glutamine. Whole E10 or E16 hearts were isolated from CD-1 mice (Charles Rivers) and treated for 48 hours with 10μM nifedipine dissolved in DMSO. Images were taken every 24 hours using a Nikon Coolpix 5000 camera attached to a Nikon Diaphot 200 microscope with a 10x lens. For E10 cell cultures, left ventricles were isolated and placed in a nominal Ca\(^{2+}\)-free digestion solution containing 0.5 mg/mL collagenase type II (Worthington) and 1mg/mL 4x USP pancreatin (Gibco) for 20 minutes. For E16 cell cultures, ventricular tissue was isolated, minced, and immediately placed in nominal Ca\(^{2+}\)-free digestion solution for two 15 minute increments. All cells were cultured on fibronectin-coated coverslips.

2.2.2 Whole-Cell Calcium Imaging

Calcium imaging was performed as described previously [98-99]. Briefly, E10 left ventricular cardiomyocytes were cultured for 48 hours with or without 10μM nifedipine. Cardiac myocytes were loaded with 2μM fura-2 for 10 minutes and de-esterfied in physiological salt solution (PSS) for 10 minutes. All recordings were performed at 37°C. Individual cells in a monolayer were windowed for recording, excited at 340nm and 380nm wavelengths, and the data was collected with IonOptix (Milton, MA) hardware and analyzed with Clampfit9, IonOptix, and Origin software. Recordings and measurements were performed
as follows. Cardiomyocytes were first placed in physiological salts solution (PSS) that included 1.8mM calcium. This allowed for the measurement of spontaneous calcium transients. Extracellular calcium was removed by washing in a PSS solution containing no calcium and 5mM of the calcium chelator EGTA (0 Ca/EGTA). This caused an abrupt cessation of spontaneous transients and cytosolic calcium levels relaxed to a new steady-state within approximately 8 seconds. Trans-SL diastolic calcium entry was measured by calculating the difference in calcium levels between the 0 Ca/EGTA level and the mean diastolic level measured in PSS (labeled ‘i’ in Figure 2.2). Next, SR calcium load was measured by adding 50mM caffeine for a 25 second interval; the amplitude of the cytosolic calcium transient (labeled ‘ii’ in Figure 2.2) acts as an index of SR load. Approximately 20 seconds post caffeine application cytosolic calcium reached a new steady-state level. The difference between this steady-state calcium level and the steady-state calcium level achieved upon initial application of 0Ca/EGTA is an index of SR calcium leak (labeled ‘iii’ in Figure 2.2).

2.2.3 Exon8/8a Splice Variant Assay

Determination of CaV1.2 exon8/8a expression was performed as in [100]. Briefly, E10 whole hearts were treated with or without 10μM nifedipine for 48 hours. cDNA was made from RNA collected using the RNAqueous 4-PCR Kit (Ambion). Exon 8/8a was amplified using PCR with Platinum Taq DNA Polymerase (Invitrogen) and the following primers for Cav1.2 Exon 8/8a:
Forward primer 5’-CACCAACTTCGACAACTTCGCC-3’
Reverse primer 5’- GCTTTGGCTTTCTCCCTCTTT-3’
The cycle conditions were as follows: 94°C for 2 min followed by 29 cycles of 94°C for 30sec, 61°C for 30sec, and 72°C for 30sec. The 218bp PCR product was digested with either BamHI (exon 8) or HaeIII (exon8a) from New England Biosciences and run out on a 5% TBE gel (Bio-Rad).
2.3 Results

2.3.1 Pharmacological Block of the L-type Calcium Channel Disrupts Embryonic Cardiac Morphogenesis

In order to look specifically at effects of L-type calcium channel block on the heart, whole excised hearts were examined in culture. Hearts excised at embryonic day 10 (E10) retained gross structural hallmarks during 48 hour culture; 79% of hearts retained a sulcus at the level of the atrioventricular canal (AVC) and 74% retained a sulcus at the site of interventricular septation (IVS). In addition, all E10 hearts retained the ability to spontaneously contract (Figure 2.1). Treatment of excised hearts with 1µM nifedipine for 48 hours resulted in gross morphological defects; only 12% and 44% of hearts retained a sulcus at the AVC and IVS, respectively (Figure 2.1B). This effect was dose dependent; 10-100 µM nifedipine decreased the preservation of sulci at the AVC and IVS to nearly 0%. Nifedipine also affected heart contraction. Nifedipine arrested beating within seconds of initial application for all doses tested (1-100 µM). However, after 24 hours of sustained L-type calcium channel block, hearts resumed spontaneous beating in a dose-dependent fashion (Figure 2.1C). In 1 µM nifedipine, 100% of hearts resumed spontaneous beating, in 10µM nifedipine only 88% of hearts resumed beating, and in 100 µM nifedipine all hearts ceased beating. Similar dose-dependent nifedipine-resistant beating was exhibited following sustained L-type calcium channel block for 48 hours (Figure 2.1C).

Nifedipine is a dihydropyridine, therefore, as a redundancy test for L-type calcium channel specificity the phenylalkylamine verapamil was also tested. Both nifedipine and verapamil are L-type calcium channel blockers, but bind to different sites of the L-type calcium channel (reviewed by [11]). Similar to nifedipine, 1µM and 5µM verapamil led to a loss of sulci at the AVC and IVS (Figure 2.1B). Furthermore, beating initially ceased then resumed in 95% of hearts after 24 hour treatment with 1µM verapamil and in 40% of hearts treated with 5µM verapamil (Figure 2.1C).
The L-type calcium channel plays an essential role in calcium handling in the heart via its ability to regulate calcium entry. Therefore, the effects of a more general inhibition of calcium entry were tested by culturing E10 hearts in calcium-free medium. Culturing in calcium-free medium for 48 hours demonstrated a unique morphology compared to L-type calcium channel block (Figure 2.1B). These hearts maintained sulci at the AVC and IVS similar to control. In addition, there was no recovery of contraction as observed at lower doses of L-type calcium channel block.

2.3.2 Sarcoplasmic Reticulum Calcium Is Altered by Pharmacological L-type Calcium Channel Block but Does Not Contribute to Cardiac Morphogenesis

In section 2.3 the observation was made that L-type calcium channel block and general removal of extracellular calcium had differential effects on heart morphogenesis as well as the ability of the heart to resume contraction. Specifically, hearts resumed contraction in the presence of L-type calcium channel block. In order for contraction to occur, cardiomyocytes must have a mechanism to allow calcium entry into the cytosol, which can in turn trigger sarcoplasmic reticulum (SR) calcium release. To determine how the cardiomyocytes may be compensating for reduced L-type calcium channel function, the effect of L-type calcium channel block on calcium dynamics in the cardiomyocyte was tested. For these experiments, whole-cell calcium imaging was used. This technique allows for the measurement of net diastolic trans-sarcolemmal (trans-SL) calcium entry, sarcoplasmic reticulum (SR) calcium load, and SR leak into the cytosol [98]. Each of these components contributes to cytosolic calcium levels.

Whole-cell calcium imaging was performed on cardiomyocytes cultured for 48 hours in the absence of nifedipine (No Drug) or in the presence of 10μM nifedipine. Consistent with the observed effect of L-type calcium channel block on whole-heart contraction (Figure 2.1), 4/11 nifedipene treated cardiomyocytes exhibited spontaneous transients. In the no drug cardiomyocytes, 11/11 exhibited
spontaneous transients. Treatment of cardiomyocytes with 48 hour nifedipine did not significantly alter trans-SL diastolic calcium entry compared to no drug control (Figure 2.3A, black bars). However, 48 hour nifedipine significantly decreased SR load and SR leak (Figure 2.3B,C; black bars). These changes were not due to a difference in spontaneous transient frequency. Figure 2.4 shows that there is no interdependency between spontaneous transient frequency and trans-SL diastolic calcium entry or SR load.

The observed decrease in SR calcium load and leak raises the possibility that L-type calcium channel block mediates its effects on morphology via an alteration in SR calcium handling. In order to test this possibility, excised E10 hearts were treated for 48 hours with micromolar ryanodine, which decreases the calcium conductance of the ryanodine receptor (RYR) [101]. As expected with a decrease in calcium release from the SR, treatment with 50µM ryanodine did not stop spontaneous transients, but did significantly decrease spontaneous transient frequency (Figure 2.5B). However, 50 µM ryanodine had no observable effect on cardiac morphology (Figure 2.5A). This finding implies that SR calcium does not play the same role in cardiac morphogenesis as L-type calcium channel block.

### 2.3.3 Pharmacological L-type Calcium Channel Block Induces Nifedipine Resistance

As described in section 2.4, a subset of 48 hour nifedipine treated cardiomyocytes exhibited spontaneous transients (48H-ST). In order to determine how these cardiomyocytes were maintaining their spontaneous transients, cardiomyocytes were challenged with acute 10µM nifedipine. Acute treatment with nifedipine, as expected, significantly decreased trans-SL diastolic calcium entry, SR load, and SR leak in control cardiomyocytes that had no prior exposure to drug (no drug; Figure 2.3, grey bars). This same effect of acute nifedipine was also observed in cardiomyocytes treated with 48 hour nifedipine that were quiescent (48H-Q). Surprisingly, 48 hour nifedipine treated
cardiomyocytes exhibiting spontaneous transients (48H-ST) were not affected by acute nifedipine challenge (Figure 2.3, grey bars).

One possible explanation for the observed nifedipine resistance is that these cardiomyocytes contain the nifedipine-resistant exon 8 splice variant of Ca\textsubscript{v}1.2 [102-103]. This nifedipine-resistant splice variant, termed exon 8a, is predominant in the vasculature, but is not normally observed in the heart. To determine if exon 8a was present in the 48 hour nifedipine treated heart, analytical digest of RT-PCR amplified cDNA was performed (Figure 2.6). Exon 8 contains a restriction site for BamHI while Exon 8a contains a restriction site for HaeIII. In both control and nifedipine treated hearts, only BamHI cleaved the RT-PCR product indicating that only nifedipine-susceptible exon 8 was present. This suggests that alternative splicing of exon 8 is not involved in the nifedipine resistance phenomenon.

2.3.4 Pharmacological L-type Calcium Channel Block Induces Nifedipine Resistance in Fetal Cardiomyocytes without Altering Morphology

The previous experiments show that the L-type calcium channel is required for normal embryonic development when the heart is undergoing distinct morphological changes. However at approximately E16 the heart has completed morphogenesis and septation and instead undergoes proliferative and hypertrophic growth. This raises the question of the role of the L-type calcium channel at this distinct stage of development. Therefore the effect of L-type calcium channel block on E16 hearts was tested in the same manner as the E10 hearts described above. E16 hearts were excised and cultured in the presence or absence of L-type calcium channel block. Treatment with nifedipine had no observable effect on heart morphology (Figure 2.7A). The highest dose of nifedipine (10µM) stopped contraction in approximately 90% of hearts, while the lowest dose stopped contraction in approximately 50% of hearts (Figure 2.7B). Because a percentage of E16 hearts contract in the presence of nifedipine, calcium imaging was performed to determine if nifedipine-treated E16 cardiomyocytes exhibited nifedipine resistance similar to E10 cardiomyocytes.
Similar to E10 control cardiomyocytes, E16 control cardiomyocytes were responsive to acute nifedipine treatment. Acute nifedipine decreased trans-sarcolemmal diastolic calcium entry and significantly decreased SR load and SR leak. The lack of significance in trans-sarcolemmal diastolic entry was most likely due to the large standard error. Sample size would need to be increased in order to resolve this issue. All 48 hour 10 µM nifedipine treated cardiomyocytes measured exhibited spontaneous transients despite the small percentage of contracting hearts observed with the treatment of 10µM nifedipine for 48 hours. Similar to 48 hour treated E10 cardiomyocytes, 48 hour treated E16 cardiomyocytes showed no significant change in trans-sarcolemmal diastolic calcium entry compared to control. However, unlike the E10 cardiomyocytes, 48 hour treated E16 cardiomyocytes showed no significant change in SR calcium load or SR calcium leak compared to control (Figure 2.8). Treatment with acute nifedipine did not significantly alter trans-SL diastolic calcium levels, SR load, or SR leak. These findings are similar to the subset of nifedipine-resistant E10 cardiomyocytes that exhibited spontaneous transients (Figure 2.3).

2.4 Discussion

The primary finding of this chapter is that pharmacological blockade of the L-type calcium channel perturbs development of the embryonic heart; this effect is not mimicked by general block of calcium entry, obtained by culturing in media lacking calcium, or by decreasing SR calcium release by addition of micromolar ryanodine. In addition, L-type calcium channel block only perturbs morphology in the earlier embryonic stage when the heart is undergoing septation and morphogenesis, not in the fetal stage when the heart is exclusively undergoing proliferation and growth. A probable explanation for this differential effect on morphogenesis is that these two stages of development vary greatly in their genetic programming. One example is the expression of the transcription factor GATA4. A role for GATA4 in the fetal E16 heart has not been established. However, GATA4 is expressed in early embryonic development and plays an
essential role in ventral morphogenesis and cardiomyocyte proliferation near E10 [88-89]. Previous work shows that L-type calcium channel block leads to a loss of GATA4 expression in the ventricle of E10 mice [87]. Therefore, it is possible that the effects of L-type calcium channel block observed in the explanted heart experiment are in part due to the loss of GATA4 expression. In fact, embryonic mice with an inducible GATA4 deletion that begins at E7.5 exhibit a singular ventricle that is similar to the morphology observed with L-type calcium channel block of explanted hearts (Figure 2.1; [88]). GATA4 is not known to be regulated by the L-type calcium channel. However, Dr. Elizabeth Schroder performed a microarray of E10 hearts treated for 48 hours with 1µM verapamil that showed a trend towards decreasing GATA4 expression (p=0.053). Real-time RT-PCR would need to be performed as a follow up experiment to determine if this decrease was significant. If these findings hold, it would be interesting to see if GATA4 over-expression could negate the effects of L-type calcium block on embryonic heart morphogenesis. However, if GATA4 shows no change with calcium channel block there are number of other possible genes and transcription factors to be studied. The microarray performed by Dr. Schroder showed significant changes in the expression of 433 genes. The greatest number of alternatively expressed genes was involved with developmental processes, followed by cellular communication, cellular differentiation, signal transduction, transcription, and calcium ion transport. An interesting future study would be to mine the microarray data for novel genes regulated by calcium that are known to be directly involved in cardiogenesis. Approximately 85 of the 433 genes that showed significant changes in microarray were involved with developmental processes. Therefore, I would expect fewer than 85 genes of these genes to have a direct and established role in cardiogenesis.

L-type calcium channel block induces pharmacological resistance that allows for contraction. This observation was made in both the embryonic and fetal heart, but was present in a larger percentage of embryonic hearts (Figures 2.1, 2.8). This finding was consistent with the observation made by Porter et al. that treatment of E7.5-E8.5 embryos with pharmacological L-type calcium channel block induces pharmacological resistance that allows for contraction.
channel block for two days did not significantly alter heart rate [87]. L-type calcium channel block resistance was not due to switching to the nifedipine-resistant splice variant of CaV1.2, suggesting that another calcium-conducting channel is involved. One possible candidate is the T-type calcium channel, which is pharmacologically resistant to nifedipine and verapamil. The role of T-type calcium channels in the embryonic heart is not well established, although there is some evidence that T-type calcium channels play a role in calcium-induced calcium release [104]. There are two types of T-type calcium channels expressed in the developing heart, CaV3.1 and CaV3.2 [5, 7]. Microarray and real time RT-PCR performed by Dr. Schroder revealed an increase in CaV3.1 expression with L-type calcium channel block (data not shown). An increase in CaV3.1 mRNA and protein was also observed in the CaV1.2 knockout model [97]. However, there was no significant change in T-type calcium channel current with CaV1.2 knockout compared to control [6]. There was also no significant change in T-type calcium channel current observed in the CaVβ2 knockout mouse [91]. These findings suggest that although CaV3.1 expression is increased with L-type calcium channel perturbation, it does not translate to a change in CaV3.1 function. Therefore, upregulation of CaV3.1 may not be an explanation as to how embryonic hearts can retain their ability to contract in the presence of L-type calcium channel block. Another possible candidate is a nifedipine-resistant calcium channel that has been identified and characterized in neonatal rat cardiomyocytes [105-107]. Little is known about this channel other than its electrophysiological properties. Similar to the L-type calcium channel, the nifedipine-resistant calcium channel is activated in the same voltage range and its current density is increased when barium is substituted for calcium as the charge carrier. However, the steady-state inactivation is negatively shifted by 16mV compared to the L-type calcium channel [106]. A future experiment would be to perform whole-cell patch clamp to determine if cardiomyocytes treated for 48 hours with nifedipine displayed an increase in nifedipine-resistant calcium channel current density.
L-type calcium channel block, in addition to inducing nifedipine resistance, also caused a significant decrease in SR load and SR leak in E10 cardiomyocytes. This finding is not consistent with the Ca\textsubscript{v}β2 knockout mouse, which showed no significant change in SR load at E10.5 [91]. The differential effects on SR load may be due to the retained presence of approximately 70% of L-type calcium channel current density in Ca\textsubscript{v}β2 knockout mice compared to control. This L-type calcium channel current is sensitive to the drug isradipine, which is in the same class of dihydropyridines as nifedipine. It is possible that the nifedipine-resistant E10 cardiomyocytes have a decrease in calcium entry into the cardiomyocyte, which in turn, would decrease SR load. However, trans-SL diastolic calcium entry is unchanged in nifedipine-resistant cardiomyocytes. While this is not a direct measurement of calcium entry into the cardiomyocyte, it may be used as an index of calcium entry as it is nifedipine-sensitive (Figure 2.3). Further evidence that the change in SR load observed in E10 cardiomyocytes may not be due entirely to a decrease in calcium entry is provided by E16 cardiomyocytes that show no significant change in SR load with 48 hour nifedipine treatment (Figure 2.8). If a decrease in calcium entry was the sole explanation for the change in SR load, then E16 cardiomyocyte should also exhibit a decrease in SR load. However, to directly address the question of whether or not a decrease in calcium entry is the reason for the decrease in SR load in E10 cardiomyocytes, a future experiment should be performed. E10 cardiomyocytes treated for 48 hours with nifedipine should be subjected to increased extracellular bath calcium, which has previously been shown to increase both calcium entry and diastolic calcium levels [98]. If a decrease in calcium entry is the reason for a decrease in SR load, then I would predict that raising extracellular bath calcium would significantly increase SR load in E10 nifedipine-treated cardiomyocytes. If there was no change in SR load with increased extracellular calcium, then I would suggest that the change may be due to differential expression of genes involved in regulation of SR calcium, such as RYR and SERCA.
Figure 2.1 The effect of pharmacological L-type calcium channel block (LTCCB) on E10 in vitro cardiogenesis. A) Representative photographs of E10 hearts treated with the LTCC antagonist nifedipine or verapamil or treated with nominal calcium free media. The black and white arrows denote the sulcus at the atrioventricular canal (AVC) and at the site of interventricular septation (IVS) respectively. (White Scale bar = 200nm) B) LTCCB dose dependently decreases the percentage of hearts that contain a sulcus at the AVC or IVS at 48 hours. C) LTCCB initially stops contraction in all hearts (as denoted by an arrow). However, by 24 hours contraction resumes in the presence of LTCCB at all doses except for 100µM nifedipine. All hearts stopped beating in Ca-free media.
Figure 2.2 Representative calcium imaging traces from embryonic day 10 cardiomyocytes. Cardiomyocytes were first placed in physiological salts solution (PSS) that included 1.8mM calcium. This allowed for the measurement of spontaneous calcium transients. Extracellular calcium was removed by washing in a PSS solution containing no calcium and 5mM of the calcium chelator EGTA (0 Ca/EGTA). This caused an abrupt cessation of spontaneous transients and cytosolic calcium levels relaxed to a new steady-state within approximately 8 seconds. Trans-SL diastolic calcium entry was measured by calculating the difference in calcium levels between the 0 Ca/EGTA level and the mean diastolic level measured in PSS (labeled ‘i’). Next, SR calcium load was measured by adding 50mM caffeine for a 25 second interval; the amplitude of the cytosolic calcium transient (labeled ‘ii’) acts as an index of SR load. Approximately 20 seconds post caffeine application cytosolic calcium reached a new steady-state level. The difference between this steady-state calcium level and the steady-state calcium level achieved upon initial application of 0Ca/EGTA is an index of SR calcium leak (labeled ‘iii’).
Figure 2.3 L-type calcium channel block (LTCCB) for 48 hours induces LTCCB resistance in a sub-population of E10 ventricular cardiomyocytes (VMs). Application of acute 10µM nifedipine to vehicle treated VMs (No Drug; n=9) significantly decreased trans-sarcolemmal diastolic calcium entry (A), sarcoplasmic reticulum (SR) load (B), and SR leak (C) (*p<0.02; ANOVA). Treatment of E10 VMs for 48 hours with 10µM nifedipine resulted in a subpopulation of VMs that were quiescent (48H-Q; n=11) and a subpopulation of VMs that exhibited spontaneous transients (48H-ST; n=4). Acute nifedipine application significantly decreased trans-sarcolemmal diastolic calcium entry in 48H-Q VMs but not in 48H-ST VMs. However, acute nifedipine did not significantly change SR load or SR leak in either population. (*p<0.02; ANOVA)
Figure 2.4 There is no interdependence between trans-sarcolemmal diastolic calcium entry and spontaneous transient frequency (A) or sarcoplasmic reticulum load and spontaneous transient frequency (B) in E10 ventricular myocytes.
Figure 2.5 Pharmacological ryanodine receptor (Ryr) block does not visibly alter E10 in vitro cardiogenesis. A) Representative photographs of E10 hearts treated with the RYR antagonist ryanodine. The black and white arrows denote the sulcus at the atrioventricular canal (AVC) and at the site of interventricular septation (IVS) respectively. (Scale bar = 200µm) B) Ryanodine decreases the rate of E10 heart contraction (Control n=19; Ryanodine n=19; *p<0.01; t-test).
Figure 2.6 Alternative splicing of Exon 8 of Ca\textsubscript{v}1.2 is not influenced by L-type calcium channel block. E10 hearts were treated for 48 hours with 10\textmu{}M nifedipine or vehicle (control). Control E10 hearts only contain the Exon 8 splice variant, as is evident by the ability of BamHI, but not HaeIII, to cleave the PCR product; 10mM nifedipine does not alter splicing. “S” indicates standard.
Figure 2.7 Pharmacological L-type calcium channel block (LTCC block) does not visibly perturb E16 in vitro cardiogenesis. A) Representative photographs of E106 hearts treated with the LTCC antagonist nifedipine. (White Scale bar = 5mm) B) LTCC block dose dependently decreases the percentage of contracting hearts at 24 hours. By 48 hours, some of the hearts have developed nifedipine resistance and have resumed contraction.
A) Trans-Sarcolemmal Diastolic Calcium Entry

B) Sarcoplasmic Reticulum Load

C) Sarcoplasmic Reticulum Leak
Figure 2.8 E16 ventricular cardiomyocytes (VMs) treated with 48 hour L-type calcium channel block exhibit resistance. E16 VMs were treated with 10mM nifedipine for 48 hours and subjected to the same calcium imaging protocol as described in Fig 2. Similar to the 48H-ST population of E10 VMs shown in Fig 2, application of acute 10µM nifedipine to 48H treated E16 ventricular myocytes did not significantly alter trans-sarcolemmal calcium entry (A), SR load (B), or SR leak (C). (No Drug, n=6; 48H Nifedipine, n=6; *p<0.05; t-test)
Chapter 3: CCt Localizes to the Nucleus in Developing Cardiomyocytes and Regulates Ca\textsubscript{v}1.2 Transcription

3.1 Introduction

Chapter 2 provided evidence that pharmacological block of the L-type calcium channel disrupts morphogenesis of the E10 heart. Altering intracellular calcium by blocking the RYR receptor on the SR or by culturing hearts in the absence of extracellular calcium did not have the same effects on E10 cardiac development as L-type calcium channel block. What is unknown is how pharmacological L-type calcium channel block can be directly regulating morphogenesis of the E10 heart. If pharmacological block were disrupting morphogenesis simply by perturbing intracellular calcium and calcium handling, then culturing hearts in the absence of calcium or in the presence of RYR receptor block should have produced a perturbed morphology similar to L-type calcium channel block. However, this was not the case. This observation suggests that the L-type calcium channel has an alternative mechanism by which it can regulated E10 heart morphogenesis. Recently it was shown that, in neurons, the cleaved portion of the Ca\textsubscript{v}1.2 carboxyl terminus (CCt) localizes to the nucleus and regulates gene transcription; moreover, localization of CCt in the neuron is regulated by pharmacological L-type calcium channel block [35]. In the heart, Ca\textsubscript{v}1.2 is also proteolytically cleaved, producing CCt [34, 108]. However, it is unknown if CCt is capable of localizing to the nucleus and regulating transcription in cardiomyocytes. Therefore, I tested the hypothesis that CCt localizes to the nucleus in cardiomyocytes, and that CCt localization is regulated by L-type calcium channel block. If CCt is indeed localized to the nucleus, and if this localization is regulated by L-type calcium channel block, then it could provide the basis for a mechanism by which the L-type calcium channel directly regulates embryonic heart morphogenesis.
3.2 Materials and Methods

3.2.1 Cell Culture

E16 cardiomyocytes were isolated and cultured as described in Section 2.2.1. HEK-293 cells were cultured in the same media described in Section 2.2.1.

3.2.2 Vector Construction

A pEGFP-C1 plasmid containing the rabbit 
\( \text{Ca}_v \)1.2 sequence between amino acids 1507-2171 (eGFP1507-2171; a kind gift from Dr. Chunyan Pang) was used as a template to amplify the sequence corresponding to amino acids 1821-2171 via PCR. The amplified PCR product was ligated into the multiple cloning site of pEGFP-C1 and verified by sequencing (eGFP1821-2171; Davis Sequencing). pEGFP-C1 containing the sequence from rabbit 
\( \text{Ca}_v \)1.2 amino acids 1906-2171 (eGFP1906-2171) and pcDNA3.1+Zeo3HAa containing the rabbit 
\( \text{Ca}_V \)1.2 amino acid sequence from 1906-2171 (HA1906-2171) were also generously provided by Dr. Chunyan Pang. Mutations of Serine 1928 were made in the eGFP1821-2171 construct were made using the QuikChange II Site-Directed Mutagenesis Kit (Strategene). A phosphodeficient mutant, S1928A, was made by mutating serine (TCC) to an alanine (GCT). A phosphomimetic mutant, S1928E, was made by mutating serine (TCC) to glutamic acid (GAG). Site-specific mutation was verified by sequencing of the entire coding region of the vector (David Sequencing).

3.2.3 Translocation Assay

E16 cardiomyocytes were transfected twenty-four hours post-isolation using Lipofectamine2000 (Invitrogen). Twenty-four hours later, cells were either treated with drug or immediately fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton, and blocked with 1% BSA in PBS. Cardiomyocytes were stained with mouse sarcomeric anti-\( \alpha \)-actinin (Sigma) and Alexa Fluor-594 goat anti-mouse IgG1 (Invitrogen) to identify cardiomyocytes and mounted with Vecta Shield with DAPI (Vector Laboratories) to identify nuclei. A LSM 5 Live (Zeiss) was used to obtain confocal images of 1\( \mu \text{m} \) thickness focused on the center of
the nuclei. Nuclear and cytosolic fluorescence was quantified using ImageJ (available at http://rsb.info.nih.gov/ij; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD) with the LSM Toolbox. The area within the nucleus and cytosol containing the greatest fluorescent intensity was used to measure the nuclear to cytosolic ratio. Representative images were obtained using a Leica TSP SP5 confocal microscope.

3.2.4. Whole-Cell Calcium Imaging

E16 cardiomyocytes were transfected with eGFP, eGFP1821-2171, or eGFP1906-2171. Whole-cell calcium imaging was performed as described in Section 2.2.2 48 hours post-transfection.

3.2.5 Immunocytochemistry with Ab55

HEK-293 cells were transfected with eGFPCaV1.2 or eGFP1821-2171 using Lipofectamine 2000 (Invitrogen). Twenty-four to forty-eight house post-transfection, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton, and blocked with 1% BSA in PBS. Cells were stained with Ab55 and goat anti-rabbit IgG(H+L) Alexa 594 (Invitrogen) and mounted with Vecta Shield with DAPI (Vector Laboratories) to identify nuclei. A LSM 5 Live (Zeiss) was used to obtain confocal images.

3.2.6 Western Blot

To probe for whole-cell CaV1.2 protein, whole cell lysates were obtained from E16 ventricular cells cultured for 48 hours with DMSO or 10μM nifedipine using Ripa buffer. Each sample (10μg) was run out on a 7.5% SDS-Page separating gel (Bio-Rad). Immunoblotting was performed using 2 μg/ml of affinity-purified L-type Ca^{2+} channel α1c-subunit polyclonal antibody (gift from Dr. Douglas Andres) visualized with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Chemicon) and Super Signal West Pico Chemiluminescence (Pierce). To confirm loading, blots were stripped using Restore Western Blot Stripping Buffer (Pierce) and re-probed with GAPDH monoclonal antibody (Ambion) and horseradish peroxidase-conjugated goat anti-
mouse secondary antibody (Invitrogen). An Epson Perfection 1650 was used to scan the immunoblots, and bands were quantified using densitometry (Scion Image, Scion).

Nuclear and cytosolic protein fractions were obtained using the NE-PER kit (Pierce). Each protein sample (60μg) was run out on a 4-15% separating SDS-PAGE gel (Bio-Rad). Immunoblots were performed with Ab55, a custom antibody against amino acids 2051-2064 in the carboxyl-terminus of Cav1.2 (ECM Biosciences, Versailles, KY), Pol II (Santa Cruz Biotechnology), or GAPDH (Ambion). Blots were visualized using horseradish peroxidase-conjugated goat secondary antibodies (Chemicon) and Super Signal West Pico Chemiluminescence (Pierce). An Epson Perfection 1650 was used to scan the immunoblots and bands were quantified using densitometry (ImageJ, available at http://rsb.info.nih.gov/ij; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

3.2.7 Luciferase Assay

The Cav1.2 promoter reporter construct was created by Dr. Schroder and these experiments were performed primarily by Dr. Schroder. Five independent wells containing E16 ventricular cardiomyocytes were transfected within 24 hours of dispersal with pGL3-Cav1.2 or the promoter-lacking vector called pGL3-Basic. To test for the effect of sustained channel block (24 hours), 1μM verapamil was added to a sub-population of cells 24 hours post-transfection. Luciferase activity was first normalized to renilla for all conditions. Luciferase/renilla level was determined in cells transfected with pGL3-basic and this value was subtracted from luciferase/renilla value in cells transfected with pGL3-Cav1.2.

3.2.8 Real-Time RT-PCR

For Real-Time RT-PCR, RNA was isolated using the RNAqueous-4PCR Kit (Ambion) and genomic DNA was removed using DNase I (Ambion). cDNA was prepared using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen), quantified using the Quant-iT OliGreen ssDNA Assay Kit (Invitrogen), and stored at -20°C. Real-time RT-PCR was performed using 10ng
of cDNA and the TaqMan Gene Expression Assay with primers for CaV1.2, CaV1.3, and CaV3.1 (Applied Biosystems). Samples were cycled and analyzed following Applied Biosystems Gene Expression Assay Protocols.

3.3 Results

3.3.1 CCt Localizes to the Nucleus in Cardiomyocytes

Previous work shows that in neurons CCt localizes to the nucleus via a nuclear retention domain located between amino acids 1821-1910 (rabbit sequence) [35]. Therefore, two constructs were used to test the ability of CCt to localize to the nucleus in cardiomyocytes. The first construct expresses the cleaved full-length portion of CaV1.2 that would be predicted to localize to the nucleus, eGFP1821-2171 [34-35]. The second construct expresses a truncated version of CCt, which lacks the proposed nuclear retention domain, eGFP1906-2171 (kindly provided by Dr. Chunyan Pang). A construct expressing eGFP alone was used as a control. E16 cardiomyocytes were transfected with one of these three constructs and the nuclear normalized to cytosolic eGFP signal intensity was measured (Figure 3.1). E16 cardiomyocytes expressing eGFP alone show a signal in both the nucleus and cytosol. The nuclear intensity is approximately twice that of the cytosol. E16 cardiomyocytes over-expressing eGFP1821-2171 have a nuclear signal intensity that is significantly increased from eGFP alone, suggesting that CCt is able to localize to the nucleus. Surprisingly, eGFP1906-2171, which should lack a nuclear retention signal, localized almost exclusively to the nucleus. These findings provide evidence that CCt localizes to the nucleus in cardiomyocytes and also suggests that regulation of CCt nuclear localization may differ between cardiomyocytes and neurons.

CCt has also been shown to form a complex with cleaved CaV1.2 and act as an autoinhibitory domain that essentially decreases CaV1.2-mediated calcium entry in an HEK over-expression system [34]. However, cardiomyocytes over-expressing eGFP1821-2171 or eGFP1906-2171 exhibited no significant change in spontaneous calcium transient frequency compared to eGFP over-expressing
cardiomyocytes (Figure 3.4A). This raises an interesting conundrum. If CCt acts as an autoinhibitor of Ca\textsubscript{v}1.2, then over-expression of CCt should decrease calcium-entry via Ca\textsubscript{v}1.2 and either decrease the frequency of or completely halt spontaneous calcium transients. This raises the question of whether or not CCt over-expression is altering calcium handling. To address this question, whole-cell calcium imaging was used to test the effect of CCt over-expression on cardiomyocyte calcium handling. Over-expression of eGFP\textsubscript{1821-2171} for 48 hours significantly increased SR load, with a trend toward increased SR leak and increased amplitude of spontaneous transients (Figure 3.4B). There was no significant change in trans-SL diastolic calcium entry. Over-expression of eGFP\textsubscript{1906-2171} had a similar, but more pronounced effect compared to eGFP\textsubscript{1821-2171}. SR load and leak were both significantly increased with eGFP\textsubscript{1906-2171} over-expression, with a trending increase in spontaneous transient amplitude. There was no change in trans-SL diastolic calcium entry. These findings differ from the effects of 48 hour nifedipine in E10 and E16 cardiomyocytes (Figures 2.3 and 2.8). In E10 cardiomyocytes, nifedipine significantly decreased SR load and leak. In E16 cardiomyocytes, nifedipine had no significant effect on SR load and leak. The differences in SR load between the over-expression model and the nifedipine-treated models suggests that CCt may be having an alternative function in cardiomyocytes that is in addition to or independent of regulating Ca\textsubscript{v}1.2 calcium current; CCt may be localizing to the nucleus and acting as a transcription factor in cardiomyocytes.

3.3.2 CCt Nuclear Localization is Regulated by L-type Calcium Channel Block

Over-expressed CCt localizes to the nucleus in cardiomyocytes. To test the hypothesis that CCt nuclear localization is regulated by pharmacological L-type calcium channel block, I first attempted to perform fluorescence after photobleaching (FRAP) experiments. FRAP experiments were utilized by Gomez-Ospina et al. to test the rate of nuclear localization of over-expressed CCt in Neuro2A cells [35]. Briefly, Gomez-Ospina et al. over-expressed Ca\textsubscript{v}1.2 with a carboxyl-terminal YFP tag (Ca\textsubscript{v}1.2-YFP) in Neuro2A cells, photobleached
the nucleus, and then measured the time it took for fluorescent YFP to reappear in the nucleus. From these experiments they concluded that it took approximately 300 seconds for the Ca\textsubscript{V}1.2-YFP to repopulate the nucleus. Purportedly, since Ca\textsubscript{V}1.2 was YFP tagged at the carboxyl-terminus, it was only the cleaved carboxyl-terminal portion of the channel that localized to the nucleus, not the entire Ca\textsubscript{V}1.2 channel. Based on their findings I hypothesized that if L-type calcium channel block regulates trafficking of CCt, then L-type calcium channel block, in comparison to control, should alter the ability of over-expressed CCt to re-localize to the nucleus after photobleaching. To test the feasibility of this experiment, I first over-expressed eGFP or eGFP\textsubscript{1507-2171} (kindly provided by Dr. Chunyan Pang) in E10 cardiomyocytes and performed FRAP. The use of eGFP\textsubscript{1507-2171} was dictated by the fact that it contains the purported nuclear retention domain described by Gomez-Ospina et al., it localizes to the nucleus in cardiomyocytes (Figure 3.2), and that the eGFP\textsubscript{1821-2171} construct was not available at the time. The FRAP experiments revealed that the time course of nuclear localization of eGFP or eGFP\textsubscript{1507-2171} after photobleaching were the same. It took approximately 60 seconds for nuclear fluorescent intensity to reach pre-bleach levels with both constructs. This finding contradicts those by Gomez-Ospina et al., where they reported that YFP alone localized to the nucleus at a faster rate than Ca\textsubscript{V}1.2-YFP. The fact the eGFP and eGFP\textsubscript{1507-2171} localize to the nucleus at the same rate suggests that at least on the order of seconds, the eGFP tag attached to the 1507-2171 piece regulates its nuclear transport.

Preliminary FRAP experiments revealed that on the order of seconds, nuclear localization of eGFP\textsubscript{1507-2171} was regulated by its eGFP tag. However, this finding does not exclude the possibility that nuclear localization of CCt is regulated on a longer time-scale, for example on the order of hours. As shown in Figure 3.1, nuclear localization of over-expressed eGFP\textsubscript{1821-2171} or eGFP\textsubscript{1906-2171} is significantly increased compared to eGFP alone at 24 hours. This suggests that nuclear localization of CCt itself is regulated over a period of 24 hours. If the eGFP tag alone was responsible for CCt nuclear localization, then there would be no significant difference in nuclear localization between
eGFP, eGFP1821-2171, or eGFP1906-2171. In light of this observation, I chose to study the effects of 24 hour L-type calcium channel block on over-expressed CCt nuclear localization in cardiomyocytes. E16 cardiomyocytes over-expressing eGFP, eGFP1821-2171, or eGFP1906-2171 were treated for 24 hours with 10µM nifedipine. Treatment with pharmacological L-type calcium channel block significantly decreased nuclear localization of eGFP1821-2171 (Figure 3.3A), but did not change eGFP localization. This suggests that L-type calcium channel block regulates CCt nuclear localization. However, L-type calcium channel block had no effect on eGFP1906-2171 nuclear localization. To confirm that the effects of nifedipine on eGFP1821-2171 nuclear localization were L-type calcium channel block specific, E16 cardiomyocytes over-expressing eGFP1821-2171 were also treated with 1µM verapamil for 24 hours. L-type calcium channel block by both nifedipine and verapamil significantly decreased nuclear localization of CCt, suggesting that the alteration in eGFP1821-2171 nuclear localization is L-type calcium channel block specific (Figure 3.3B).

Nuclear localization of over-expressed eGFP1821-2171 was altered by L-type calcium channel block. However, this does not necessarily mean that endogenous CCt is altered by L-type calcium channel block in cardiomyocytes. In order to study the effects of calcium channel block on endogenous CCt nuclear localization, a custom antibody against the distal portion of CCt, Ab55, was used. To test the ability of Ab55 to probe for endogenous CCt in individual cells, immunocytochemistry was performed. HEK-293 cells were used as a negative control since they have very little CaV1.2 [109]. For positive controls, full-length eGFPCaV1.2 (green) or eGFP1507-2171 (green) were over-expressed in HEK-293 cells. Ab55 association was visualized using a secondary antibody that excites at 594nm (red). Figure 3.5A shows representative confocal images from HEK-293 cells over-expressing eGFPCaV1.2 (top) or eGFP1507-2171 (bottom). Ab55 interacted with an unknown epitope in HEK-293 cells in addition to recognizing eGFPCaV1.2 over-expressing HEK-293. Additionally, Ab55 did not recognize eGFP1507-2171 in all over-expressing HEK-293 cells. These findings indicate that Ab55 is not suitable for immunocytochemistry. Next, Ab55 was
tested for its ability to recognize CCt via immunoblot by Dr. Schroder (Figure 3.5B). Full-length Cav1.2 or eGFP1821-2171 was over-expressed in tsA-201 cells. Ab55 detected full-length Cav1.2 (~240kDa) and over-expressed CCt (~37kDa). Probing with an eGFP-specific antibody detected a similarly sized band as Ab55 (data not shown). In E16 cardiomyocytes, Ab55 detected a band at ~37kDa that increased in intensity with HA1906-2171 over-expression. A similarly sized band was detected with anti-HA antibody (data not shown). A ~37kDa band was also detected in adult ventricular myocytes with Ab55. Additionally, probing with an antibody against the II-III linker of Cav1.2 shows that E16 and adult ventricular myocytes only express the proteolytically cleaved Cav1.2 (~190kDa) [29-32]. These findings indicate that Ab55 is able to detect endogenous CCt via immunoblot.

To test the effects of L-type calcium channel block on CCt localization in the cardiomyocytes, Ab55 was used to probe western blots of cytosolic and nuclear protein fractions from E16 cardiomyocyte cultures. Cultured E16 cardiomyocytes were treated for 24 hours with 10µM nifedipine, 1µM verapamil, or a calcium free solution containing 0.3mM EGTA (0Ca+0.3mM EGTA). Western blot confirmed that the percent of nuclear CCt probed with Ab55 significantly decreased with nifedipine treatment (Figure 3.6). Verapamil and 0Ca+0.3mMEGTA had similar but non-significant effects. This may have been due to the inherent confounds of this experiment, which is the availability of only a relatively small amount of tissue, ultimately leading to low protein yields. These results provide further support that nuclear localization of CCt is regulated by calcium in developing cardiomyocytes.

3.3.3 Phosphomimetic Mutation of Serine 1928 Significantly Decreases CCt Nuclear Localization

Calcium regulates endogenous and over-expressed CCt nuclear localization in embryonic cardiomyocytes. However, the question remains of the exact mechanism regulating CCt localization. Nucleocytoplasmic transport of transcription factors can be regulated by numerous mechanisms, including
phosphorylation and dephosphorylation [110]. It is established that CCt can be phosphorylated at Serine 1928 by both β-adrenergic receptor activated PKA and α-adrenergic receptor activated PKC [47-48]. Conversely, CCt can also be dephosphorylated at Serine 1928 by protein phosphatase 2A (PP2A) [111]. Therefore I hypothesized that mutation of Serine 1928 would alter over-expressed CCt nuclear localization in embryonic cardiomyocytes. Mutations were made in the eGFP1821-2171 vector. A phosphodeficient mutation was created by mutating Serine 1928 to an alanine (S1928A) and a phosphomimetic mutation was created by mutating Serine 1928 to glutamic acid (S1928E). Nuclear localization of over-expressed eGFP1821-2171, S1928A, and S1928E was compared in E16 cardiomyocytes. S1928E showed a significant decrease in nuclear localization compared to eGFP1821-2171 (Figure 3.7). To confirm that phosphorylation of Serine 1928 decreased nuclear localization of CCt, eGFP1821-2171 over-expressing cardiomyocytes were treated with 1µM isoproterenol for 10 minutes. Treatment with isoproterenol should cause phosphorylation of Serine 1928 via PKA. However, isoproterenol treatment did not significantly alter CCt nuclear localization (data not shown). One possible explanation for this lack of effect is that a majority of over-expressed eGFP1821-2171 is already phosphorylated at Serine 1928; therefore, β-adrenergic stimulation has no large effect on nuclear localization. This may explain why over-expressed CCt is not exclusively localized to the nucleus in untreated cardiomyocytes. A future experiment to test this supposition would be to acutely treat cardiomyocytes over-expressing eGFP1821-2171 with a PKA and/or PKC inhibitor in order to block phosphorylation of Serine 1928. I hypothesize that acute treatment with a PKA or PKC inhibitor would significantly increase eGFP1821-2171 CCt nuclear localization.

3.3.4 CCt Regulates Cav1.2 Expression in Fetal Cardiomyocytes

The previous data suggests that CCt is capable of localizing to the nucleus in cardiomyocytes. However, the question remains of whether or not CCt nuclear localization has functional relevance in the cardiomyocyte. To begin answering this question, I chose to test the hypothesis that, similar to neurons,
CCt functions as a transcription factor in cardiomyocytes. An intriguing, potential transcriptional target of CCt is Ca\(_{\text{v}}\)1.2. Previous work published by Dr. Schroder showed that forty-eight hour treatment of adult mice with the L-type calcium channel blocker verapamil significantly increased Ca\(_{\text{v}}\)1.2 expression [112]. My data provides evidence that in embryonic cardiomyocytes, twenty-four hour pharmacological L-type calcium channel block decreases nuclear localization of CCt. Therefore it is possible that there is a correlation between Ca\(_{\text{v}}\)1.2 expression and CCt nuclear localization, and that, furthermore, CCt regulates Ca\(_{\text{v}}\)1.2 expression. To begin establishing this correlation, I first wanted to confirm that L-type calcium channel block was indeed increasing Ca\(_{\text{v}}\)1.2 expression in the developing heart. E16 cardiomyocytes were treated with 10\(\mu\)M nifedipine for 48 hours. At 48 hours, Ca\(_{\text{v}}\)1.2 protein and Ca\(_{\text{v}}\)1.2 mRNA were both significantly increased (Figure 3.8A,B), confirming that L-type calcium channel block increases Ca\(_{\text{v}}\)1.2 expression in the fetal heart. Next, to begin to determine if CCt is capable of regulation Ca\(_{\text{v}}\)1.2 expression, the effects of CCt over-expression on Ca\(_{\text{v}}\)1.2 mRNA were measured. At 72 hours, over-expression of eGFP1821-2171 significantly decreased Ca\(_{\text{v}}\)1.2 mRNA expression compared to eGFP over-expressing controls (Figure 3.9B). These findings support the postulate that CCt is capable of regulating Ca\(_{\text{v}}\)1.2 message, and furthermore, suggest that CCt may be directly regulating Ca\(_{\text{v}}\)1.2 transcription.

To test the hypothesis that CCt directly regulates Ca\(_{\text{v}}\)1.2 transcription, luciferase assays were first performed to determine if CCt can regulate the Ca\(_{\text{v}}\)1.2 promoter. These experiments were graciously performed by Dr. Schroder. First, Dr. Schroder tested whether or not L-type calcium channel block increased Ca\(_{\text{v}}\)1.2 promoter activity in correlation with the increase in Ca\(_{\text{v}}\)1.2 mRNA and protein that I observed. Figure 3.8C shows that 48 hour treatment with the L-type calcium channel inhibitor verapamil significantly increased Ca\(_{\text{v}}\)1.2 promoter activity in E16 cardiomyocytes compared to vehicle treated control. These findings correlate with the observed increase in Ca\(_{\text{v}}\)1.2 mRNA and protein expression with verapamil treatment (Figures 3.8A,B). Next, Dr. Schroder tested whether or not over-expressed CCt decreased Ca\(_{\text{v}}\)1.2 promoter activity in
correlation with the decrease in CaV1.2 mRNA that I observed. **Figure 3.9A** shows that over-expression of CCT significantly decreased CaV1.2 promoter activity compared to over-expressed eGFP control. These data provided by Dr. Schroder suggest that CCT is capable of regulating the CaV1.2 promoter to repress transcription of CaV1.2; furthermore, Dr. Schroder has published Chromatin immunoprecipitation (ChIP) data showing that CCT is able to bind to the CaV1.2 promoter [29]. Together, these findings provide evidence that, similar to neurons, CCT is capable of acting as a transcription factor in cardiomyocytes.

### 3.4 Discussion

One of the primary findings in this chapter is that over-expressed CCT and endogenous CCT localize to the nucleus in cardiomyocytes, and that this nuclear localization is regulated by L-type calcium channel block. However, there are some differences between these findings and the published work of CCT's role in neurons [35]. First, it was shown in neurons that a nuclear retention signal is located between amino acids 1821-1910. However, in cardiomyocytes, over-expression of eGFP1906-2171, which lacks the purported nuclear retention domain, had more pronounced nuclear localization than eGFP1821-2171 (**Figure 3.1**). One possible explanation for this difference is that Gomez-Ospina et al. performed their experiments to determine the nuclear retention domain in human embryonic kidney (HEK-293) cells. HEK-293 cells have a very small calcium current density and lack the calcium handling properties and spontaneous activity of cardiomyocytes [109]. While their findings are valid in HEK-293 cells, they may not necessarily translate to a cardiomyocyte where calcium regulation is likely overriding a lack of the purported nuclear retention domain. In fact, in cardiomyocytes the region between amino acids 1821-1906 may act as a calcium-regulated nuclear targeting domain. L-type calcium channel block decreased nuclear localization of eGFP1821-2171, but had no effect on eGFP1906-2171. To identify the potential calcium-regulated nuclear targeting domain, constructs would need to be made that successively deleted regions
starting from amino acid 1821. If the domain was identified, it would be interesting to test mutations of the amino acids in that domain to see if those mutations would alter the ability of L-type calcium channel block to regulate eGFP1821-2171 nuclear localization. A second difference between my work and that performed by Gomez-Ospina et al. was the effect that L-type calcium channel block had on CCt nuclear localization. Gomez-Ospina et al. reported an increase in CCt nuclear localization in cortical neurons with L-type calcium channel block. I observed a decrease in CCt nuclear localization with L-type calcium channel block in cardiomyocytes. The experiments performed by Gomez-Ospina et al. utilized immunocytochemistry to look at CCt localization in cortical neurons. In contrast, the experiments in this chapter were initially performed in E10 cardiomyocytes using over-expressed eGFP tagged CCt. A limitation to over-expression experiments is that expression levels cannot be controlled in individual cells. It is possible that eGFP tagged CCt was over-expressed at a level that overwhelmed the ability of individual cardiomyocytes to fully regulate CCt localization. However, population differences were seen in eGFP tagged CCt nuclear localization in response to L-type calcium channel block and zero calcium (Figure 3.2). These findings corresponded to the results obtained via western blot where antibody was used to measure endogenous CCt nuclear localization in E16 cardiomyocytes (Figure 3.4). Therefore, while the findings in embryonic and fetal cardiomyocytes differ to those in cortical neurons, they are robust and suggest that the mechanisms controlling CCt nuclear localization may differ between the two cell types.

Another finding in this chapter that differs from the current literature is that over-expression of CCt in E16 cardiomyocytes does not alter calcium handling in a way that is consistent with L-type calcium channel block. Hulme et al. have shown that an over-expressed distal CCt construct (based on the rabbit sequence from amino acid 1822-2171) associates with and inhibits over-expressed Ca\textsubscript{v}1.2 channels truncated at amino acid 1821 in tsA-201 cells [34]. However, Hulme et al. did not explore the effects of over-expressing distal CCt in cardiomyocytes. There is evidence to support that CCt is associates with the
channel in intact cells [32]. Therefore, it is possible that in E16 cardiomyocytes, a majority of native CCT remains associated with CaV1.2; only a small percentage of native CCT localizes to the nucleus. Therefore, native CCT would already be regulating CaV1.2 channel function in cardiomyocytes, and over-expressed CCT would have little to no additional effect. One future experiment that could be performed to test this supposition would be to over-express CCT in cardiomyocytes and measure $I_{Ca,L}$ to see if it is altered. I predict that over-expression of CCT in cardiomyocytes would not alter $I_{Ca,L}$.

In this chapter, evidence was provided that CCT nuclear localization is regulated by calcium and L-type calcium channel block in cardiomyocytes, and that CCT is capable of regulating transcription, specifically transcription of CaV1.2. Chapter 2 showed that L-type calcium channel block perturbs embryonic morphogenesis. It is possible that L-type calcium channel block mediates its effects on cardiogenesis, in part, by decreasing CCT nuclear localization. Since CCT is capable of acting as a transcription factor, decreasing CCT nuclear localization could potentially alter the expression of genes that are essential for heart development. Based on this assumption, I hypothesized that over-expression of CCT would negate the effect of L-type calcium channel block on heart development. I attempted to transfect hearts with eGFP1821-2171 in order to test this hypothesis. However, I only observed eGFP1821-2171 positive cells in small regions of the heart, and the regions transfected were never identical between hearts. Therefore these experiments would need to be repeated in a model where CCT expression could be regulated throughout the heart. An ideal model would be a mouse that had a cardiac-specific deletion of amino acids of 1822-2171 of CaV1.2. In this model, CaV1.2 would still be expressed, but not the CCT portion of CaV1.2. I hypothesize that this CCT deletion mouse would exhibit perturbed heart development, similar to that seen with L-type calcium channel block. I would also hypothesize that this deletion would be embryonic lethal since mice with disrupted expression of the entire CaV1.2 channel exhibit embryonic lethality[6]. Therefore, I would ideally want to make a mouse with an inducible,
cardiac-specific deletion of CCt so the effects of CCt deletion could be measured throughout development and into adulthood.
Figure 3.1 Nuclear localization of over-expressed CCt in E16 cardiomyocytes. A) Representative confocal images of E16 cardiomyocytes over-expressing CCt. α-actinin (red) positive cells are cardiomyocytes. B) eGFP1821-2171 and eGFP1906-2171 are both nuclear localized when over-expressed in E16 ventricular cardiomyocytes. (t-test performed)
Figure 3.2 eGFP1507-2171 localizes to the nucleus in E16 cardiomyocytes. Representative confocal images of E16 cardiomyocytes over-expressing CCl. Cells with positive α-actinin (red) staining are cardiomyocytes.
Figure 3.3 L-type calcium channel block decreases over-expressed CCT nuclear localization in E16 cardiomyocytes. A) 10μM Nifedipine treatment for 24 hours significantly decreases the nuclear/cytosolic intensity of over-expressed eGFP1821-2171. Nifedipine had no effect on the localization of over-expressed eGFP or eGFP1906-2171. B) Treatment with nifedipine or verapamil for 24 hours significantly decreased the nuclear/cytosolic intensity of eGFP1821-2171. (t-test performed)
Figure 3.4 CCt over-expression does not alter spontaneous calcium transient amplitude or trans-sarcolemmal diastolic calcium entry in E16 cardiomyocytes. A) CCt over-expression had no significant effect on spontaneous calcium transient amplitude, or trans-sarcolemmal calcium entry. CCt over-expression significantly increase sarcoplasmic reticulum calcium load and eGFP1906-2171 over-expression significantly increased sarcoplasmic reticulum calcium leak (*p<0.05; t-test). B) A plot of spontaneous transient frequency and spontaneous transient amplitude indicates no interdependence between the two under all over-expression conditions.
Figure 3.5 Evaluation of Ab55 for immunocytochemistry and immunoblotting. A) Representative images of HEK-293 cells transfected with eGFPCa\textsubscript{v}1.2 and Ca\textsubscript{v}2A (top) or eGFP1507-2171 (bottom) and stained using Ab55 with an AlexaFluor594 (red) secondary antibody. Arrows note cells of interest. (White scale bar = 50µm) B) Representative western blots performed by Dr. Schroder with CCt over-expression in tsa-201 cells and E16 ventricular myocytes, as well as adult ventricular myocytes. Western blots were probed with Ab55 (CCt-specific antibody) and/or II-III Linker Antibody (Ca\textsubscript{v}1.2-specific antibody).
Figure 3.6 L-type calcium channel block significantly decreases endogenous CCt nuclear localization. E16 cardiomyocytes were cultured for 24 hours in the presence of L-type calcium channel block (nifedipine or verapamil) or zero calcium with 0.3mM EGTA. CCt nuclear localization was significantly decrease with nifedipine treatment (*p<0.05; t-test). CCt trended toward a decrease with verapamil or zero calcium treatment.
Figure 3.7 Phosphomimetic mutation of Serine 1928 significantly decreases over-expressed CCt nuclear localization. The nuclear localization of over-expressed eGFP1821-2171 lacking a serine 1928 mutation (1821) was compared to nuclear localization of eGFP1821-2171 containing a phosphodeficient mutation (S1928A) or a phosphomimetic mutation (S1928E) at Serine 1928. (t-test performed)
Figure 3.8 L-type calcium channel block induces compensatory up-regulation of \(\text{Cav1.2}\). A) Western blot was performed on 48H 10\(\mu\)M nifedipine treated E16 cardiomyocytes. \(\text{Cav1.2}\) protein expression was quantified using densitometry and normalized to GAPDH. 10\(\mu\)M nifedipine significantly increased \(\text{Cav1.2}\) protein compared to control. (*p=0.02; n=3 for each condition; experiment performed by Dr. Schroder) B) \(\text{Cav1.2}\) mRNA in E16 hearts treated with 10\(\mu\)M nifedipine. (*p<0.001; n=3 for each condition; t-test) C) Luciferase assay performed by Dr. Schroder shows that 48H 1\(\mu\)M verapamil significantly increased \(\text{Cav1.2}\) promoter reporter activity in E16 cardiomyocytes compared to vehicle. (p=0.03; n=5 for each condition)
Figure 3.9 eGFP1821-2171 decreases Ca\textsubscript{v}1.2 promoter activity and mRNA.

A) E16 ventricular myocytes were co-transfected for 72 hours with the Ca\textsubscript{v}1.2 promoter driving luciferase and either eGFP1821-2171 or eGFP only as a control. The luciferase assay was performed by Dr. Schroder (n=3; p<0.05) B) E16 ventricular myocytes were transfected with eGFP1821-2171. All data is normalized to GAPDH and shown as fold difference from eGFP-only control. (*p<0.05; t-test)
Chapter 4: Regulation of CCt Nuclear Localization in the Adult Heart

4.1 Introduction

L-type calcium channel blockers are used clinically in the treatment of adult hypertension, hypertension associated with preeclampsia in pregnancy, and pediatric hypertension [113-115]. These blockers act on the L-type calcium channels present in smooth muscles of the vasculature, ultimately causing relaxation. L-type calcium channels are also present in the heart, however little is known about the long-term effects of L-type calcium channel blockers on cardiac myocytes. Sustained hypertension is a known cause of pathological cardiac hypertrophy that can ultimately lead to congestive heart failure, coronary heart disease, and stroke [116]. L-type calcium channel blockers are capable of decreasing left ventricular mass in tandem with decreasing blood pressure in patients with hypertension-induced left ventricular hypertrophy [117-119]. However, patients with chronic heart failure due to left ventricular dysfunction exhibited a higher incidence of heart failure deterioration when treated with the L-type calcium channel blocker nifedipine [120]. A meta-analysis of studies where patients with coronary heart disease were treated with L-type calcium channel block showed that mortality rate increased in a nifedipine dose-dependent manner [121]. It is unknown exactly how calcium channel blockers are causing beneficial effects in hypertension induced left ventricular hypertrophy, but deleterious effects in left ventricular dysfunction or coronary heart disease. One possibility is that L-type calcium channel blockers are acting directly on the heart and causing changes in calcium handling and gene expression. Evidence to support this theory was recently shown by our lab. Treatment of mice with 48 hour L-type calcium channel block (verapamil, 3mg/kg/day) induced a significant compensatory up-regulation of CaV1.2 expression and protein levels in the heart [112]. However, our lab was unable to establish the mechanism of this compensatory up-regulation at the time. In Chapter 3, I provided evidence that L-type calcium channel block decreases nuclear localization of the transcription factor CCt in cardiomyocytes. Furthermore, Dr. Schroder provided evidence that CCt acts as a transcriptional repressor of CaV1.2. Therefore, it is plausible that
the observed up-regulation of CaV1.2 expression in the 48 hour verapamil treated mice was due to a decrease in CCT nuclear localization, which relieved repression of the CaV1.2 promoter. Therefore, in this chapter I will test the hypothesis that L-type calcium channel block decreases CCT nuclear localization in adult cardiomyocytes. The possibility that L-type calcium channel blockers regulate nuclear localization of a transcription factor, CCT, in the heart has important clinical implications. It is possible that CCT nuclear localization is differentially regulated in left ventricular hypertrophy and left ventricular dysfunction, explaining the differential effects of L-type calcium channel blockers on clinical outcome.

4.2 Materials and Methods

4.2.1 Adult Ventricular Cardiomyocyte Isolation

Hearts from 8-10 week old female ICR mice were excised and perfused via an aortic cannula with perfusion buffer (113mM NaCl, 4.7mM KCl, 0.6mM KH2PO4, 0.6mM Na2HPO4, 1.2mM MgSO4-7H2O, 12mM NaHCO3, 10mM KHCO3, 10mM HEPES, 30mM taurine, 6mM glucose) also containing 1mM of the contractile inhibitor 2,3-butanedione monoxime (BDM). Constant flow of the perfusion buffer with 1mM BDM was continued until there was no visible blood remaining in the heart. Hearts were next perfused with digestion buffer which consisted of perfusion buffer, 20µM CaCl2, and 10µg/mL Liberase TH (Roche) for approximately 40 minutes. Ventricle was excised and ventricular tissue was teased apart using forceps to promote adult ventricular myocyte dissociation. Digestion was halted placing cardiomyocytes in Stop 1 buffer (perfusion buffer, 12.5µM CaCl2, 1% fetal bovine serum) followed by Stop 2 buffer (perfusion buffer, 12.5µM CaCl2, 5% fetal bovine). Calcium was re-introduced to adult ventricular myocytes over a period of 30 minutes using a calcium ladder that began at 40µM and ended at 1.8mM CaCl2. Adult ventricular myocytes were next transferred to and maintained in Dulbecco’s Minimal Essential Media (DMEM) supplemented with 10% fetal bovine serum (FBS), 100µg/ml penicillin,
100µg/mL streptomycin, and 2µM L-glutamine. Adult ventricular myocytes were paced using field stimulation at 1Hz with 10ms durations of approximately 30 volts.

### 4.2.2 Whole Heart Langendorff Perfusion

Hearts from 8-10 week old female ICR mice were excised and perfused via the aorta with perfusion buffer lacking calcium (113mM NaCl, 4.7mM KCl, 0.6mM KH2PO4, 0.6mM Na2HPO4, 1.2mM MgSO4-7H20, 12mM NaHCO3, 10mM KHCO3, 10mM HEPES, 30mM taurine, 6mM glucose). Hearts were paced at 1Hz in perfusion buffer containing 1.8mM calcium. Verapamil (1µM), isoproterenol (1µM), phenylephrine (20µM), H-89 (1µM), and PD-98059 (20µM) were obtained from Sigma-Aldrich. Bim-I (1µM), Cylosporine A (10µM), and KN-93 (1µM) were obtained from Calbiochem. FK-506 (1µM) was obtained from Tocris. Left ventricular free wall tissue was excised and frozen on dry ice prior to protein isolation.

### 4.2.3 Nuclear and Cytosolic Protein Extraction and Western Blot

Nuclear and cytosolic protein fractions were obtained using the NE-PER kit (Pierce). Each protein sample (60µg) was run out on a 4-15% separating SDS-PAGE gel (Bio-Rad). Immunoblots were performed with Ab55, a custom antibody against amino acids 2051-2064 in the carboxyl-terminus of CaV1.2 (ECM Biosciences, Versailles, KY), Pol II (Santa Cruz Biotechnology), or GAPDH (Ambion). Blots were visualized using horseradish peroxidase-conjugated goat secondary antibodies (Chemicon) and Super Signal West Pico Chemiluminescence (Pierce). An Epson Perfection 1650 was used to scan the immunoblots and bands were quantified using densitometry (ImageJ, available at [http://rsb.info.nih.gov/ij]; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

### 4.2.4 Serum-Induced Hypertrophy and Translocation Assay

E16 cardiomyocytes were isolated as described in Section 2.2.1 and cultured in media containing 10% fetal bovine serum or in media lacking serum.
Translocation assays were performed as described in Section 3.2.3. Cell area was measured using ImageJ.

4.2.5 Osmotic Mini Pumps and Echocardiography

These procedures were performed by Carmen Kiper. Osmotic mini pumps from Alzet (model no. 2002, 2004) were used for delivery of isoproterenol (Sigma, 30mg/kg/day) or vehicle (0.02% ascorbic acid) in 10-12 week old female ICR mice. Pumps were primed overnight and then inserted into the mouse subcutaneously for either two or three weeks. Mice were sedated with intraperitoneal ketamine-xyzaline prior to insertion of the pump. Echocardiography was conducted using a VisualSonics ultrasound machine on female ICR mice prior to pump insertion and at two or three weeks post pump insertion to look for the presence of cardiac hypertrophy. Isofluorane (2ml/min) was used to sedate the animals and their heart rate was monitored while on a heated platform (37°C). Images were taken on the parasternal short-axis of the left ventricle at a rate of 7mHz using a 707 transducer. Measurements were made in M-mode. Hypertrophy was defined as an increase in the heart weight to body weight ratio as well as an increase in left ventricular wall thickness. VisualSonics calculates the fractional shortening and ejection fraction by the following formulas: $FS = (LV_d - LV_s)/LV_d$ and $EF = (LV-Vol_d - LV-Vol_s)/LV-Vol_d$.

4.2.6 Real-Time RT-PCR

These experiments were performed as described in Section 3.2.8.
4.3 Results

4.3.1 Calcium Regulates CCt Nuclear Localization

Isolated adult ventricular myocytes (AVMs) were first used to test the hypothesis that L-type calcium channel block decreases nuclear localization of CCt. The advantage of this model is that it mimics the isolated embryonic ventricular myocytes model utilized in Chapter 3. The effect of pacing was first tested on isolated AVMs. The hypothesis was that paced AVMs would have the same percentage of nuclear-localized CCt as immediately excised whole left ventricle. AVMs were isolated from 4 or 3 individual mice and paced or not paced for 30 minutes or 90 minutes respectively. Figure 4.1 shows that pacing AVMs for 30 minutes (black squares) significantly decreased CCt nuclear-localization compared to freshly excised left ventricular tissue (black diamonds). AVMs from the same hearts that were left quiescent for 30 minutes (grey squares) consistently showed an increase in CCt nuclear localization compared to paced AVMs (dotted lines). This finding suggests that pacing, which should increase cardiomyocyte calcium entry compared to quiescent cardiomyocytes, decreases CCt nuclear localization. However, the results from the 90 minute paced AVMs tell a different story. Similar to 30 minute pacing, AVMs paced for 90 minutes (black triangles) showed a significant decrease in CCt nuclear localization. Conversely, AVMs from two of the 90 minute paced mice that were left quiescent (grey triangles) showed a decrease in CCt nuclear localization that was even greater than the 90 minute paced AVMs (dotted lines). The findings at 90 minutes suggest that pacing increases CCt nuclear localization in 2 out of 3 mice, which contradicts the 30 minute pacing findings. In conclusion, isolated AVMs are an unsatisfactory model for measuring CCt nuclear localization. Culturing of isolated AVMs in and of itself alters CCt nuclear localization, which is exemplified by 90 minute quiescent AVMs exhibiting a significant decrease in CCt nuclear localization compared to excised left ventricular tissue. Further evidence that isolated AVMs are an unsatisfactory model can also be seen in the large data scatter between individual mouse AVM isolations at the 30 minute and 90 minute time points. This large scatter is not observed in excised left ventricular tissue.
from individual mice. This difference in data scatter suggests that the quality of each individual AVM isolation may also affect CCt nuclear localization, further complicating this model system.

Isolated AVMs are not an ideal model system for studying CCt nuclear localization due to inconsistent results between individual isolations and between the times of cultures. Therefore, an alternative model was used to test the effects of L-type calcium channel block on CCt nuclear localization in the adult heart, paced Langendorff-perfused whole mouse hearts. The primary advantage of this model system compared to AVMs is that the heart remains intact. Therefore, cardiomyocytes remain in an environment that is highly similar to in-vivo conditions, with the exception that sympathetic and parasympathetic innervations are removed.

To evaluate whether CCt nuclear localization in the Langendorff-perfused model would mimic CCt nuclear localization in the in-vivo heart, endogenous CCt nuclear localization was compared between Langendorff-perfused left ventricular tissue and left ventricular tissue from hearts that were excised and flash frozen. Langendorff-perfused mouse hearts were paced at 1Hz for 20 minutes in the presence of 1.8mM calcium. Left ventricular tissue was collected and compared to left ventricular tissue from hearts that were excised and immediately flash-frozen. Endogenous CCt nuclear localization was measured via western blot with the CCt antibody, Ab55. Figure 4.2 shows that CCt nuclear localization in the Langendorff-perfusion model mimics endogenous CCt nuclear localization in the excised and flash-frozen heart. Approximately 33% of CCt is nuclear localized under both conditions. This finding suggests that the Langendorff-perfused heart model is an adequate substitute for the in-vivo heart for measuring CCt nuclear localization.

Chapter 3 provided evidence that pharmacological L-type calcium channel block decreases endogenous CCt nuclear localization in embryonic cardiomyocytes. Therefore, I chose to test the hypothesis that L-type calcium channel block also decreases CCt nuclear localization in the adult heart.
Treatment with 1µM verapamil visibly blunted contraction of the heart in the presence of pacing and significantly decreased the percentage of endogenous CCt in the nuclear fraction to 16%. In Chapter 3, L-type calcium channel block as well as removal of extracellular calcium was shown to decrease endogenous CCt nuclear localization in E16 cardiomyocytes. Therefore, the effect on CCt nuclear localization of removing calcium from the perfusate (0Ca) was tested. Perfusion with a 0Ca solution visibly stopped contraction, even in the presence of pacing, and decreased the percentage of CCt nuclear localization to nearly zero. These findings suggest that calcium entry into the cardiomyocyte is required for CCt nuclear localization, and mimic the results from embryonic and fetal heart (Chapter 3).

4.3.2 Pharmacological Inhibition of CCt Phosphorylation Does Not Alter Nuclear Localization in the Adult Paced Heart

Similar to embryonic cardiomyocytes, calcium regulates CCt nuclear localization in the adult heart. Therefore, the hypothesis was tested that similar to over-expressed CCt in embryonic cardiomyocytes (Section 3.5), nuclear localization of CCt in the adult heart would be regulated by Serine 1928 phosphorylation. Specifically, phosphorylation of Serine1928 would significantly decrease CCt nuclear localization in the adult heart. Ideally, these experiments would have initially been done by over-expressing the Serine1928 mutants in the adult heart and evaluating nuclear localization. However, unlike embryonic cardiomyocytes, adult cardiomyocytes cannot be transfected. Therefore, the effects of Serine1928 phosphorylation on endogenous CCt nuclear localization were tested indirectly by pharmacological manipulation. Agonist stimulation of β- and α-adrenergic receptors was used to activate PKA and PKC, respectively, which should cause Serine 1928 phosphorylation. Stimulation of adrenergic receptors is known to alter intracellular calcium; therefore these experiments were performed on a zero calcium background. Adult hearts were perfused for 10 minutes with 0Ca and then stimulated with the β-adrenergic receptor agonist isoproterenol (ISO) or the α-adrenergic receptor agonist phenylephrine (PE) for 10 minutes. Treatment with either agonist significantly increased nuclear
localization of CCt to a level equivalent to what was observed in hearts paced in the presence of calcium. This suggests that activation of PKA and PKC promotes CCt nuclear localization, possibly via Serine 1928 phosphorylation (Figure 4.3).

To test if active PKA and PKC were responsible for nuclear localization in the beating heart, CCt nuclear localization was measured in hearts paced at 1Hz in the presence of calcium with the addition of the PKA inhibitor H-89 or the PKC inhibitor BIM-I. Treatment with either antagonist did not significantly alter CCt nuclear localization compared to the paced heart, suggesting that PKA and PKC may not be involved in regulating CCt nuclear localization in the contracting heart (Figure 4.4). Calmodulin kinase II (CaMKII) is another kinase present in the heart that is known to phosphorylate CCt, and furthermore, is regulated by calcium. Therefore, the effects of the CaMKII inhibitor KN-93 were tested on CCt nuclear localization in the paced heart. Similar to H-89 and Bim-I, treatment with KN-93 did not significantly alter nuclear localization in the paced heart, suggesting that CaMKII does not regulate nuclear localization of CCt in the contracting heart (Figure 4.4).

4.3.3 The Phosphatase Calcineurin May Regulate CCt Nuclear Localization in the Paced Adult Heart

The previous section suggests that the kinases CaMKII, PKA, and PKC do not regulate CCt nuclear localization in the paced heart. However, nucleocytoplasmic transport can also be regulated by dephosphorylation. In Section 3.5 it was shown that a phosphodeficient mutation of Serine 1928 led to decreased localization of over-expressed CCt. Therefore it is possible that dephosphorylation of CCt is a signal for nuclear exclusion in the cardiomyocyte. Calcineurin is known to be activated by calcium and is capable of binding to and dephosphorylating CCt [10, 122]. Therefore, the effects of calcineurin inhibition on CCt nuclear localization were examined. Hearts paced at 1Hz were treated with the calcineurin inhibitor cyclosporine for 10 minutes. Cyclosporine caused a dramatic decrease of CCt nuclear localization to 4% (Figure 4.5), which was not significantly different from 0Ca treatment. To confirm the effect of cyclosporine on CCt nuclear localization, an alternative pharmacological inhibitor of calcineurin,
FK-506, was tested. However, treatment with FK-506 did not significantly decrease CCt nuclear localization compared to paced hearts. Future studies will need to be performed to clarify whether or not calcineurin regulates CCt nuclear localization (discussed in Section 4.5).

Pharmacological stimulation of β-adrenergic or α-adrenergic receptors significantly increased CCt nuclear localization in the absence of calcium (Figure 4.3); it is possible that in this model, adrenergic stimulation regulates CCt nuclear localization via calcineurin. Therefore, the effects of the calcineurin inhibitors cyclosporine and FK-506 were tested on β-adrenergic stimulated hearts in the absence of calcium. Figure 4.6 shows that cyclosporine and FK-506 significantly decreased nuclear localization compared to isoproterenol (ISO) treated hearts in the absence of calcium, suggesting that calcineurin may regulate CCt localization. This raises the question of how adrenergic stimulation is regulating calcineurin and in turn regulating CCt nuclear localization. Previous work provides evidence that both the β-adrenergic and α-adrenergic receptors can stimulate the MAPK pathway leading to activation of ERK1/2 [123-125]; ERK1/2 in turn can activate calcineurin [126]. To determine if ERK1/2 may be involved in adrenergic regulation of CCt nuclear localization, the effect of the pharmacological inhibitor PD-98059 on CCt nuclear localization was tested in paced hearts and hearts treated with ISO in the absence of calcium. PD-98059 is an inhibitor of MEK1/2, which is directly upstream of ERK1/2; PD-98059 has been used extensively in the literature [127-131]. Treatment with PD-98059 decreased nuclear localization of CCt in the paced heart (Figure 4.5) and in the ISO treated heart (Figure 4.6). This finding suggests that active ERK1/2 promotes accumulation of CCt in the nucleus, perhaps via activation of calcineurin. To further test this supposition, the ratio of active (phospho-ERK1/2) to total ERK1/2 was measured via western blot in 0Ca hearts versus ISO treated hearts. The hypothesis was that isoproterenol treatment would significantly increase the ratio of active (phosphorylated) ERK1/2. Surprisingly, the ratio of phospho-ERK1/2 to total ERK1/2 was unchanged with isoproterenol treatment (Figure 4.7). This finding suggests that in this system, β-adrenergic stimulation
may not increase nuclear CCt via a pathway that involves phosphorylation of ERK1/2. However, the opposing results from the PD-98059 studies and the ERK1/2 western blot render these studies inconclusive. One future study that needs to be performed is verification that PD-98059 treatment is indeed significantly decreasing phospho-ERK1/2 under the same conditions where it is shown to decrease nuclear localization of CCt. This could be done by performing a western blot similar to Figure 4.7. I intended to perform this experiment; however, there was not sufficient lysate left over from the CCt nuclear localization experiment to successfully perform the western blot.

4.3.4 CCt Overexpression Attenuates Serum-Induced Cardiomyocyte Hypertrophy

Calcineurin is known to regulate the hypertrophic response in the heart [57, 132]. Pharmacological inhibition of calcineurin attenuates in vitro cardiomyocyte hypertrophy [133], suggesting that an upregulation of calcineurin is an important mediator of cardiomyocyte hypertrophy. In the previous section I provided evidence that pharmacological inhibition of calcineurin decreases nuclear localization of CCt. Therefore, I wanted to test the hypothesis that CCt nuclear localization would be increased in in vitro cardiomyocyte hypertrophy. Dispersed E16 cardiomyocytes were cultured for 24 hours in media containing 10% fetal bovine serum (serum) or media lacking serum (serum free). Serum induced E16 cardiomyocyte hypertrophy. This was evident by serum increasing cardiomyocyte cell area by 25%(+/-5%) and increasing ANF promoter activity. To test the effect of serum-induced hypertrophy on CCt localization, eGFP1821-2171 and eGFP1906-2171 were over-expressed in E16 cardiomyocytes treated with or without serum. Figure 4.8 shows that serum did not significantly alter nuclear localization of eGFP1821-2171 or eGFP1906-2171. However, over-expression of CCt did significantly attenuate serum-induced hypertrophy. As shown in Table 1, eGFP1821-2171 and eGFP1906-2171 significantly decreased cell size compared to eGFP transfected cells treated with serum. Representative images are shown in Figure 4.9. Dr. Schroder showed that over-expression of
CCt significantly decrease ANF promoter activity, providing a plausible mechanism by which CCt attenuates hypertrophy [29].

Serum-induced hypertrophy did not significantly alter nuclear localization of over-expressed CCt. However, this may have been due to the confound of over-expressed CCt attenuating the hypertrophic response. Dr. Schroder performed an experiment comparing endogenous CCt nuclear localization in E16 cardiomyocytes treated for 24 hours. Dr. Schroder’s results indicated that serum-induced hypertrophy significantly increased endogenous CCt nuclear localization [29].

4.3.5 Pathological Hypertrophy Induced by Isoproterenol does not Alter CCt Nuclear Localization

Serum-induced cardiomyocyte hypertrophy significantly increases nuclear localization of endogenous CCt [29]. This finding suggests that CCt nuclear localization may also be altered in whole-heart pathological hypertrophy. Therefore, I wanted to test the hypothesis that CCt nuclear localization is increased in a whole-animal model of pathological cardiac hypertrophy. Isoproterenol-induced cardiac hypertrophy is a well described and well studied model of pathological cardiac hypertrophy [134-136]. Additionally, our lab has previously published work using a model of osmotic pump delivered isoproterenol in mice [112]. Therefore, a mouse model of isoproterenol-induced pathological hypertrophy was utilized to study the effects of pathological hypertrophy on CCt nuclear localization. Osmotic pump implantation and echocardiography were performed by Carmen Kiper. Mice treated for 2 weeks with isoproterenol exhibited a significant increase in heart weight, heart weight to body weight ration, and left ventricular wall thickness, indicating the presence of cardiac hypertrophy. To confirm that hypertrophy was also present at a molecular level, ANF expression levels were measured. I predicted that expression would be increased since ANF is a marker of cardiac hypertrophy. However, 2 week treatment with isoproterenol did not significantly alter ANF expression compared to control (Figure 4.10); this may be explained by the fact that apex tissue was
used for measuring ANF expression, as all left ventricular tissue was used for evaluating CCt localization. Endogenous CCt nuclear localization was measured using left ventricular free wall from isoproterenol treated animals. CCt nuclear localization was not significantly different between isoproterenol treated animals and control (Figure 4.11). In tandem, Ca\textsubscript{v}1.2 expression was also unchanged in isoproterenol treated mice (Figure 4.10).

4.3.6 Physiological Hypertrophy Induced by Pregnancy May Alter CCt Nuclear Localization

In addition to pathological hypertrophy, the heart can also undergo physiological hypertrophy in response to chronic exercise or pregnancy. In pregnant rats, expression of the L-type calcium channel subunit increases throughout the duration of pregnancy and then decreases during parturition [137-138]. In Chapter 3, I provided evidence that CCt acts as a repressor of Ca\textsubscript{v}1.2 transcription. Therefore, I tested the hypothesis that CCt nuclear localization is decreased in a murine model of pregnancy. Left ventricular tissue was isolated from mice at day 18 of pregnancy. Parturition occurs at day 20 of pregnancy. Endogenous CCt nuclear localization was measured via western blot. Mean CCt nuclear localization was not significantly altered in pregnant mice compared to non-pregnant female mice (Figure 4.12). However, there was a large variation in CCt nuclear localization between individual pregnant mice, as evident by the large standard deviation. Physiological hypertrophy in the heart is induced by an increase in workload on the heart. It is possible that different litter sizes produce different amounts of workload on the heart, thereby inducing differing degrees of physiological hypertrophy. Therefore, the hypothesis was tested that litter size directly correlates with CCt nuclear localization in the pregnant mouse heart. Litter size was plotted versus CCt nuclear localization. Figure 4.12B indicates that there is no correlation between litter size and CCt nuclear localization. Upon further analysis of the individual data it was discovered that all of the pregnancy nuclear CCt values fell outside of the range of the mean nuclear CCt (30%+/−4%). Nine of the pregnant mice showed decreased CCt nuclear localization compared to the mean while only five showed increased CCt nuclear localization.
It is possible that the five mice with increased CCt nuclear localization were undergoing premature parturition, where Ca\textsubscript{v}1.2 expression is decreased. A future experiment to test this supposition would be to measure Ca\textsubscript{v}1.2 expression in the pregnant mice. I would predict that the nine mice with decreased CCt nuclear localization would have increased Ca\textsubscript{v}1.2 expression, consistent with pregnancy. In turn, the five mice with increased CCt nuclear localization would have decreased Ca\textsubscript{v}1.2 expression, consistent with parturition.

4.4 Discussion

The primary finding of this chapter is that L-type calcium channel block and perfusion with a calcium-free buffer significantly decrease endogenous CCt nuclear localization in the adult modified Langendorff-perfused heart (Figure 4.2). This is in tandem with the finding in Chapter 3 that L-type calcium channel block and treatment with 0Ca+0.3mM EGTA decrease endogenous CCt nuclear localization in embryonic cardiomyocytes (Figure 3.6). However, there is a significant difference in the time-course that was utilized in the two models studied. In the adult heart, CCt nuclear localization was measured on the order of minutes, while changes in embryonic and fetal cardiomyocyte CCt nuclear localization were observed on the order of days. Therefore, it would be interesting to test both a short and long time course in both model systems. An attempt was made to test changes in nuclear localization of over-expressed eGFP1821-2171 on the order of minutes using FRAP (Section 3.4). However, these experiments were not feasible due to the fact that eGFP and eGFP1821-2171 localized to the photobleached nucleus of E16 cardiomyocytes over the same time course. If a CCt antibody suitable for immunocytochemistry was available, then it would be possible to study the regulation of endogenous CCt nuclear localization in embryonic cardiomyocytes over a short time course. It would also be interesting to study the effects of long-term calcium channel block on CCt nuclear localization in the adult heart. Unfortunately, adult cardiomyocytes cannot be cultured for an extended period. Our lab previously studied a mouse model where verapamil was delivered via an osmotic pump for 48 hours [112]. An interesting future study would be to examine nuclear
localization of CCt in these mouse hearts, especially since these hearts exhibited an increase in Ca\textsubscript{v}1.2 protein and expression. I would hypothesize that 48 hour verapamil treatment would significantly decrease CCt nuclear localization compared to control animals.

In this chapter, the role of PKA and PKC in regulating CCt nuclear localization was measured indirectly by 10 minute application of adrenergic receptor agonists. Pharmacological stimulation of β-adrenergic and α-adrenergic receptors significantly increased CCt nuclear localization in the absence of external calcium and pacing. This finding was opposite of the observed decrease in nuclear localization of over-expressed CCt with a phosphomimetic mutation of S1928 (Figure 3.7). It is possible that in the over-expression model, the excess amount of CCt that is inherently produced is transported in and out of the nucleus by mechanisms other than those that regulate physiological nuclear localization of endogenous CCt. To determine if PKA or PKC phosphorylation regulated endogenous CCt nuclear localization in the paced heart, pharmacological inhibitors were utilized, with the result that inhibition of either kinase did not alter CCt nuclear localization. This finding suggested that β-adrenergic and α-adrenergic receptors regulate CCt nuclear localization via a mechanism that is independent of PKA or PKC phosphorylation. I hypothesized that the β-adrenergic and α-adrenergic receptors regulate CCt nuclear localization via activation of ERK1/2. However, experiments to test this hypothesis were inconclusive. Pharmacological inhibition of β-adrenergic stimulated hearts with PD-98059 significantly decreased CCt nuclear localization, suggesting that ERK1/2 regulates CCt nuclear localization. In contrast, immunoblot with antibodies specific to phosphorylated ERK1/2 indicated that β-adrenergic stimulated did not induce a significant change in the amount of phosphorylated ERK1/2. A follow-up experiment that needs to be performed is to test whether or not PD-98059, at the concentration used, does indeed decrease the amount of phosphorylated (active) ERK1/2. If PD-98059 does not significantly decrease the amount of phosphorylated ERK1/2, then the conclusion can be made that PD-
98059 alters CCt nuclear localization via a mechanism that does not involve ERK1/2.

Another potential regulator of CCt localization studied in this chapter was the phosphatase calcineurin. Unfortunately, these studies did not provide conclusive evidence as to whether or not calcineurin regulates CCt nuclear localization. Treatment with the calcineurin inhibitors cyclosporine and FK-506 significantly decreased CCt nuclear localization in isoproterenol treated hearts on a calcium free background (Figure 4.5). However, only cyclosporine decreased nuclear localization in paced hearts (Figure 4.4). Upon review of the literature, it was discovered that FK-506 can increase SR calcium release at rest and during contraction in rat ventricular myocytes [139]. An increase in cytosolic calcium alone should increase CCt nuclear localization since L-type calcium channel block and 0Ca decrease CCt nuclear localization. However, if calcineurin was the only pathway by which calcium regulates CCt nuclear localization, than the FK-506 co-effect of calcineurin blockades should have decreased nuclear localization despite an increase in calcium. This raises the possibility that calcium regulates CCt nuclear localization via multiple pathways, including calcineurin. It is also possible that calcineurin is not directly involved in CCt nuclear localization and that the pharmacological effects observed were due to unknown non-specific effects on the heart.

In this chapter I tested the hypothesis that hypertrophy increases nuclear localization of CCt. Endogenous CCt nuclear localization was increased in a model of serum-induced hypertrophy in both adult cardiomyocytes and embryonic cardiomyocytes. Over-expression of CCt in embryonic cardiomyocytes attenuated the serum-induced hypertrophic response within 24 hours. A possible mechanism for over-expressed CCt attenuation of hypertrophy is that over-expressed CCt transcriptionally regulates genes involved in the hypertrophic response. Evidence to support this theory is provided by Figure 4.10, which shows that CCt suppresses ANF promoter activity. The theory that CCt transcriptionally regulates genes involved in the hypertrophic response warrants further testing, as it could potentially have important clinical implications.
for treating pathological cardiac hypertrophy. It would be interesting to see if CCt was capable of regulating the promoters of other genes involved in the hypertrophic response, such as the embryonic β-myosin heavy chain or skeletal α-actinin [69]. It would also be interesting to perform a microarray study to compare gene expression profiles of cardiomyocytes treated with serum versus CCt over-expressing cardiomyocytes treated with serum. This may lead to the identification of novel genes regulated by CCt. Another possible mechanism for over-expressed CCt attenuation of hypertrophy is that over-expressed CCt attenuates hypertrophy via its ability to alter sarcoplasmic reticulum calcium load (Figure 3.4). The calcium imaging experiments in Figure 3.4 were all performed in the presence of serum. This warrants future exploration as to the effects of CCt over-expression on calcium handling under serum versus serum free conditions to determine if they are the same.

Serum-induced hypertrophy was shown to significantly increase nuclear localization of CCt in cultured cardiomyocytes. This led to the question of whether CCt nuclear localization would also be altered in an animal model of pathological cardiac hypertrophy. A murine model of isoproterenol-induced cardiac hypertrophy revealed that endogenous CCt nuclear localization was unchanged. While echocardiography revealed structural changes in the heart consistent with hypertrophy, ANF expression from apex tissue was unchanged. Ideally, these experiments would need to be repeated and ANF measured from left ventricular tissue to determine if there was indeed an induction of hypertrophic gene expression in this animal model. However, upon reflection, isoproterenol-induced cardiac hypertrophy may not be the best model for studying the effects of pathological cardiac hypertrophy on nuclear localization of CCt. In Figure 4.3, I provided evidence that acute adrenergic stimulation with isoproterenol increases nuclear localization of CCt in the absence of calcium. It is possible that long-term adrenergic stimulation is adding an extra confound and ultimately masking any hypertrophic-specific effects on CCt nuclear localization. Therefore, instead of pursuing the isoproterenol-induced cardiac hypertrophy model I would like to look at CCt nuclear localization in a non-pharmacologically
induced model of cardiac hypertrophy, such as the aortic-banding model or spontaneous hypertensive rat model [140]. I would also like to measure CCT nuclear localization in cardiac tissue from human subjects exhibiting cardiac hypertrophy to establish the clinical relevancy of CCT.

In this chapter, CCT nuclear localization was also studied in a whole-animal model of physiological cardiac hypertrophy. Pregnancy-induced hypertrophy was used as a model because previous studies have shown that these hearts exhibit an increase in Cav1.2 expression, which would hypothetically correlate with a decrease in CCT nuclear localization. Mean values collected from fourteen pregnant mice showed no significant difference in CCT nuclear localization compared with non-pregnant controls. However, there may have been the complication of premature parturition affecting the data. A future experiment would be to look at pregnant animals from an earlier time-point, such as day 16 of pregnancy, to decrease the possibility of premature parturition. Additionally, Cav1.2 message should be measured along with CCT nuclear localization in order to determine if there is a direct correlation between the two in this animal model.
Table 1 Average Percent Change in Cell Size Compared to Serum eGFP-Transfected Cells.

<table>
<thead>
<tr>
<th>Range</th>
<th>Average Percentage Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1821 to 2171 (n=3; 62)</td>
<td>−28±9%*</td>
</tr>
<tr>
<td>1906 to 2171 (n=3; 93)</td>
<td>−27±5 %*</td>
</tr>
</tbody>
</table>

*P<0.04.
Figure 4.1 Pacing and culturing time affect CCt nuclear localization in isolated adult ventricular myocytes. For each condition, the individual data points are plotted with the mean value +/- the standard deviation to the right. In the excised heart (n=4) the mean percentage of nuclear Ab55 is ~30%. Isolated cardiomyocytes paced for 30 minutes show a significant decrease in the percentage of nuclear Ab55 compared to cardiomyocytes that were left quiescent for 30 minutes (n=4 for each condition; *p<0.05). Isolated cardiomyocytes paced for 90 minutes show no significant change in the percentage of nuclear Ab55 compared to cardiomyocytes that were left quiescent for 90 minutes (n=3 for each condition).
Figure 4.2 L-type calcium channel block and 0Ca decrease endogenous CCt nuclear localization in the adult left ventricle. A) Adult mouse hearts were langendorff-perfused and paced at 1Hz for 20 minutes (n=4) in order to mimic CCt nuclear localization observed in the immediately excised heart. Treatment with verapamil (n=3) or zero calcium (n=3) in the presence of pacing significantly decreased nuclear localization (*p<0.03 vs paced; t-test). B) Representative cytosolic and nuclear western blots from each condition probed with Ab55. GAPDH is primarily in the cytosolic fraction, while PolII is primarily in the nuclear fraction. “Cyt” indicates cytosolic fraction and “Nuc” indicates nuclear fraction. The CCt band, which is ~37kDa is indicated by an arrow.
Figure 4.3 Adrenergic stimulation regulates CCt nuclear localization independent of calcium. Hearts perfused with 0Ca solution for 10 minutes in the absence of pacing showed a significant decrease in nuclear localization compared to hearts paced at 1Hz in the presence of 1.8mM Ca (*p=0.01; t-test). Treatment with 1µM isoproterenol (ISO) or 20µM phenylephrine (PE) in the presence of 0Ca and no pacing restored CCt nuclear localization to levels not significantly different from control. (Note: these treatments are independent of Figure 4.2)
Figure 4.4 Inhibition of CaMKII, PKA, or PKC does not affect CCt nuclear localization in the paced adult heart. Adult mouse hearts were langendorff-perfused and paced at 1Hz for 10 minutes in the absence or presence of pharmacological agent. Treatment with the CaMKII inhibitor KN-93, the PKA inhibitor H-89, or the PKC inhibitor BIM-I did not significantly later CCt nuclear localization. (Note: 1Hz Paced data is from Figure 4.3)
Figure 4.5 Calcineurin and ERK1/2 inhibition decrease CCt nuclear localization in the paced adult heart. Hearts were paced at 1Hz for 10 minutes and treated with the calcineurin inhibitors cyclosporine and FK-506 or the ERK1/2 inhibitor PD98059 for 10 minutes. Cyclosporine and PD98059 decrease CCt nuclear localization while FK-506 had no effect. (*p<0.05; t-test). (Note: 1Hz Paced data is from Figure 4.3)
Figure 4.6 Calcineurin and ERK1/2 inhibition significantly reduce the effect of isoproterenol on CCT nuclear localization. Hearts were perfused with a 0Ca solution for 10 minutes then treated with isoproterenol with or without the addition of a calcineurin inhibitor (cyclosporine or FK-506) or an ERK1/2 inhibitor (PD98059). Treatment with any of the inhibitors significantly decreased CCT nuclear localization compared to isoproterenol treatment alone. (*p<0.05; t-test). (Note: 0Ca, Not Paced and 1µM ISO are from Figure 4.3)
Figure 4.7 Isoproterenol treatment does not Increase the ratio of phospho-ERK1/2. Hearts were perfused in a 0Ca solution for 10 minutes then treated with 1µM isoproterenol for 10 minutes. Western blots were probed with phospho-ERK1/2 and ERK1/2. There was no significant difference in the relative phospho-ERK1/2 integrated density in 0Ca solution versus 0Ca+ISO (n=3 for each condition; p>0.1).
Figure 4.8 Serum treatment does not significantly alter nuclear localization of over-expressed CCT. The ratio of nuclear to cytosolic eGFP intensity was measured in E16 cardiomyocyte cultured in serum (FBS) or cultured in serum free media (SF) for 24 hours. eGFP transfected controls exhibited a significant increase in nuclear/cytosolic intensity that was not mimicked by eGFP1821-2171 (1821) or eGFP1906-2171 (1906) over-expression.
Figure 4.9 Representative confocal images. E16 cardiomyocytes were transfected with eGFP, eGFP1821-2171, or eGFP1906-2171 and cultured in the presence or absence of serum for 24 hours. α-actinin is a cardiomyocyte-specific marker. (white scale bar = 40µM).
Figure 4.10 Expression of Cav1.2 and ANF are not changed in isoproterenol-induced induced pathological cardiac hypertrophy. Real-time RT-PCR was performed on cDNA collected from the apex of 2-week isoproterenol treated animals or saline controls. Data is represented as a fold change in expression from control. There was no significant statistical change in expression of either gene.
Figure 4.11 Isoproterenol-induced pathological cardiac hypertrophy does not significantly alter CCh nuclear localization. A) Isoproterenol was delivered for 2 weeks via osmotic pump at a rate of 3mg/kg/day (performed by Carmen Kiper). Saline was used as a control. The results from western blots performed by myself indicate that there was no statistically significant difference in left ventricular CCh nuclear localization between the two conditions.
Figure 4.12 Pregnancy-induced physiological cardiac hypertrophy does not conclusively alter CCT Nuclear Localization. A) Left ventricular tissue collected from non-pregnant female mice (excised control heart) or female mice pregnant for 18 days (Day 18 Pregnant Heart) showed no significant difference in mean CCT nuclear localization. B) A plot of litter size versus CCT nuclear localization indicates no correlation between the two. However, CCT nuclear localization in all pregnant mice fall outside range of the mean (black line, 30%) +/- standard deviation (dotted line, 4%) nuclear localization in the non-pregnant female mice. See Section 4.3.6 for further explanation.
Chapter 5: Dissertation Summary

The work presented in this dissertation project provides evidence in support of my global hypothesis; CCt localizes to the nucleus in embryonic and adult cardiomyocytes via a calcium-mediated mechanism and regulates transcription.

Chapter 2 established an in-vitro model for studying the effects of pharmacological L-type calcium channel block on cardiac development at E10. Utilizing this model, evidence was provided that cardiogenesis is not perturbed by a general perturbation of intracellular calcium (i.e. removal of extracellular calcium or pharmacological inhibition of sarcoplasmic reticulum calcium release). Instead, cardiogenesis appears to be perturbed by a mechanism that directly involves the L-type calcium channel; pharmacological L-type calcium channel block perturbed embryonic cardiogenesis. An additional observation made was that L-type calcium channel block induced a resistance response in a subset of embryonic ventricular cardiomyocytes. This resistance response consisted of all ventricular cardiomyocytes exhibiting no change in sarcoplasmic reticulum calcium load and a subset of ventricular cardiomyocytes exhibiting spontaneous calcium transients and no change in trans-sarcolemmal diastolic calcium entry in response to acute L-type calcium channel block challenge. The mechanism for this L-type calcium channel block resistance response is unknown. However, this compensatory resistance response does enforce the idea that the L-type calcium channel is important in heart development; the developing heart attempts to compensate for pharmacological L-type calcium channel block.

Interestingly, pharmacological L-type calcium channel block does not visibly perturb the embryonic heart at E16. This is most likely due to the fact that at E16, the heart has completed cardiogenesis and is instead undergoing growth. However, L-type calcium channel block does induce the resistance response observed in E10 cardiomyocytes. The presence of this resistance response again emphasizes the importance of the L-type calcium channel heart.
Chapter 3 examined whether or not CCt is able to localize to the nucleus in embryonic cardiomyocytes. Over-expression of two pieces of the Ca\textsubscript{v}1.2 carboxyl-terminus, one containing (eGFP1821-2171) and one lacking (eGFP1906-2171) a purported nuclear retention domain observed in neurons, revealed that both localized to the nucleus of embryonic cardiomyocytes. In fact, eGFP1906-2171 exhibited greater nuclear localization than eGFP1821-2171; according to findings in the neuronal model eGFP1906-2171 should not have localized to the nucleus. A possible explanation for this contradictory result in cardiomyocytes is that nuclear localization of CCt is regulated by different pathways in neurons and cardiomyocytes. This raised the question of whether or not calcium regulates CCt nuclear localization in cardiomyocytes similar to neurons. Treatment of embryonic cardiomyocytes with pharmacological L-type calcium channel block significantly decreased over-expressed eGFP1821-2171 localization and trended towards decreasing over-expressed eGFP1906-2171. This result was the exact opposite of that observed in neurons; in neurons, pharmacological calcium channel block increased CCt nuclear localization. This finding suggests that CCt nuclear localization is, in fact, regulated differently in cardiomyocytes versus neurons. To further confirm this postulate, endogenous CCt nuclear localization in embryonic cardiomyocytes was also examined via immunoblot. These results mimicked what was observed with the over-expression system.

Chapter 3 also examine whether or not CCt plays a function role in the nucleus. Over-expression of eGFP1821-2171, which as mentioned above is localized to the nucleus, correlated with a decrease in Ca\textsubscript{v}1.2 expression. Conversely, treatment with L-type calcium channel block, which decreases nuclear localization of over-expressed and endogenous CCt, correlated with an increase in Ca\textsubscript{v}1.2 expression. These findings suggested that CCt may be regulating Ca\textsubscript{v}1.2 transcription. A follow-up study examining the ability of over-expressed CCt to regulate the CaV1.2 reporter revealed that CCt represses the Ca\textsubscript{v}1.2 promoter. This experiment was performed by Dr. Schroder after I attempted the experiment numerous times. Dr. Schroder provided further
evidence that CCt regulates the Ca\textsubscript{v}1.2 in published work showing that CCt binds to the Ca\textsubscript{v}1.2 promoter [29].

To further determine the mechanism regulating CCt nuclear localization, I tested the hypothesis that calcium regulates CCt nuclear localization by mediating CCt phosphorylation or dephosphorylation. In Chapter 3, overexpression of Serine1928 mutated CCt constructs in E16 cardiomyocytes suggested that phosphorylation of Serine1928 decreased nuclear localization of CCt. This would suggest that activation of PKA and PKC, two kinases that phosphorylate Serine1928, would increase nuclear localization. This was indeed the finding in Chapter 4; adult hearts treated with \(\alpha\)- and \(\beta\)-adrenergic receptor agonists showed increased CCt nuclear localization in the absence of calcium. However, in the paced adult heart, inhibition of PKA and PKC did not conversely decrease CCt nuclear localization. This suggests that while PKA and PKC may be able to regulate CCt nuclear localization, it is probable that these two kinases are not the primary physiological regulators of CCt nuclear localization in the heart where calcium is present.

An alternative mediator of CCt nuclear localization explored was the protein phosphatase calcineurin. In Chapter 4, pharmacological block of calcineurin with cyclosporine decreased CCt nuclear localization in both paced and adrenergic receptor agonist treated hearts. Furthermore, indirect pharmacological block of the known calcineurin activator ERK1/2 decreased CCt nuclear localization in paced and adrenergic receptor agonist treated hearts. Collectively, these findings suggest that both calcium and adrenergic receptors regulate CCt nuclear localization via a pathway involving ERK1/2 and calcineurin. However, a western blot of active (phosphorylated) ERK1/2 versus total ERK1/2 did not support this posit. While the findings in Chapter 4 support that calcineurin regulates CCt nuclear localization, they are not conclusive as to whether or not ERK1/2 is involved. The components of the pathway linking the L-type calcium channel and adrenergic receptors to calcineurin and CCt nuclear localization still need to be determined.
Another question studied in this dissertation was the physiological relevance of CCt nuclear localization. In Chapters 2 and 3, I showed that pharmacological L-type calcium channel block, specifically, perturbs embryonic cardiogenesis and that pharmacological L-type calcium channel decreases nuclear localization of the transcription factor CCt in embryonic cardiomyocytes. This correlation suggests that L-type calcium channel block perturbs embryonic cardiogenesis by decreasing CCt nuclear localization. However, what is lacking is data showing that CCt in and of itself is capable of regulating embryonic cardiogenesis.

Chapter 4 provided evidence that CCt nuclear localization plays a role in the serum-induced hypertrophic response in embryonic and adult cardiomyocytes. Work published by Dr. Schroder indicates that endogenous CCt nuclear localization is increased in the serum-induced hypertrophic model [29]. When I over-expressed CCt in the serum-induced hypertrophic model, surprisingly, the hypertrophic response was attenuated; cell size and ANF expression decreased. This model suggests that over-expression of CCt may prove to be a future therapeutic tool for cardiac hypertrophy. However, the mechanism by which over-expressed CCt attenuates hypertrophy first needs to be determined.

To further establish a role for CCt in cardiac hypertrophy, CCt nuclear localization was measured in a model of adult, isoproterenol-induced pathological hypertrophy and adult, pregnancy-induced physiological hypertrophy. Unfortunately, these models had confounding problems that made their results inconclusive. In the model of pathological hypertrophy, two week treatment with isoproterenol significantly increased heart weigh, heart weight to body weight ration, and left ventricular wall thickness as indicated by echocardiography performed by Carmen Kiper. These are all hallmarks of hypertrophy. However, measurement of expression of the hypertrophic gene ANF in the apex of these hearts showed that there was no significant change in expression with isoproterenol treatment. These conflicting results suggest that this isoproterenol treatment model was not a clear model of pathological hypertrophy. Therefore
the lack of an effect on CCt nuclear localization that was observed is not conclusive. Future experiments will need to be performed in a pathological hypertrophy model that exhibits both structural and hypertrophic gene expression changes in order to determine if CCt nuclear localization is altered in hypertrophy. The confounding problem in the pregnancy-induced physiological hypertrophy model was the possibility that premature parturition was affecting the data. Therefore, these experiments will need to be repeated in an alternative model, such as an earlier time point of pregnancy when the likelihood of premature parturition is very low. In summary, while there is evidence to support a role for CCt in a model of cellular hypertrophy, it is unclear whether CCt plays a role in an animal model of whole-heart hypertrophy. Future studies will need to be performed to clarify this issue.

Based on the results from this dissertation there are two models that I propose for the role of CCt in the embryonic and adult heart. In the first model, CCt acts as an auto-regulation of CaV1.2 expression in cardiomyocytes (Figure 5.1). Calcium enters via CaV1.2 and promotes nuclear localization of CCt via calcineurin. In the nucleus, CCt acts as a suppressor of the CaV1.2. Therefore, more CaV1.2 channel is not made. However, if calcium entry via CaV1.2 is blocked, CCt is not localized to the nucleus. There is no suppression of the CaV1.2 promoter; therefore more CaV1.2 channel is made. The second model I propose is that CCt regulates genes that are important for both embryonic development and the induction of pathological cardiac hypertrophy (Figure 5.2). In the embryonic heart, developmental cues, which may include calcium entry via CaV1.2, promote nuclear localization of CCt. Nuclear CCt regulates the transcription of genes that are essential for embryonic development. In the adult heart, stimuli of pathological hypertrophy, which may include adrenergic receptor stimulation and CaV1.2 calcium entry, promote nuclear localization of CCt. Nuclear CCt regulates the transcription of genes that are involved in the development of pathological cardiac hypertrophy. Future studies will need to be performed to establish the validity of these two proposed models. These studies should include investigations as to the exact mechanism(s) by which CCt nuclear
localization is regulated and also the specific physiological role(s) that CCt plays in the heart. A mouse model that would be extremely useful for these future studies would be an inducible, transgenic mouse that exclusively expressed CaV1.2 lacking CCt in the heart. In context of my first proposed model (figure 5.1), I hypothesize that this mouse model would display increased expression of CaV1.2 compared to wild-type mice. In context of my second model (Figure 5.2), I hypothesize that this mouse model, when induced during development, would display perturbed cardiogenesis. In the adult heart, I hypothesize that induction of a CCt deletion would diminish the ability of the adult heart to hypertrophy in response to pathological hypertrophic stimuli.

There are two important questions that are raised by this dissertation work that need to be addressed in future studies. First, my dissertation finding suggests that calcium entering through the L-type calcium channel regulates CCt nuclear localization (Figures 5.1 and 5.2). This raises the question of how this calcium, which enters cardiomyocytes on a beat-to-beat basis, can also signal to CCt. This is a question that has been raised by the field, but has yet to be answered [10, 141-142] One hypothesis is that CaV1.2 is located in two separate domains in cardiomyocytes [10]. In one domain, CaV1.2 is in complex with RYR and sarcoplasmic reticulum where it functions solely in excitation-contraction coupling. In the second domain CaV1.2 is not in complex with RYR and is solely involved in signaling, not in excitation-contraction coupling. If there was a CCt antibody available that was suitable for immunocytochemistry, then embryonic and adult cardiomyocytes could be probed for CCt along with CaV1.2 and RYR to see if CCt associates with CaV1.2 in complex with RYR.

The second question this dissertation work raises is how CCt can both be in a complex with CaV1.2, influencing channel function, and localized to the nucleus regulating transcription. One hypothesis is that not all cleaved CCt is associated with channel, leaving a portion of CCt available to be shuttled between the nucleus and cytosol. A second hypothesis is that CCt is transcribed separately from full-length CaV1.2, thereby allowing for production of independent CCt protein that is able to act as a transcription factor in the cardiomyocyte.
begin testing the first hypothesis, co-immunoprecipitation experiments could be performed to see if there is $\text{Ca}_v1.2$ present that is not associated with Cct. For testing of the second hypothesis, a mouse $\text{Ca}_v1.2$ knockout model would be ideal [90]. Using this model, Real-time RT-PCR could be performed to determine if a Cct transcript was present when no $\text{Ca}_v1.2$ is being made.
Figure 5.1 Proposed model of CCt nuclear localization acting as an auto-regulatory feedback mechanism for CaV1.2 transcription and channel production in the cardiomyocyte. Calcium enters via CaV1.2 and promotes nuclear localization of CCt via calcineurin. In the nucleus, CCt acts as a suppressor of the CaV1.2. Therefore, more CaV1.2 channel is not made. However, if calcium entry via CaV1.2 is blocked, CCt is not localized to the nucleus. There is no suppression of the CaV1.2 promoter; therefore more CaV1.2 channel is made.
Figure 5.2 Proposed model of CCT nuclear localization regulating gene expression in response to cardiac developmental cues and stimuli of pathological cardiac hypertrophy.
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Published Paper


Published Abstracts

**Byse M**, Schroder E, Satin J. Abstract 286: Cardiac L-type Calcium Channel (Ca\textsubscript{v}1.2) Activity Modulates Ca\textsubscript{v}1.2 C-terminus Auto-regulatory Transcription. *Circulation*, Aug 2009. Poster.


**Byse M**, Schroder E, Satin J. L-type Calcium Channel (LTCC) Function Contributes to Transcriptional Regulation of the LTCC Revealing a Critical


