AUTOIMMUNE ASSOCIATED CONGENITAL HEART BLOCK: PATHOGENIC CROSS REACTIVITY WITH CARDIAC L-TYPE CALCIUM CHANNELS

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ABBREVIATIONS

AVB	Atrio-ventricular block
AVN	Atrio-ventricular node
Ca	Calcium
CHB	Congenital heart block
EC Coupling	Excitation-Contraction Coupling
ECG	Electrocardiogram
ELISA	Enzyme-Linked Immunosorbant Assay
FITC	Fluorescein isothiocyanate
$I_{Ca\text{-}L}$	L-type Ca current
I.P.	Intraperitoneal
I-V	Current-Voltage
LTCC	L-Type Calcium Channel
mV	Millivolt
Negative IgG	IgG from healthy mothers with healthy children
NTG	Non-Transgenic
Postivie IgG	IgG from mothers with CHB children
SAN	Sinoatrial node
S.C.	Subcutaneous
SLE	Systemic Lupus erythrematosus
SR	Sarcoplasmic reticulum
SSA/Ro	SSA/Ribonucleoprotein
SSB/La	SSA/Ribonucleoprotein
TG	Transgenic
TRITC	Tetramethylrhodamine isocyanate

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INTRODUCTION

ABSTRACT

Congenital heart block (CHB) is an autoimmune disease associated with autoantibodies against ribonucleoproteins SSB/La, and SSA/Ro. Atrioventricular block is the hallmarks of CHB, however sinus bradycardia has been reported in animal models of CHB. Interestingly, knockout of the neuroendocrine Cav1.3 Ca channel results in significant sinus bradycardia and AV block. The hypothesis being tested here is that Cav1.3 Ca channel is a target for maternal autoantibodies and thus plays a role in the development of CHB.

RT-PCR and immunostaining showed expression of Cav1.3 Ca channel in the human fetal heart. IgG containing ant-SSA/Ro –SSB/La autoantibodies from mothers with CHB children but not from mothers with healthy children inhibited Cav1.3 Ca current and cross-reacted with Cav1.3 Ca channel protein. Because Cav1.3 and Cav1.2 Ca channels co-exist in native cardiomyocytes, silencing of the Cav1.2 Ca channel was performed to allow characterization of Cav1.3 Ca channel in cardiac myocytes. The use of a lentiviral vector-based system to deliver siRNA to rat neonatal myocytes (RNM) resulted in ~100% transfection efficiency and 91% silencing of the Cav1.2 gene. Electrophysiological recording using the patch-clamp technique of the silenced myocytes also showed the respective inhibition of the Cav1.2 current which correlated with the biochemical results. GST Fusion proteins of the extracellular regions (S5-S6) of each of the four domains I-IV of Cav1.3 Ca channel protein were prepared and tested for reactivity with sera from mothers with CHB children using enzyme-linked immunosorbent assay (ELISA). Serum samples from 17 of 118 (14.4%) patients who had a child with CHB reacted with the Extra-cellular loop E1 corresponding to Domain I S5-S6 region. Rescue and worsening of electrocardiographic abnormalities was achieved in two immunized transgenic mouse models of CHB (Cav1.2 overexpression and Cav1.3 knockout mice).

The data demonstrate 1) the expression of Cav1.3 Ca channel in human fetal heart; 2) the inhibition of Cav1.3 Ca Channel by maternal IgG, and direct cross-reactivity of with the Cav1.3 protein; 3) the extracellular loop Domain I S5-S6 of Cav1.3 Ca channel is a possible antigenic target of pathological autoantibodies in neonatal lupus; 4) overexpression of cardiac Cav1.2 reduces the electrocardiographic abnormalities seen in the pups of immunized mothers, whereas Cav1.3 knockout exhibited severe conduction abnormalities and increased mortality supporting a central role of L-type Ca channel in pathogenesis of CHB.

INTRODUCTION

II.1 Background

Congenital heart block has emerged as an important model of passively acquired autoimmune disease since its first discovery over 100 years ago (Chameides et al., 1977; McCue et al., 1977). CHB is strongly associated with autoantibodies against the intracellular ribonucleoproteins SSA/Ro (52 and 60 kDa) and SSB/La (48 kDa) (Waltuck and Buyon, 1994). The transplacental passage of these autoantibodies from the mother to the infant circulation during gestation damages the fetal heart resulting in various degrees of AV block and sinus bradycardia (Izmirly et al., 2007; Lee, 1993; Waltuck and Buyon, 1994).

The goal of this study is to extend upon the previous studies in delineating the cross reactivity between L-type Ca channels and maternal autoantibodies (Adler et al., 2005; Boutjdir, 2000; Boutjdir et al., 1997; Boutjdir et al., 1998; Hu et al., 2004; Mazel et al., 1999; Miranda-Carus et al., 1998; Qu et al., 2001; Ramadan et al., 2009; Xiao et al., 2001a; Xiao et al., 2001b). A pathological role of anti-SSA/Ro and –SSB/La autoantibodies and L-type Ca channels is well documented (Boutjdir, 2000; Boutjdir et al., 1998; Hu et al., 2004; Mazel et al., 1999; Qu et al., 2005a; Qu et al., 2001; Xiao et al., 2001a) , further clarification is warranted. Both biochemical and functional data from our lab established that both anti-SSA/Ro and –SSB/La autoantibodies from mothers with CHB children, cross-react with the Cav1.2 and Cav1.3 proteins by Western blot (Qu et al., 2005a; Qu et al., 2001) and functionally inhibit individually expressed Cav1.2 and Cav1.3 Ca channels in tsA201 cells (modified HEK293 cells) (Qu et al., 2005a; Qu et al., 2001). Similarly, using native myocytes, we demonstrated that both anti-SSA/Ro and –SSB/La autoantibodies from mothers with CHB children inhibited the total L-type Ca current (Boutjdir et al., 1998). Complete AV block was also reproduced in Langendorff perfused human fetal hearts

(Boutjdir et al., 1997; Boutjdir et al., 1998; Garcia et al., 1994). Based on the previous work, the working hypothesis proposed is that during gestation, maternal autoantibodies cross the placenta into the fetal circulation, cross react and inhibits the L-type Ca channels which are critical for SA and AV nodal electrogenesis. Chronic exposure of the Ca channels to maternal autoantibodies leads to a decrease in the Ca channel density and eventually cell death. Cell death, per se, could trigger inflammation, fibrosis and calcification of the conduction system.

II.2 Physiology of the heart

Heart physiology will be introduced with an explanation of the conduction system in order to understand the pathology of CHB. The heart is thought of as two entities: the contractile myocardium consisting of the atria and ventricles, and the conduction system consisting of the SA node, AV node, the His bundle and the purkinje fibers that propagate the impulse and coordinates contraction (Figure 1).

The SA node (1) is the hearts pacemaker characterized by spontaneous diastolic depolarization that generates impulses conducted to the AV node (2) via branches that innervate and depolarize the atria. Atrial depolarization causes atrial contraction. Conduction through

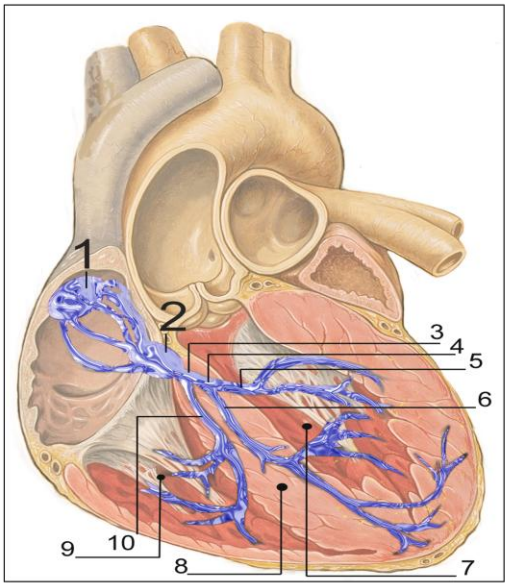

Figure 1: Schematic of the conduction system of the heart. Impulse generated in the SA node (1) stimulate the atria to contract and travel to the AV node (2). After a delay, the stimulus is conducted through the bundle of His to the bundle branches and then to the purkinje fibers and the ventricular triggering contraction. From Wikipedia.com.

the AV node is delayed to allow for complete ventricular filling. Once the impulse passes the AV node, conduction is transmitted rapidly through the ventricular conduction system which consists of the His bundle and the purkinje fibers ensuring coordinated contraction of the ventricle from apex to base.

Development of the conduction system of the heart: The human heart becomes fully developed by 6-8 weeks gestation (James, 1970). By the first trimester, the SA node can be detected and by 10 weeks it acquires its own artery. During the second month of gestation, the three intermodal pathways from the SA to AV nodes appear. The AV node develops from the bundle of His to which it joins to by 8 weeks gestation. The His bundle continues its development postnatally where it undergoes extensive remodeling to achieve its adult form. Around 16 weeks gestation, the fetal conduction system reaches functional maturity. This coincides with the period of heightened transfer of autoantibodies to the fetal circulation and the initial identification of bradycardia (James, 1970; Stiehm, 1975).

An electrocardiogram (ECG) consists of a P wave, a PR interval, a QRS wave, ST segment, T wave, QT interval, and possibly a U wave. The P

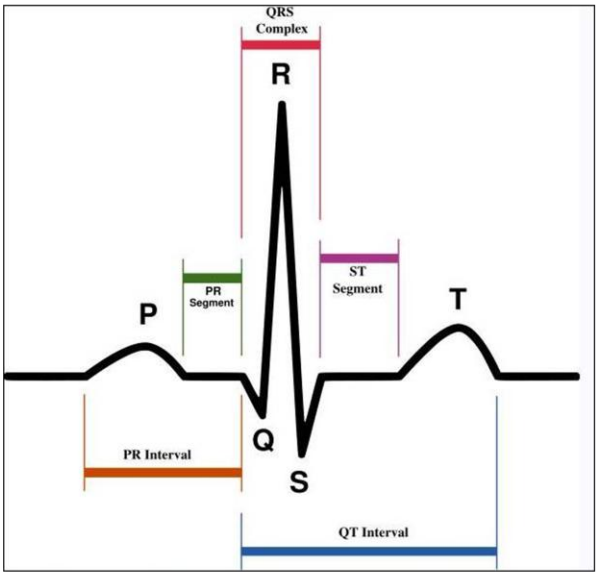

Figure 2: A normal electrocardiogram (ECG) of the heart. An ECG is the best way to measure and diagnose abnormal rhythms of the heart. P wave is atrial depolarization. The PR interval is measured from the beginning of the P wave to the beginning of the QRS complex. The QRS complex corresponds to the depolarization of the ventricles. The T wave represents the repolarization of the ventricles. From Wikipedia.com.

wave represents the atrial depolarization. The QRS complex corresponds to the ventricular depolarization and the T wave (and U wave) to the repolarization. The PR interval is measured from the beginning of the P wave to the beginning of the Q wave (Figure 2).

CHB is a conduction abnormality which affects the SA node as well as the AV node of the fetal heart. Maternal autoantibodies' effects on the SA node are manifested as sinus bradycardia and those on the AV node as different degrees of AV block. AV block is a block or delay in the signal propagation through the heart (John and Fleisher, 2004). First degree AV block is characterized by a prolonged AV conduction time measured by PR interval in an ECG. In first degree AV block the impulse is conducted but it is just delayed. Second degree AV block refers to a failure to conduct some of the atrial impulses to the ventricles. Type I (Wenckeback) second degree AV block is characterized by lengthening of the AV conduction time until conduction is blocked. Type II (Mobitz II) second degree AV block refers to an occasional block without prior lengthening of the PR interval. In third degree (complete) AV block, there are no impulses conducted through the AV node. In the presence of complete AV block, another pacemaker, either at the AV junction, the bundle of His or the ventricle itself stimulates the rest of the ventricles.

It is hypothesized that CHB progresses gradually from a first degree AV block to second and finally to the irreversible third degree AV block (Sonesson et al., 2004). However, this observation still remains to be validated.

II.3 Clinical Manifestations of CHB

Various degrees of AV block as well as bradycardia represent the clinical findings in CHB children. Third degree AV block is irreversible with mortality approaching 30%. Some of the reported CHB deaths are due to heart failure (Anandakumar et al., 1996; Waltuck and Buyon, 1994). Sinus bradycardia unrelated to AV block is also present in a fraction of CHB children. This observation has been shown in animal CHB model (Boutjdir et al., 1997; Mazel et al., 1999; Suzuki et al., 2005; Xiao et al., 2001a), in Langendorff perfused rat heart model (Restivo et al., 2001) and subsequently reported in infants born to mothers carrying the autoantibodies (Brucato et al., 2000; Menon et al., 1998b). Interestingly, despite being exposed to the same autoantibodies, no complete AV block has ever been reported in the mother's heart (Behan et al., 1989; Dorner et al., 1992; O'Neill et al., 1993).

CHB is the most serious manifestation of a syndrome called Neonatal Lupus Erythrematosus (McCauliffe, 1995). The non cardiac manifestations of NLE are skin rash, cytopenias and hepatitis (Buyon, 1998). All manifestations except CHB are transient and resolve at about 6 months with the disappearance of maternal autoantibodies from the neonatal circulation (Laxer et al., 1990; Watson et al., 1984). The transient features reflect the effect of the autoantibodies in organs that have the capacity of continual regeneration.

II.4 Diagnosis

CHB is believed to be gradually progressive; therefore, early diagnosis before progression to third degree AV block is necessary. CHB can be diagnosed by auscultation, sonogram, M-mode echocardiogram (in utero or after birth), electrocardiogram (ECG), and holter monitoring. Fetal

magnetocardiography has also been used to monitor CHB with high precision. Transmaternal fetal electrocardiography is difficult to interpret due to low voltage signals making the P wave inseparable from noise and maternal signals (Andelfinger et al., 2001). Magnetocardiography requires expensive equipment and is not suitable for routine surveillance (Horigome et al., 2000). Ultrasonography is the routine method most widely used to diagnose fetal arrhythmias. Combining the standard fetal echocardiographic mechanical (m-mode Doppler) or hemodynamic (blood flow Doppler) allows detection of first degree AV block (Andelfinger et al., 2001; Glickstein et al., 2000; Sonesson et al., 2004).

II.5 Histology

The pathogenic autoantibodies causing CHB are associated with progressive destruction of the AV node (Jaeggi et al., 2002; Jaeggi et al., 2005). Autopsy revealed fibrosis and calcification of the AV node (Ho et al., 1986; Litsey et al., 1985). Histological studies demonstrated antibodies in cardiac tissue (Lee et al., 1987; Litsey et al., 1985). Deposition of complement, lymphocytic infiltrates, calcification and fibrosis has also been found in fetuses dying from CHB (Clancy et al., 2004; Ho et al., 1986; Lee et al., 1987; Litsey et al., 1985). CHB fetal hearts eluates were found to contain autoantibodies to SSA/Ro 52 and 60 kDa (Reichlin et al., 1994).
The observation of antibody deposition, fibrosis and calcification has been found in the entire myocardium and not restricted to the AV node (Lee et al., 1987; Litsey et al., 1985; Meckler and Kapur, 1998; Piercecchi-Marti et al., 2003). The overall effect these autoantibodies cause include myocarditis and dialated cardiomyopathy (Eronen et al., 2001; Jaeggi et al., 2005; Nield et al., 2002a; Nield et al., 2002b; Taylor-Albert et al., 1997); a feature found in few cases of CHB.

Prolongation of the QT interval has also been described (Cimaz et al., 2000; Gordon et al., 2001).

II.6 Treatment

Current therapies for CHB include in utero administration of steroids (Saleeb et al., 1999), sympathomimetics (Carpenter et al., 1986; Groves et al., 1995), plasmaphoresis (Buyon et al., 1987), intravenous immunoglobulin (IVIG) therapy (Buyon et al., 2001), and cardiac pacing (Carpenter et al., 1986). Maternal steroids are used to prevent the inflammatory damage leading to fibrosis and calcification of the AV node. Betamethazone and dexamethazone are the agents of choice. However, several adverse reactions are reported with steroid therapy including neurodevelopmental impairment, diminished birth weight as well as adverse obstetric complications. A choice is made as far as questioning the positive effects of steroids on CHB compared to the negative effects on the fetus. Once complete AV block has developed, maternal administrations of steroids are not useful since complete AV block is irreversible (Breur et al., 2004; Saleeb et al., 1999; Shinohara et al., 1999). Sympathomimetics (β-adrenergic agonists) are effective in the therapy of bradycardia (Jaeggi et al., 2004). IVIG and plasmaphoresis, to date, offer limited success (Arroyave et al., 1995; Buyon et al., 1987; Herreman et al., 1985; Kaaja and Julkunen, 2003; Tsai et al., 2001; Wong et al., 2001). The only effective treatment is cardiac pacemaker implantation (Buyon and Clancy, 2003). Despite adequate pacing, about 10% develop cardiomyopathies. Unfortunately, none have significantly altered mortality justifying the need for more aggressive support for both basic and clinical investigation.

II.7 Incidence

Because of the rarity and complex etiology of CHB, the incidence is not well established. A generally accepted mean incidence is 1:17,000 in the 1970s (Michaelsson and Engle, 1972) and 1:11,000 in the latter decade (Buyon et al., 1998; Hamilton RM, Neonatal Heart Disease). However, this incidence dramatically increases to about 5% in Lupus patients and to 18% in subsequent pregnancies. This indicates that the incidence of CHB in the latter decades (Buyon et al., 1998; Siren et al., 1998) was higher than previously reported likely due to more effective detection of CHB during pregnancy using fetal ultrasound and to the improved diagnostics. Recurrence is defined as any child with CHB whose mother harbors the anti-SSA/Ro and/or anti-SSB/La and an immediately preceeding pregnancy that resulted in a child with CHB. According to the National Registry for Neonatal Lupus, the recurrence rate is 18%, (Brucato et al., 1995; Buyon et al., 1998; Eronen et al., 2000) supporting the need for close echocardiographic monitoring in all subsequent pregnancies with heightened surveillance between 18 and 24 weeks of gestation. Despite the persisting antibodies, such a recurrence level indicates that the antibodies are necessary but other fetal factors are involved in the susceptibility to CHB.

II.8 Mortality and Morbidity

The National Registry for Neonatal Lupus documented a mortality rate of 20% in CHB children of mothers seropositive for anti-SSA/Ro and anti-SSB/La.The survival is proportional to the gestational age at birth; as such, the children born at a later gestational age had a lower mortality. Infants who survive the neonatal period have an excellent prognosis. In fact, 100% of affected children require lifelong pacemakers before entering adulthood (Buyon et al., 1998; Michaelsson et al., 1997). Most deaths reported thereafter seem to be related to heart failure (Guereta et al.,

1997). Therefore, most deaths in CHB occur in utero or in the first 3 months of life leading to a mortality of 20%, justifying the need for additional research in hope of better understanding this fatal disease.

II.9 Vulnerability Window

The initial detection of CHB is the first prenatal record of bradyarrhythmia. It was noted that the timing of AV block is not random but was clustered between 20-24 weeks gestation (Buyon et al., 1998). In fact, 80 % of the CHB cases were detected before 30 weeks gestation and only 18 % thereafter (Figure 3). The vulnerability period coincided with heightened transplacental passage of autoantibocdies to the fetus. Fetal concentration of total IgG are barely detectable in the first trimester (<100mg/dl) and remain low until 17 weeks when they increase to 400 mg/dl by week 24 and to 800 mg/dl by week 32 gestation (Stiehm, 1975).

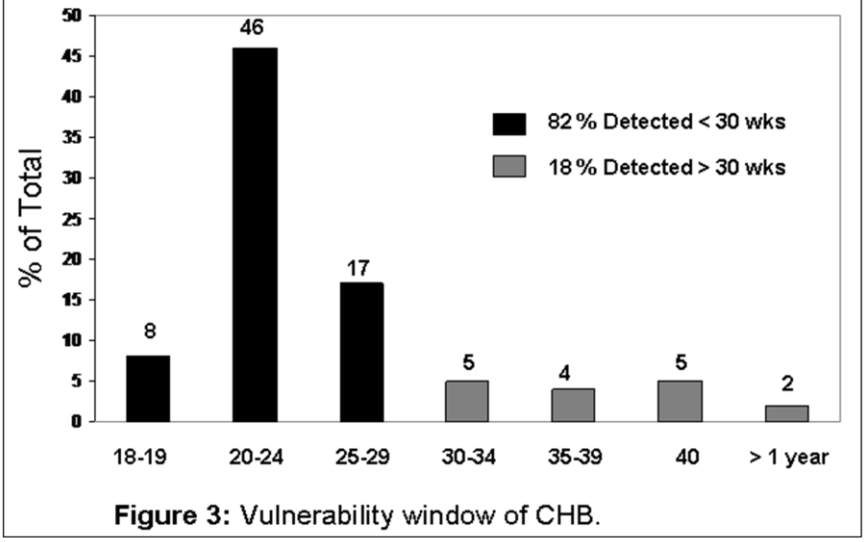

Figure 3: Vulnerability window of CHB.

Figure 3: Vulnerability window of CHB. The timing of AV block is not random but is clustered between 20-24 weeks gestation. Eighty % of the CHB cases were detected before 30 weeks gestation and only 18 % thereafter. (Buyon et al., 1998).

II.10 Autoimmunity

An important attribute of the immune system is the ability to distinguish self from nonself. This "self/nonself recognition" allows the body to defend itself against pathogenic microorganisms and at the same time not attack its own body; a concept of equal significance. When the immune system fails, it will generate an immune reaction to self resulting in an autoimmune disease.

II.11 SSA/Ro 52 kDa Autoantigen

The SSA/Ro autoantigen 52 kDa is a member of the tripartite motif (TRIM) proteins also known as the RING/N-box/Coiled-coil family (Borden, 1998; Reymond et al., 2001).

It consists of a RING finger, a B-box motif, a coiled coil region (Leucine zipper motif) and also has B30.2 (also known as SPRY) domain at the carboxyl terminal (Figure 4). The TRIM family is involved in a wide range of processes including apoptosis and cell cycle regulation.

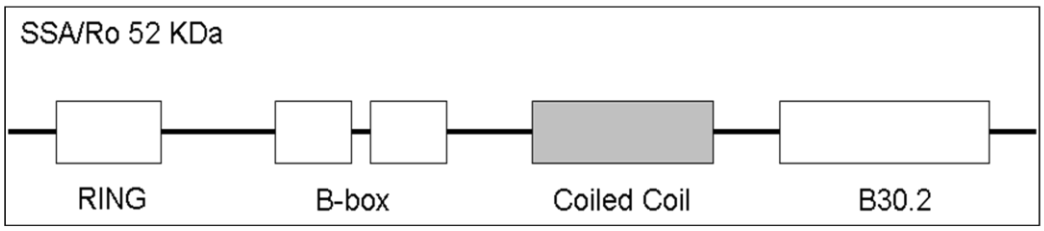

Figure 4: SSA/Ro 52 kDa structure. The SSA/Ro autoantigen 52 kDa is a member of the tripartite motif (TRIM) proteins. It consists of a RING finger, a B-box motif, a coiled coil region (Leucine zipper motif) and also has B30.2 (also known as SPRY) domain at the carboxyl terminal. (Borden, 1998; Reymond et al., 2001).

It was thought that the SSA/Ro 52 kDa may be involved in regulation of gene expression since it contained the RING finger (Zinc finger) and the leucine zipper, which are motifs frequently found in proteins involved in gene regulation. However, recently it has been shown that RING finger proteins act as E3 ubiquitin ligases and participate in the proteosome mediated proteolysis

(Jackson et al., 2000; Liu, 2004). Later on, SSA/Ro 52 kDa was specifically shown to be associated with ubiquitination and function as a E3 ubiquitin ligase (Linares and Scheffner, 2003; Trockenbacher et al., 2001; Urano et al., 2002). Human SSA/Ro 52 kDa is 475 amino acids long and is more than 70% homologous to its murine counterpart. The homology becomes even higher in the functional domains described (Keech et al., 1996). An alternatively spliced SSA/Ro 52 kDa mRNA was found to be expressed in the fetal heart. The mRNA transcript encodes a smaller protein refered to as 52β (45 kDa) that is maximally expressed at 14-16 weeks gestation which coincides with the time of development of CHB (Chan et al., 1995). The function of the spliced SSA/Ro 52 kDa is not yet known.

II.12 SSA/Ro 60 kDa Autoantigen

SSA/Ro 60 kDa contains a zinc finger and an RNA binding motif, both could account for its direct interaction with small noncoding RNAs of unknown function called Y RNAs (cytoplasmic RNAs) (Ben-Chetrit et al., 1989; Lerner et al., 1981). The SSB/La protein has also been found in a fraction of the SSA/Ro 60/Y RNA complexes but studies failed to demonstrate any association with the SSA/Ro 52 (Boire et al., 1995; Kelekar et al., 1994). SSA/Ro 60 has been found to bind misfolded noncoding RNAs that are eventually degraded. Therefore, the function of this protein is in the quality control of imperfect noncoding RNA and regulates the degredation of the 5S rRNA precursors (O'Brien and Wolin, 1994).

II.13 SSB/La 48 kDa Autoantigen

SSB/La does not share antigenic determinants and is not homologous to the SSA/Ro proteins (Chambers et al., 1988; Chan et al., 1990). It facilitates the maturation of RNA polymerase III

transcripts, binds to small RNAs and correctly folds them suggesting an RNA chaperone, and associates with the SSA/Ro 60/ Y RNA complex (Gottlieb and Steitz, 1989; Wolin and Cedervall, 2002).

There has been much debate over the relationship of these autoantigens since most often the antibodies to these antigens are found together. It has been shown that mice immunized with one antigen leads to epitope spreading and production of antibodies to the other 2 antigens which hinders the ability to attribute which antibody is responsible for the etiology of CHB (Topfer et al., 1995).

II.14 Maternal antibody profile

Not all mothers with anti-SSA/Ro and anti-SSB/La antibodies give birth to children with CHB which raises the question whether profiling the mother antibody status can predict pregnancies complicated with CHB. A study addressing the antibody fine specificity in the sera of mothers with CHB children showed that the SSA/Ro autoantibodies were found in more than 80% of the mothers while the anti-SSB/La antibodies were detected in more than 60% (Buyon et al., 2009; Friedman et al., 2007). Anti-SSA/Ro 52 was significantly higher in mothers with CHB children compared to mothers with healthy children. Most recent studies show that SSA/Ro 52 antibodies seem to be more associated with CHB than SSA/Ro 60 or SSB/La (Julkunen et al., 2004; Salomonsson et al., 2002), however the presence of the SSB/La antibodies has been suggested to increase the risk of CHB (Gordon et al., 2004). An antibody to the amino acids 200-239 (p200) of the SSA/Ro 52 has been associated with CHB; however, the target of this p200 antibody is still unclear (Salomonsson et al., 2005; Strandberg et al., 2008).

II.15 Proposed hypothesis of CHB

The association between the anti-SSA/Ro and anti-SSB/La autoantibodies and CHB is universally accepted. However, the mechanism by which the antibodies initiate the pathological cascade is not clear. Two plausible explanations can be considered: either the intracellular antigens SSA/Ro -SSB/La are trafficked to the cell surface or anti-SSA/Ro -SSB/La autoantibodies cross react with a surface antigen on the sarcolemma of cardiomyocytes. A considerable amount of evidence has been proposed to explain the accessibility of the intracellular antigens and circulating antibodies. These include viral infection (Baboonian et al., 1989), ultraviolet light exposure (LeFeber et al., 1984) and apoptosis (Clancy et al., 2006) which all lead to the translocation of SSA/Ro -SSB/La to the cell surface. The second possibility is that maternal antibodies react with cell surface components. To date, sarcolemmal proteins have been identified including the muscarinic receptor (Bacman et al., 1994), laminin (Horsfall et al., 1996), the serotonin receptor 5-HT_4 (Eftekhari et al., 2000), and the L-type Ca channels (Boutjdir et al., 1997; Boutjdir et al., 1998; Garcia et al., 1994; Qu et al., 2005a).

Based on previous work from our group, it is proposed that L-type Ca channels are direct targets for maternal anti-SSA/Ro –SSB/La autoantibodies. Initially, Garcia et al., showed AV block in rabbit hearts perfused with IgG from mothers with CHB children and showed a reduction in $I_{Ca\text{-}L}$ of isolated rabbit ventricular myocytes (Garcia et al., 1994). Our group confirmed and extended upon this observation. Specifically, we reproduced the complete AV block in Langendorf perfused human fetal hearts and correlated the findings to the inhibition of $I_{Ca\text{-}L}$ which is responsible for electrogenesis at the AV and SA nodes. This implies interaction between the anti-SSA/Ro -SSB/La and Ca channels resulting in perturbation of Ca influx with subsequent development of CHB.

II.16 Voltage Gated Calcium Channels

An increase in intracellular Ca triggers a wide variety of physiological processes ranging from muscle contraction, secretion, neurotransmission, chemotaxis and gene expression. Although there are many channels involved in controlling intracellular Ca levels, voltage gated Ca channels (VGCC) play a key role in the process. Calcium channels were first purified and biochemically characterized from the transverse tubule membranes of skeletal muscles. The channels are complex proteins composed of several subunits encoded by multiple genes. The α1 subunit is the largest subunit (190-250 kDa) and is organized into 4 homologous domains I-IV, each with 6 transmembrane segments (S1-S6). It includes the ion conducting pore, the voltage sensor, as well as all known sites of channel regulation by second messengers, drugs and toxins. The α1 subunit is associated with the $\alpha_2\delta$, a transmembrane disulfide linked subunit, and the β subunit which is attached to the cytosolic surface of the α1 subunit. The β subunit is involved in the cell surface trafficking of the channel making its expression a necessity, however it is still controversial whether the other auxiliary subunits are needed.

The α1 subunit is encoded by 10 distinct genes. They are classified according to activation threshold into high-voltage activated (HVA) and low-voltage activated (LVA). The HVA are divided into L-type (Cav1.1, Cav1.2, Cav1.3, and Cav1.4) and non L-type (Cav2.1, Cav2.2, and Cav2.3). The LVA or the T-type Ca channels include Cav3.1, Cav3.2, and Cav3.3. This thesis is involved in elucidating the role of L-type Ca channels in NLE, therefore, a more detailed background will be given. The L-type Ca channel family includes four members designated as Cav1.1 (α1S), Cav1.2 (α1C), Cav1.3 (α1D), and Cav1.4 (α1F). These four Cav1 subunits have distinct distributions. Cav1.1 is exclusively expressed in skeletal muscles and functions in excitation contraction coupling. The Cav1.2 Ca channel, the most widely distributed, is found in

the heart, smooth muscles, brain and pancreas. The Cav1.3 Ca channel was originally thought of as purely of neuroendocrine origin until the generation of the Cav1.3 knockout mice that revealed expression in the heart mainly in the conduction system. Inactivation of the channel resulted in deafness and cardiac rhythm abnormalities. The Cav1.4 gene is the only calcium channel located on the X-chromosome. This channel is exclusively expressed in the retina.

From the L-type Ca channels discussed, the only L-type Ca Channels expressed in the heart are: Cav1.2 (α_{1C}) and Cav1.3 (α_{1D}). Cav1.2 or α_{1C} is ubiquitously expressed in the heart and mediates cardiac excitation-contraction coupling. Cav1.3 or α_{1D} on the other hand is restricted to the supraventricular tissue in the adult with the highest expression in the SAN and AVN (Figure 13). Cav1.2 I_{Ca-L} activates at more positive (–40 and –30 mV) potentials, and accounts for the conduction electrogenesis at the AVN, whereas Cav1.3 I_{Ca-L} activation occurs between –60 and –40 mV at a range in which diastolic depolarization at the SAN operates (Koschak et al., 2001). Thus, Cav1.3 I_{Ca-L} seems to be essential for normal cardiac pacemaker activity. In fact, Cav1.3 -/- phenotype is sinus bradycardia and AVBs similar to what is noted in CHB (Mangoni et al., 2003). It is therefore logical that Ca channel blockade at the SA and AV nodes by maternal anti-SSA/Ro –SSb/La antibodies will be expected to interrupt the sinus rhythm and conduction of the impulse to the ventricles (Figure 5).

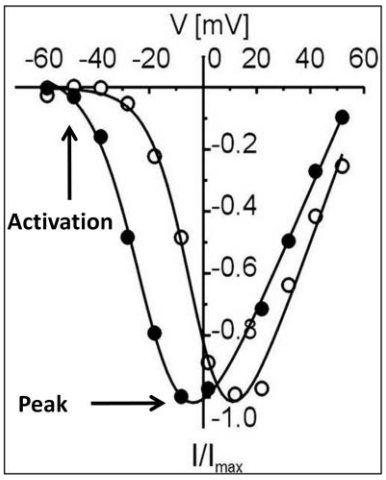

Figure 5: Current-Voltage relationship comparing Cav1.2 and Cav1.3 Ca channels. Cav1.2 I_{Ca-L} activates at more positive (–40 and –30 mV, unfilled circles) potentials whereas Cav1.3 I_{Ca-L} activation occurs between –60 and –40 mV (Filled black circles). (Koschak et al., 2001).

II.17 Previous related work

II.17.1 Experimental models of congenital heart block

There are a number of in vivo and ex vivo models developed to understand the mechanism of pathogenesis in CHB. The ex vivo studies reproduce CHB by adding the anti-SSA/Ro –SSB/La autoantibodies to langendorff perfused hearts. The in vivo models are divided into passive or active immunization of female mice, rats or rabbits. This section will describe each model used with an overview of the data and their success in inducing CHB.

Passive model of CHB

A passive model of CHB was developed in BALB/c mice. Timed pregnancies were injected with anti-SSA/Ro –SSB/La autoantibodies purifed from human sera from mothers with CHB children. ECG screening of the pups from the injected mothers showed sinus bradycardia and AV block as compared to controls. Affinity purified IgG from 2 mothers with CHB children induced AV block in 88% (14/16), 90% (9/10) and 47% (14/30) in pups injected at 8, 11 or 16 days gestation, respectively (Mazel et al., 1999). Bradycardia was also present in 44% (7/16), 70% (7/10) and 33% (10/30) of pups injected at 8, 11, or 16 days gestation (Mazel et al., 1999).

Active model of CHB

An active model of CHB was created by immunizing female mice with human or murine SSA/Ro 52, SSA/Ro 60 or SSB/La 48 antigens. Results from various studies are shown below in Table 1. A high incidence of advanced degrees of AV block (II and III) was observed with the immunization using SSA/Ro 52. The average of the incidence of I degree AV block in these models was around 21% which is similar to the incidence reported in humans. However the

incidence of III degree AV block was 2.5% indicating that advanced degrees of AV block are uncommon in animal models.

Using a peptide from the SSA/Ro 52 spanning amino acides 200-238 refered to as p200, I degree AV block was induced in 19% (10/52) rat pups (Salomonsson et al., 2005). Eftekhari et al. proposed that the autoantibodies cross react with the 5-HT4 receptor. The group demonstrated that pups immunized with 5-HT4 peptides displayed bradycardia and AV block (Eftekhari et al., 2000). Immunization with SSA/Ro 52β induced AV block in 12% (10/86) in which 5/86 pups had complete III AV block (Miranda-Carus et al., 1998).

SSA/Ro 60 and SSB/La 48 induce I degree AV block in 17% (16/97) for SSA/Ro 60 and 7% (4/55) for SSB/La (averaged total from 2 studies Miranda-Carus 1998 and Suzuki 2005). As suggested from human studies, SSA/Ro 60 and SSB/La 48 seem to be less associated with CHB induction (Julkunen et al., 2004; Salomonsson et al., 2002).

The results provide strong evidence for the strong pathogenic role of anti-SSA/Ro –SSB/La autoantibodies, particularly anti-SSA/Ro 52 autoantibodies in the development of CHB.

Table 1: Comparison of AV block induced in animal models. Ro52 appears to be the most associated with AV block induction and higher degrees of AV block as compared to the other antigens.

Antigen	Species (Strain)	Pups	AVB %	I° AVB	II° AVB	III° AVB	Reference
Ro 52	Mouse (BALB/c)	20	25	3	0	2	Boutjdir 1997
Ro52α	Mouse (BALB/c)	56	9	3	1	1	
Ro52β		86	12	5	0	5	
Ro 60		54	19	10	0	0	Miranda-Carus 1998
La 48		27	7	2	0	0	
Mouse Ro 52		22	9	2	0	0	
G21V (5-HT4 Peptide)	Mouse (BALB/c)	29	3	2	1	0	
C15Q (5-HT4 Peptide)		24	3	0	3	0	Eftekhari 2001
R18L (5-HT4 Peptide)		20	0	0	0	0	
C15T (5-HT4 Peptide)		19	0	0	0	0	
Ro52	Rabbit (New Zealand)	121	12	13	1	0	Xiao 2001
Ro52 (p200 Peptide)	Rat	52	19	10	0	0	Salomonsson 2005
Ro60	Mouse (C3H/HEJ)	43	14	6	0	0	
La		28	7	2	0	0	Suzuki 2005
Calreticulin		6	33	2	0	0	

Anti-SSA/Ro –SSB/La induce sinus bradycardia and AV block in Langendorff perfused whole hearts

The application of IgG from mothers of CHB children resulted in sinus bradycardia and 2:1 AV block which was followed by complete AV block (Figure 6). The same IgG resulted in complete inhibition of action potentials recorded from isolated AV nodal cells (Figure 7). The sinus bradycardia and AV block was similarly demonstrated in langendorff perfused human hearts (Boutjdir et al., 1997). The same effects of sinus bradycardia and AV block were seen in

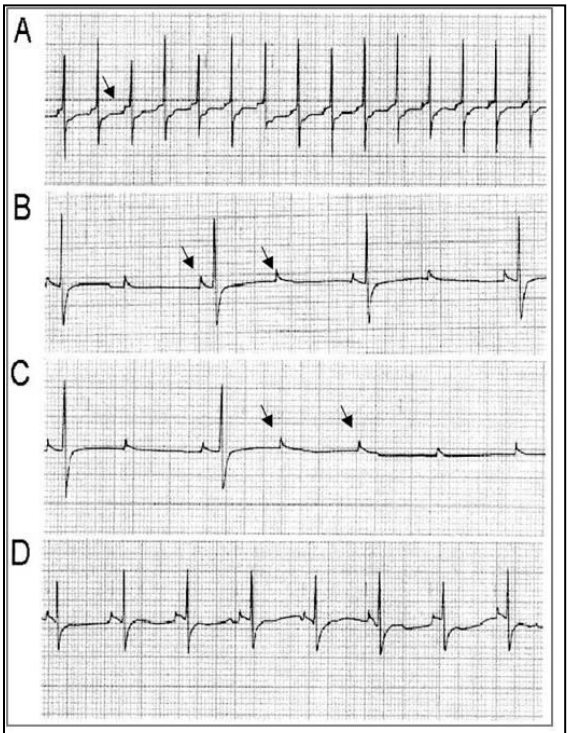

Figure 6. Effects of maternal antibodies on ECG recordings of isolated rat heart perfused by the Langendorff technique. ECG was recorded by the conventional ECG machine in lead I (A) Regular sinus rhythm (horizontal scale, 50 mm/s and vertical scale: 5 mm/mV) at 300 beats/min in Tyrode's solution. After 5 min of perfusion with positive IgG(800 µg/mL), there was bradycardia associated with a 2:1 second degree AV block (B) that degenerated into complete AV block by 15 min of IgG perfusion (C). Return to Tyrode's solution, resulted in only partial recovery (Fig. 1D).

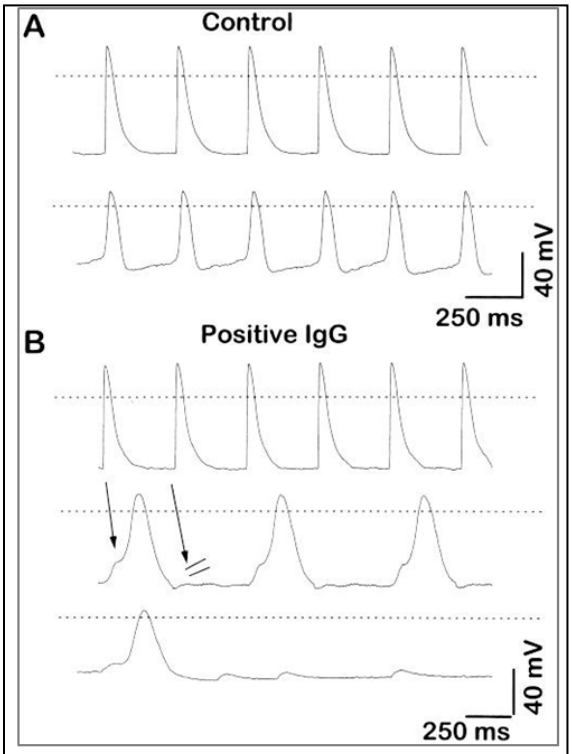

Figure 7. Effects of maternal antibodies on an isolated multicellular AV nodal preparation. (A) Simultaneous control action potentials from the crista terminalis (upper tracing) and the AV node area (lower tracing). (B) Superfusion of the preparation with positive IgG (800 µg/mL) for 10 min resulted in 2:1 AV block (indicated by the arrows) which progressed to near complete inhibition of the AV node action potential by 15 min (B, bottom). Note the time scale difference between (A) and (B).

different degrees (Garcia et al., 1994; Hamilton et al., 1998; Restivo et al., 2001; Viana et al., 1998).

Anti-SSA/Ro –SSB/La specifically inhibit L-type Ca current (I_{Ca-L})

The effect of maternal IgG on the L-type Ca current was tested in ventricular cardiomyocytes, SA and AV nodal cells. Maternal IgG inhibited the I_{Ca-L} by 62%, 46.2% and 51% in ventricular myocytes, SA and AV nodal cells respectively. The maternal IgG had no effect on the transient outward K current (Ito), the inward rectifier K current (I_{k1}) and the fast Na current (I_{Na}) (data not shown). Similarly, negative IgG had no significant effect on the L-type Ca current.

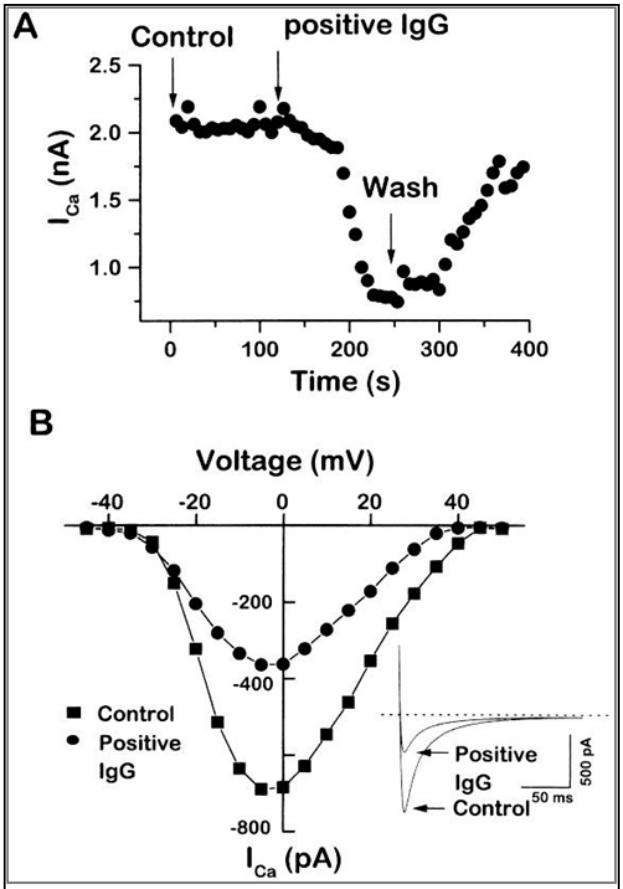

Figure 8. Effects of maternal antibodies (IgG) on L-type ICa recorded from rat ventricular myocytes. (A) The time course of ICa inhibition by positive IgG (80µg/mL). IgG reduced ICa from 2057 to 765 pA (at 0 mV). Washout was only partial. (B) The current-voltage relations during control and after cell superfusion with positive IgG. Current-voltage relations of peak ICa were recorded every 8 s by a series of depolarizing pulses to -50 through 60 mV with 10-mV increments. IgG decreased ICa and scaled down the current-voltage relations.

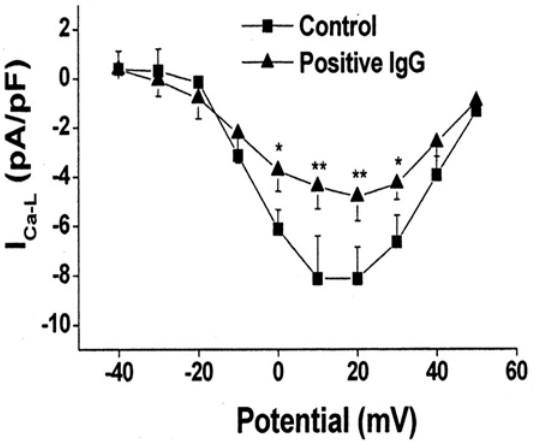

Figure 9. Positive IgG inhibited L-type Ca current in SAN. Positive IgG fractions (80μg/mL) significantly inhibited I_{Ca-L} (46.2±5.6%), $p < 0.05$, $n = 6$).

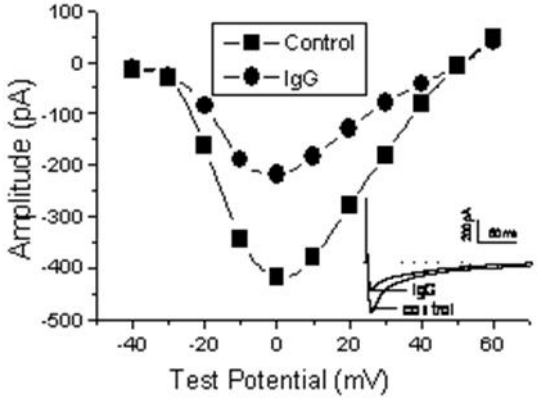

Figure 10. Positive IgG inhibited L-type Ca current in AV node. Positive IgG fractions (80μg/mL) significantly inhibited I_{Ca-L} (51±7.2%), $p < 0.05$, $n = 8$).

Anti-SSA/Ro –SSB/La directly recognize the L-type Ca channel proteins (Cav1.2 and Cva1.3)

To demonstrate that positive IgG binds directly to the L-type Ca channels α_{1C} and α_{1D}, western blot following immunoprecipitation was used. Immunoprecipitated α_{1C} from human fetal hearts was probed using commercial anti- α_{1C} antibody (Lane 1) and positive IgG (lane 2) (Figure

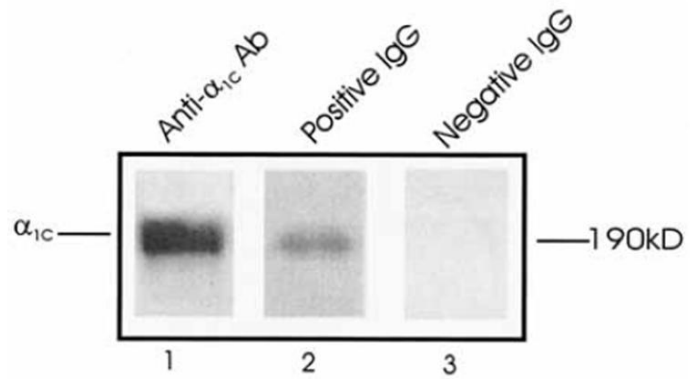

Figure 11. Immunoblot of human fetal cardiac Ca channel α_{1C}-subunit. Immunoblots were performed under non-reducing conditions on 8–16% Tris-Glysine gel. Human fetal hearts (16–20-week gestation) were used. Immunoprecipitated proteins with anti-α_{1C} antibody were separated by SDS-PAGE, transferred to PVDF membrane and then probed with anti-α_{1C} antibody (lane 1), positive IgG (lane 2) and negative IgG (lane 3).

11). Both recognized the same molecular weight band. Using negative IgG lacking the anti-SSA/Ro –SSB/La antibodies, no band was detected (Qu et al., 2001). Similarly, Cross-reactivity of positive IgG with L-type Ca channel α_{1D} protein expressed in tsA201 is shown in figure 12. Western blot experiments were performed on proteins extracted from $\alpha_{1D}/ß_{2a}/ \alpha_2\delta$-transfected tsA201 cells with positive IgG and anti- α_{1D} antibody as shown in Figure 12. Anti- α_{1D} antibody recognized the 190-kDa band corresponding to the α_{1D} Ca channel protein in all 4 lanes (Figure 12A). The membrane blot was stripped (Figure 12B) and reprobed with positive and negative IgGs, respectively. Positive (Figure 12C) but not negative IgG recognized the same 190-kDa α_{1D} Ca channel protein band (Figure 12D). This confirms that the results are not due to the residual anti- α_{1D} antibody, because no bands were seen on the stripped membrane (Figure 12B) (Qu et al., 2005a).

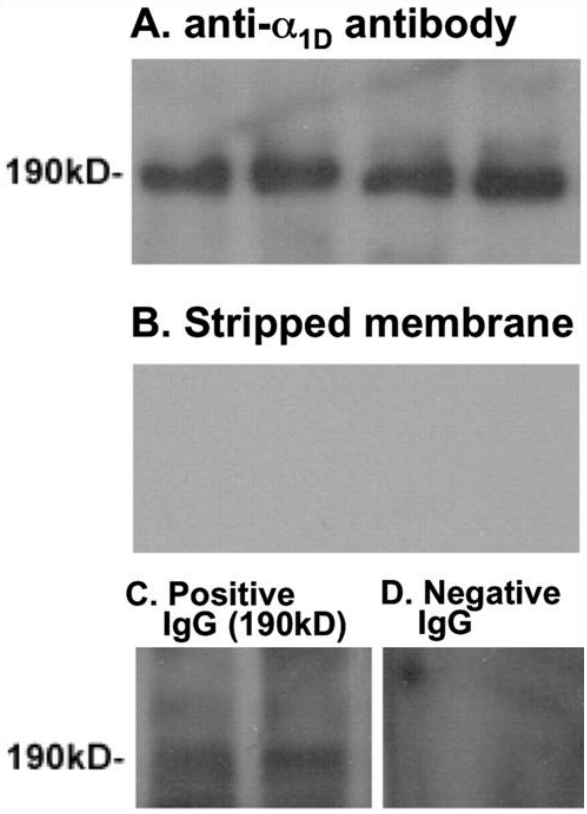

Figure 12. Cross-reactivity of positive IgG with L-type Ca channel α_{1D} protein expressed in tsA201 cells by Western blot. A, α_{1D} Ca channel protein was detected in all 4 lanes with anti-α_{1D} Ca channel antibody (Sigma) on proteins extracted from transfected tsA201 cells. B, Anti-α_{1D} Ca channel antibody was stripped from membrane. C, Same membrane was split in 2. One part was reprobed with positive IgG. Positive IgG recognized same 190-kDa band corresponding to α_{1D} Ca channel protein. D, Remaining half of stripped membrane was reprobed with negative IgG. Negative IgG did not cross-react with α_{1D} Ca channel protein.

II.18 Summary and Specific aims in this thesis

Autoimmune-associated congenital heart block (CHB), an important cause of morbidity and mortality in newborns, is established as a passively acquired autoimmune disease in which transplacental passage of autoantibodies against intracellular ribonucleoproteins SSB/La (48kDa), and SSA/Ro (52kD and 60kD) results in damage to the developing fetal heart. Other neonatal abnormalities affecting the skin, liver, and blood are also reported to be associated with anti-SSA/Ro and anti-SSB/La autoantibodies in the maternal and fetal circulation and are grouped under the heading of Neonatal Lupus Syndrome. Neonatal lupus was termed because of the cutaneous lesions in the neonate resemble those seen in Systemic Lupus Erythrematosus (SLE). The hallmark of CHB is characterized by irreversible atrioventricular block, not accompanied by any cardiac structural or anatomic abnormalities. Sinus bradycardia has also been reported in animal models of CHB and in clinical settings. However, the non cardiac manifestations are transient, resolving at about six months of age coincident with the disappearance of maternal autoantibodies from the neonatal circulation. Although significant effort has been made to understand the pathophysiology of CHB, the exact mechanism by which these antibodies contribute to the conduction abnormalities is not completely understood. The main objective of this study is to test the general hypothesis that, in CHB, L-type Ca channels are the target for anti-SSA/Ro and SSB/La autoantibodies from mothers with Systemic Lupus Erythrematosus, Sjogrens' syndrome or even asymptomatic mothers harboring the respective autoantibodies. Two types of L-type Ca Channels are expressed in the heart: Cav1.2 (α_{1C}) and Cav1.3 (α_{1D}). This thesis will focus on Cav1.3 due to its unique expression in the

supraventricular tissue. We propose to define the role of these maternal autoantibodies in the pathogenesis of CHB by the following specific aims:

Specific Aim #1. To demonstrate the expression and localization of Cav1.3 Ca channel in human fetal heart and characterize the effects of anti-SSA/Ro -SSB/La autoantibodies on Cav1.3 Ca current in human embryonic kidney cells.

Specific Aim #2. To characterize the effects of anti-SSA/Ro -SSB/La autoantibodies on Cav1.3 Ca current in rat neonatal cardiomyocytes.

Specific Aim #3. To molecularly map the anti-SSA/Ro -SSB/La target region on Cav1.3 Ca channel.

Specific Aim #4. To rescue or wosen the electrocardiographic abnormalities seen in CHB in a murine model by immunizing two transgenic mouse lines: Cav1.2 overexpression and Cav1.3 knockout transgenic models.

CHAPTER 1

EXPRESSION OF CaV1.3 L-TYPE CALCIUM CHANNEL IN THE HEART AND THE CHARACTERIZATION OF THE EFFECTS OF ANTI-SSA/Ro-SSAB/La AUTOANTOBODIES ON THE CAV1.3 CALCIUM CURRENT IN tsA201 CELLS

Because the hallmark phenotype of CHB is AVB, the AV node has been the focus of previous work from our lab and not the SAN. In this regard, sinus bradycardia has been reported in animal models and in some clinical settings (Brucato et al., 2000) (Menon et al., 1998a). The high incidence of sinus bradycardia in our mouse models of CHB indicates that the spectrum of electrocardiographic abnormalities extends beyond the AVN to affect the SAN. Previously, we showed that IgG from mothers with a CHB infant inhibited L-type (I_{Ca-L}) Ca current and that this inhibition could account for the AV block and sinus bradycardia in CHB(Qu et al., 2005a). The rationale behind the proposed hypothesis is that impulse generation at the SAN and conduction at the AVN critically depends on $I_{Ca-L.}$ Cardiac L-type Ca channels are multisubunit complexes composed of α_{1C} and $\alpha_{1D.}$ Cav1.2 or α_{1C} is ubiquitously expressed in the heart and mediates cardiac excitation-contraction coupling. Cav1.3 or α_{1D} on the other hand is restricted to the supraventricular tissue in the adult with the highest expression in the SAN and AVN (Figure 6). Cav1.2 I_{Ca-L} activates at more positive (–40 and –30 mV) potentials, and accounts for the conduction electrogenesis at the AVN, whereas Cav1.3 I_{Ca-L} activation occurs between –60 and –40 mV at a range in which diastolic depolarization at the SAN operates (Koschak et al., 2001). Thus, Cav1.3 I_{Ca-L} seems to be essential for normal cardiac pacemaker activity. In fact, Cav1.3 -/- phenotype is sinus bradycardia and AVBs similar to what is noted in CHB (Mangoni et al., 2003). Therefore, in this chapter, the expression and localization of Cav1.3 Ca channel was

sought and the effect of anti-SSA/Ro –SSB/La antibodies was tested in human embryonic kidney cells.

Methods

Human Fetal Heart Tissue

Human fetal hearts (15- to 20-week gestation) are obtained after elective termination of normal pregnancy from National Institutes of Health–sponsored tissue banks in Baltimore, MD, and Seattle, WA. The use of human fetal heart tissue received an exemption from the VA New York Harbor Healthcare System Institutional Review Board.

Reverse Transcription–Polymerase Chain Reaction

Total cellular RNA is isolated from human fetal tissue, and reverse transcription is performed. The sense primer was 5'-TTAGTGACGCCTGGAACACG-3', and the antisense primer was 5'-CCTGTATCAGGAAAGTGG-3'. The primers are chosen from conserved regions among different species and are unique to the Cav1.3 Ca channel. The expected PCR amplification size is 1047 bp. Final PCR products are evaluated on ethidium bromide–stained 1% agarose gel. Sequencing of the PCR products is performed by Genemed.

Isolation of Human and Rat Fetal Cardiac Myocytes

Cardiac myocytes are obtained from Langendorff-perfused human fetal hearts. Hearts are perfused at 37°C with 100% O_2 gassed Tyrode's solution followed by Ca-free Tyrode's solution with 0.5 mg/mL collagenase type B (Boehringer Mannheim). Cells are then dispersed in a Kraft-Brühe solution containing (in mmol/L): potassium glutamate 70, KCl 30, KH_2PO_4 10, $MgCl_2$ 1, taurine 20, glucose 10, HEPES 10. Isolated 17- to 19-day-old fetal Sprague-Dawley rat myocytes

are obtained and cultured on coverslips in Dulbecco's modified Eagle's medium containing 10% calf serum (Gibco) overnight before being subjected to the immunostaining procedures.

Indirect Immunofluorescent Staining

Indirect immunostaining is performed on isolated human fetal myocytes. Cells are fixed and permeabilized with 4% paraformaldehyde and 0.1% Triton. After they are blocked with 5% normal goat sera, the cells are incubated overnight at 4°C with anti-Cav1.3 Ca channel antibody (1:200) and detected with FITC-conjugated anti-rabbit IgG (1:200, Jackson ImmunoResearch Laboratories, Inc). A confocal scanning laser microscope (MRC-600; Bio-Rad) is used for visualization.

Protein Extraction and Western Blot

Membrane proteins are prepared using the following protocol; heart tissue is homogenized in a seven-fold volume of buffer (in mmol/l: HEPES 20, EGTA 4, and dithiotreitol 1, pH 7.5) complete with protease inhibitors (in mmol/l: Leupeptin 0.1 and phenylmethylsulfony fluoride 0.3) and stirred for 45 min at 4°C. A first spin of 1000 *g* at 4°C is performed for 10 min to remove cell debris and nuclei. The supernatant is collected and subjected to a second centrifugation at 100,000 *g* for 60 min at 4°C. The supernatant is then removed, and the pellet containing the particulate fraction is re-suspended in the same buffer. The protein concentration is determined in triplicate by a Bio-Rad D_C protein assay Kit. Same amount of membrane proteins (50 μg and 100 μg) are loaded for each lane of standard 4-12% SDS polyacrylamide gels as previously described (Qu et al., 2005a). After electrophoresis, proteins are transferred to a PVDF membrane. Membranes are blocked in PBS containing 0.1% Tween and 5% non-fat milk. Membranes are incubated overnight at 4 °C in 1:200 primary anti-Cav1.3 Ca channel antibody

(sigma and Calbiochem). The immunoblots are developed with horseradish peroxidase-labeled goat anti-rabbit IgG (Chemicon) in PBS-Tween as a secondary antibody (1:2000) for 1 hr, followed by detection by ECL (Amersham).

Expression of Cav1.3 Ca channel and Electrophysiological recording of Cav1.3 Ca current in tsA201 cells

tsA201 cells are grown in culture media consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100ug/ml) as previously reported (Chahine et al., 2008; Qu et al., 2005a; Qu et al., 2005b; Qu et al., 2007). The cells are grown at 37 ºC, 5% CO_2 and transiently transfected with 4 µg of a mix of α_{1D}, ß$_{2a}$, $\alpha_{2\delta}$, and lymphocyte surface antigen (CD8-a) cDNAs (in pCMV6b vector, kindly provided by Drs J. Striessnig, Innsbruck, Austria; S. Seino, Kobe, Japan; and Dr. M. Chahine, Quebec, Canada) by the Ca phosphate method as described previously (Baroudi et al., 2006; Chahine et al., 2008; Qu et al., 2005a; Qu et al., 2005b). Cells expressing surface CD8-a fixes the beads and are visually distinguishable from non-transfected cells. Whole-cell voltage-clamp recording is performed with the Axopatch 200B (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA) at 48 hours after transfection. The internal solution contains (in mmol/L): 135 CsCl, 4 $MgCl_2$, 4 ATP, 10 HEPES, 10 EGTA, and 1 EDTA, adjusted to pH 7.2 with tetraethylammonium hydroxide (TEAOH). The bath solution contains (in mmol/L): 135 choline chlorine, 1 $MgCl_2$, 2 $CaCl_2$, and 10 HEPES, adjusted to pH 7.4 with TEAOH. Signals are sampled at 20 kHz and low-pass filtered at 2 kHz. Data are leak-subtracted online with a P/4 protocol and analyzed with pClamp version 9.0 (Axon Instruments). For α_{1D} $I_{Ca\text{-}L}$ current-voltage (I-V) relations, tsA201 cells are depolarized from a holding potential (HP) of –100 mV to test potentials between –80 and 60 mV with increments of 10 mV. The rate of diffusion of

autoantibodies was measured using trypan blue and was estimated to reach the patch clamped cell in approximately 20-30 seconds.

RESULTS

Cav1.3 Ca Channel Is Expressed in Human Fetal Cardiac Myocytes

To establish that Cav1.3 Ca channel is a target for maternal autoantibodies, one major criterion must be met; that Cav1.3 Ca channel should be expressed in the human fetal heart. In this chapter, I combined reverse transcription polymerase chain reaction (RT-PCR), confocal immunofluorescent staining, Western blot analysis, and immunohistochemistry to address this criterion. RT-PCR was performed to investigate the expression of Cav1.3 mRNA in human fetal hearts (16-24 weeks gestation). Using Cav1.3 Ca channel specific primers, Cav1.3 Ca channel mRNA was amplified from atria, ventricles, and AV node of human fetal hearts (Figure 1A). To detect the Cav1.3 Ca channel protein in human fetal hearts, total proteins were extracted from the human fetal hearts and used to examine the expression of Cav1.3 Ca channel. Figure 1B shows a 190 kDa band corresponding to Cav1.3 Ca channel. Human fetal cardiomyocytes were isolated and stained using indirect immunofluorescence for Cav1.3 Ca channel. Cav1.3 Ca channel was localized on the sarcolemma of the cardiomyocytes as shown in Figure 1C. Interestingly, nuclear staining was also observed. To complement the immunostaining experiments, we also used immunohistochemistry on adult mouse hearts to detect the Cav1.3 Ca channel. The results confirmed the RT-PCR in which the Cav1.3 Ca channel was detected in the supraventricular tissue and was absent in the adult ventricles (Figure 2).

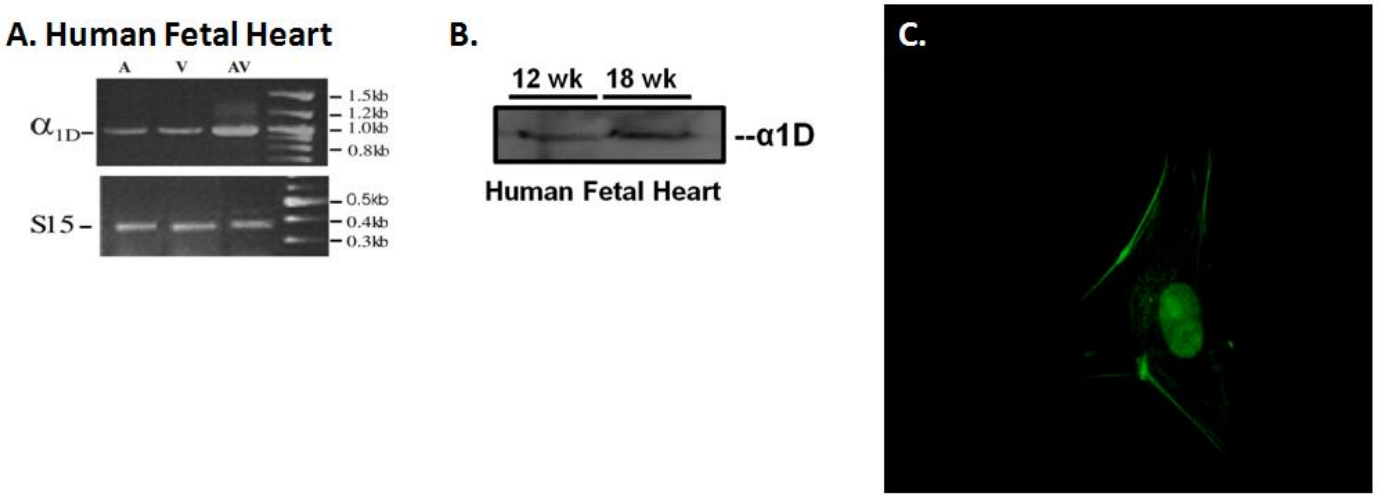

Figure 1. Expression and localization of Cav1.3 Ca channel in human fetal heart. Panel A. represent an RT-PCR from human fetal heart gestation age 16-24 weeks. RNA extracted using TRIZOL reagent, quantified and reverse transcribed using Retroscript kit from Ambion. PCR using Cav1.3 specific primers amplified 1015bp band specific for Cav1.3. S15 was used as a positive control. Panel B. represent a western blot using Cav1.3 polyclonal antibody showing the presence of Cav1.3 in human fetal heart. Panel C represent a confocal image of human fetal cardiomyocytes stained with Cav1.3 specific antibody showing the membrane/sarcolemmal staining as well as nuclear staining. (Panel A. from Qu et al. Circulation 2005).

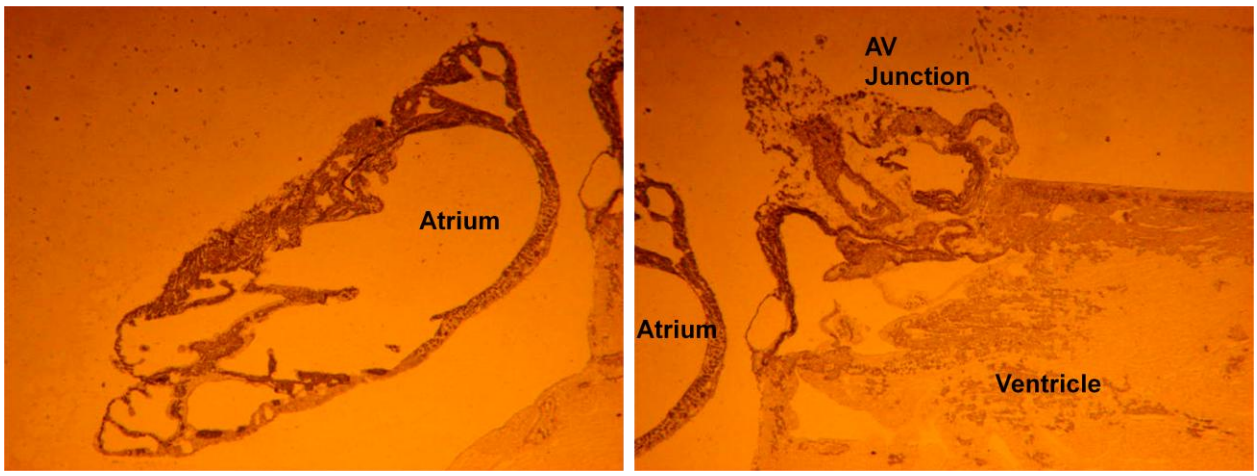

Figure 2. Immunohistochemistry of Cav1.3 Ca channel in mouse heart. Staining using Cav1.3 specific primers on 5-10µm sections from mouse adult hearts showed atrial and AV junction presence of Cav1.3. Adult ventricles lack Cav1.3 as shown by the absence of staining.

Anti-SSA/Ro –SSB/La inhibited Cav1.3 $I_{Ca\text{-}L}$ in tsA201 cells

Following the confirmation of the presence of Cav1.3 in the supraventricular tissue, we sought to examine the effects of the autoantibodies on Cav1.3. However, there are no pharmacological agents or biophysical approaches to separate Cav1.3 from Cav1.2 $I_{Ca\text{-}L}$ in native cardiomyocytes. Therefore, we used a mammalian heterologous expression system (tsA201, Human Embryonic Kidney cells) as an alternative to study the effects of anti-SSA/Ro and anti-SSB/La autoantibodies on Cav1.3 Ca channel. Coexpression of Cav1.3 with its accessory subunits yielded a functional Cav1.3 $I_{Ca\text{-}L}$ that activated at approximately -60 to -50 mV and peaked at -10 mV with 2 mM Ca as a charge carrier. We tested whether the autoantibodies could inhibit Cav1.3 $I_{Ca\text{-}L}$. Application of anti-SSA/Ro and anti-SSB/La autoantibodies (100μg/ml) from mothers with CHB children (also termed positive IgG) reduced peak current of Cav1.3 $I_{Ca\text{-}L}$. Averaged data showed an inhibition of 42.2±3% at -10 mV (Figure 3A and B). The inhibitory effect was partially restored following washout of the autoantibodies as shown in Figure 3 C. This response was specific since denatured autoantibodies didn't have any effect on Cav1.3 $I_{Ca\text{-}L}$ (Figure 3D).

Discussion

In summary, the data demonstrate that the α_{1D} Ca channel is expressed in human fetal heart, and α_{1D} $I_{Ca\text{-}L}$ is inhibited by positive IgG. Given that the α_{1D} Ca channel plays a critical role in SA node pacemaking, blockade of α_{1D} $I_{Ca\text{-}L}$ in human fetal heart may contribute to the genesis of sinus bradycardia in CHB.

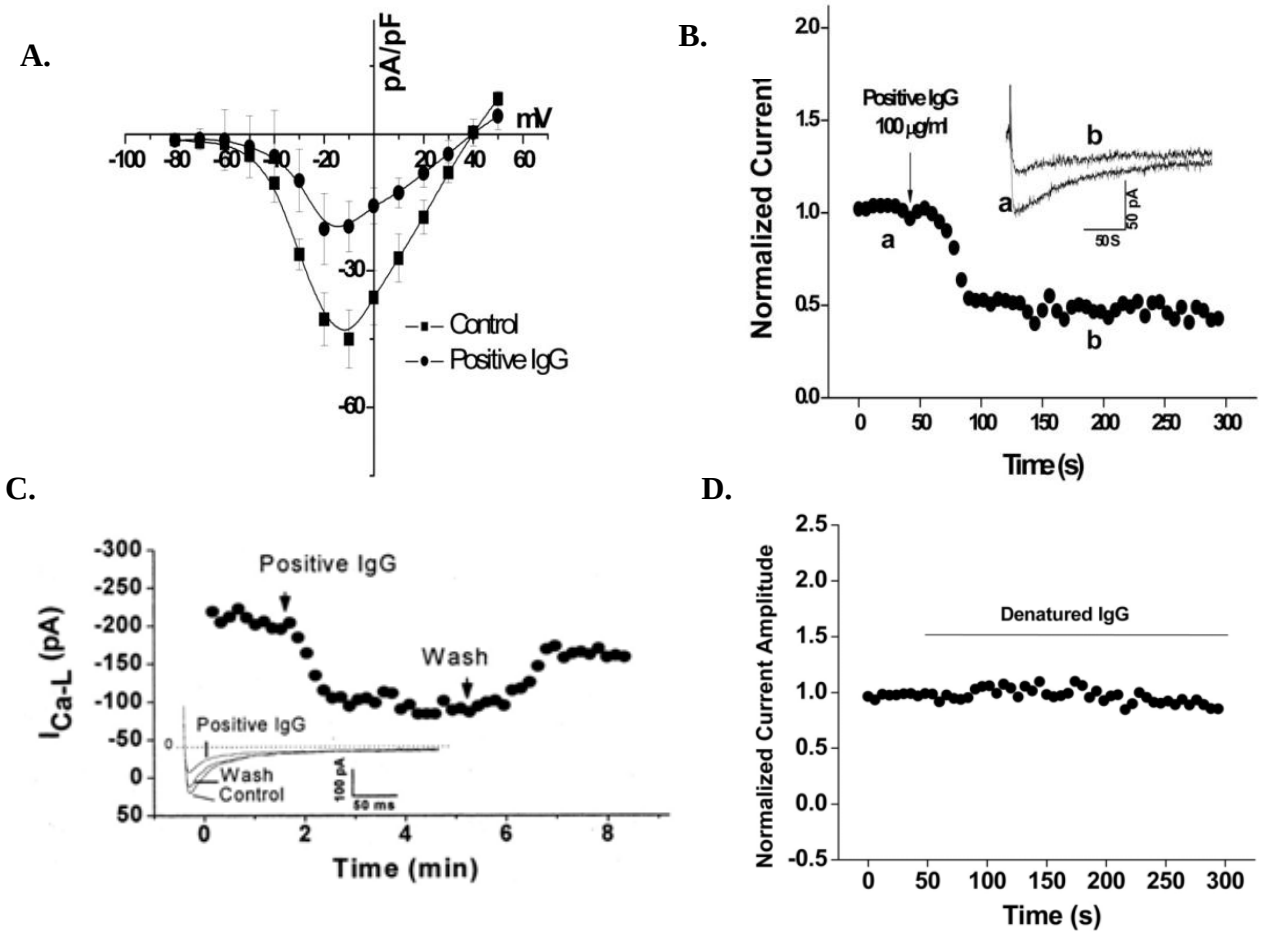

Figure 3. Functional characterization of Cav1.3 Ca channel expressed in tsA201 cells. Panel A. shows an IV curve with a functional Cav1.3 Ca current before and after the addition of positive IgG (anti SSA/Ro and SSB/La). Panel B. shows the time course following consecutive stimulation at -10mV before and after the addition of 100µg/ml positive IgG. Panel C. Time course of peak $I_{Ca.-L}$ at 10 mV before, during application of positive IgG, and after washout of positive IgG (100 µg/mL). Original traces for control, positive IgG, and washout are shown in the inset. Panel D. Denatured IgG (used as a control) had no effect on the $I_{Ca.-L}$.

CHAPTER 2

Silencing of Cav1.2 gene in Neonatal Cardiomyocytes by Lentiviral Delivered shRNA

ADDENDUM

To date, there are no data concerning the effects of maternal anuantibodies on Cav1.3 Ca channels in native cardiomyocytes. To characterize the effects of anti-SSA/Ro -SSB/La autoantibodies on Cav1.3 Ca channel in native cardiomyocytes, RNAi will be used to substantially silence the Cav1.2 Ca channel (see paper). Similarly, the effect of anti-SSA/Ro -SSB/La autoantibodies on the remaining Cav1.3 Ca current will be carried out by patch-clamp technique.

Two types of L-type Ca Channels are expressed in the heart: Cav1.2 (α_{1C}) and Cav1.3 (α_{1D}). Unlike Cav1.2, Cav1.3 is highly expressed in sinoatrial node (SAN) and atria, but not in the adult ventricles and is involved in diastolic depolarization and thus essential for the generation of normal cardiac rhythm and for induction of impulse propagation at the AVN. Cav1.3 knockout mouse presents with a phenotype of sinus bradycardia and different degrees of AV blocks supporting a critical role of Cav1.3 Ca channel in the SAN and AVN. On the other hand, Cav1.2 is the predominant form in cardiomyocytes and is essential for excitation-contraction (EC) coupling. Deletion of the Cav1.2 gene results in embryonic lethality before embryonic day

E14.5, thus making it difficult to study Cav1.3 in its native setting in cardiomyocytes. As mentioned earlier, there are no pharmacological or biophysical approaches to separate Cav1.3 from Cav1.2 Ca currents in native cardiomyocytes, heterologous mammalian expression systems such as the non cardiac tsA201 cell line was used. However, these expression systems do not represent the native environment of the Cav1.3 Ca channel. To study the Cav1.3 Ca channel in its own native environment, we used RNA interference to silence Cav1.2 Ca channel thereby allowing for the study the effects of anti-SSA/Ro and anti-SSB/La autoantibodies on the Cav1.3 Ca channel.

Anti-SSA/Ro –SSB/La autoantibodies inhibited Cav1.3 $I_{Ca\text{-}L}$ in native cardiomyocytes.

Modified figure 4 from the silencing paper shows the effect of the addition of anti-SSA/Ro – SSB/La autoantibodies following the silencing of the Cav1.2 gene. The autoantibodies inhibited the Cav1.3 $I_{Ca\text{-}L}$ by 35%.

This observation provided the first evidence that the Cav1.3 $I_{Ca\text{-}L}$ is inhibited in native cardiomyocytes.

MODIFIED FIGURE 4.

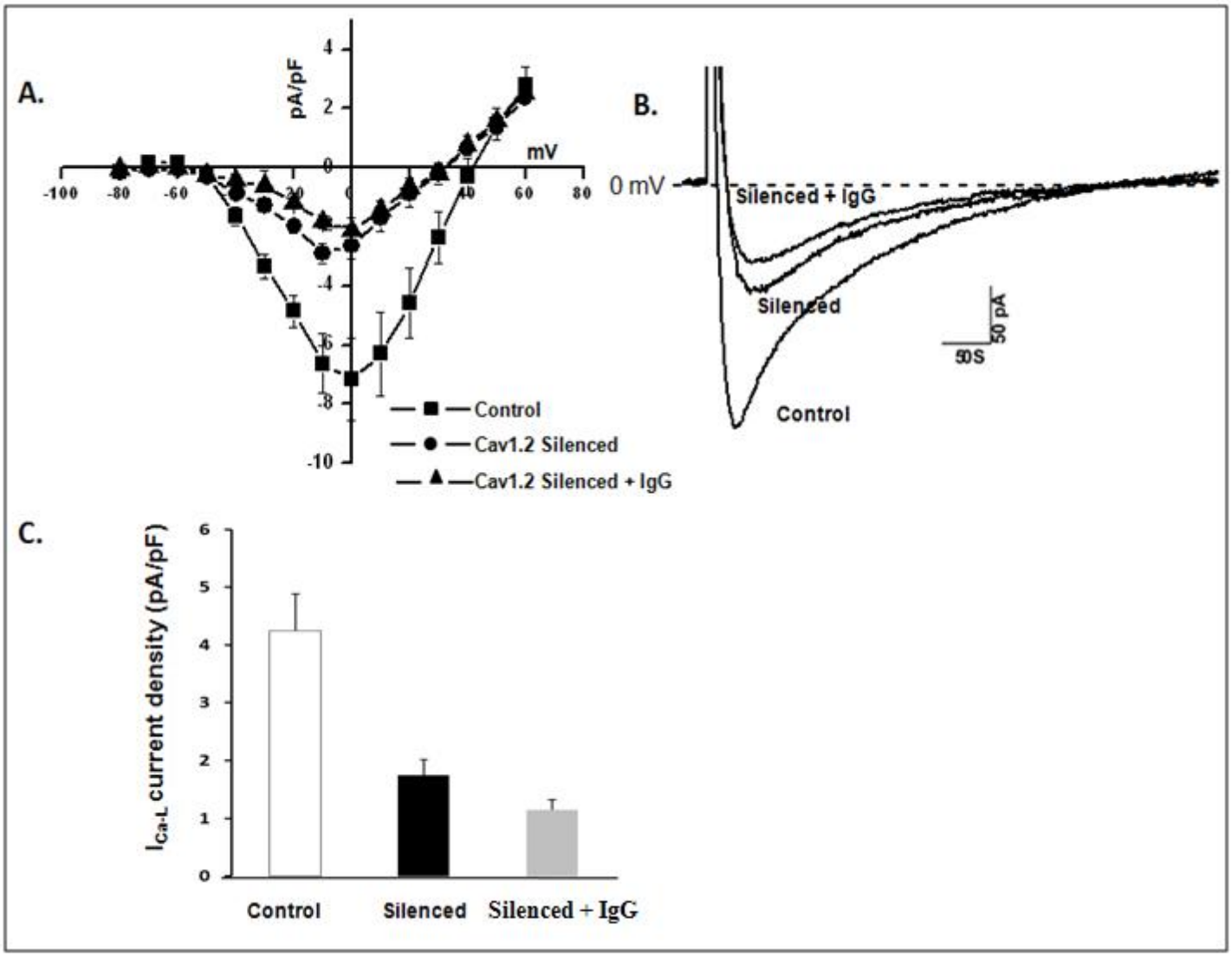

Figure 4: Effect of IgG on the remainng Cav1.3 Ca current following silencing of Cav1.2 in neonatal cardiomyocytes. The autoantibodies inhibited the Cav1.3 I_{Ca-L} by 35% in the Silenced + IgG group.

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Silencing of Cav1.2 gene in neonatal cardiomyocytes by lentiviral delivered shRNA

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ABSTRACT

Cav1.2 (α_{1C}) and Cav1.3 (α_{1D}) L-type Ca channels are co-expressed in the heart. To date, there are no pharmacological or biophysical tools to separate α_{1D} from α_{1C} Ca currents ($I_{Ca\text{-}L}$) in cardiomyocytes. Here, we established a physiological model to study α_{1D} $I_{Ca\text{-}L}$ in native myocytes using RNA interference. Transfection of rat neonatal cardiomyocytes (RNC) with α_{1C} specific siRNA resulted in low silencing efficiency (50–60%) at the mRNA and protein levels. The use of lentivirus shRNA resulted in 100% transfection efficiency and 92% silencing of the α_{1C} gene by real-time PCR and Western blot. Electrophysiological experiments showed that the total $I_{Ca\text{-}L}$ was similarly reduced by 80% in lentivirus transfected cells. Both biochemical and functional data demonstrated high transfection and silencing efficiency in the cardiomyocytes using lentiviral shRNA. This novel approach allows for the assessments of the roles of α_{1C} and α_{1D} Ca channels in native myocytes and could be used to examine their roles in physiological and pathological settings.

Introduction

Ca influx through the L-type Ca channels modulates numerous cellular physiological processes ranging from muscle contraction, neuronal excitability, immune regulation, to gene expression [1]. Activation of L-type Ca channels results in the increase in cytosolic Ca thus triggering different cellular events under physiological and pathological settings.

Cardiac L-type Ca channels are complexes of multisubunits composed of α, β_2, and $\alpha_{2\delta}$ subunits [2,3]. Two types of L-type Ca channels are expressed in the heart: Cav1.2 (α_{1C}) and Cav1.3 (α_{1D}) and both contribute to the total $I_{Ca\text{-}L}$. Cav1.2 or α_{1C} is ubiquitously expressed in the cardiovascular system and mediates cardiac excitation–contraction coupling [3]. Cav1.3 or α_{1D} is exclusively restricted to the supraventricular tissue of the adult with higher expression in the sino-atrial node (SAN) and the atrio-ventricular node (AVN) [4]. Up until recently, α_{1D} Ca channel has been thought to be of neuroendocrine origin but recent data confirmed its expression in the heart [4,5].

Electrophysiologically, α_{1D} $I_{Ca\text{-}L}$ differs from α_{1C} $I_{Ca\text{-}L}$ in that α_{1D} $I_{Ca\text{-}L}$ activates at a more negative membrane potentials [6]. Thus, because of its unique localization in the conduction tissue and the range of activation potential, it has been suggested that α_{1D} $I_{Ca\text{-}L}$ is essential for normal cardiac pacemaker activity and for impulse propagation to the AVN [5,7,8].

Transgenic mice were generated for both Cav1.2 and Cav1.3. α_{1D} knockout (KO) mice are viable and exhibit sinus bradycardia and different degrees of AV blocks [4,5,7,8]. However, deletion of the α_{1C} gene, the predominant form in cardiomyocytes, results in embryonic lethality at day E14.5 [9]. The study of α_{1D} $I_{Ca\text{-}L}$ has been limited to expression systems such as tsA201 cells because of the lack of efficient pharmacological blockers and biophysical methods to separate α_{1D} $I_{Ca\text{-}L}$ from α_{1C} $I_{Ca\text{-}L}$.

To study α_{1D} Ca channel in a physiological native environment, RNA interference (RNAi) was used to silence the α_{1C} gene and test the effectiveness of RNAi silencing of Cav1.2 in primary rat neonatal cardiomyocyte in culture.

Methods

Animal protocol

All experiments were performed in accordance with animal studies subcommittee regulations at VA New York Harbor Health-

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care System and all procedures related to animal use conform to the NIH guidelines (Guide for the Care and Use of Laboratory Animals, NIH Publication 86-23).

Preparation of primary neonatal rat cardiomyocyte cultures

Hearts were removed from 1- to 3-day-old Sprague–Dawley rats and ventricles were minced and subjected to consecutive digestion with 0.75 mg/ml trypsin and 20 μg/ml DNase I in CBFHH buffer (137 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.6 mM dextrose, 0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 20.1 mM HEPES, pH 7.4). Cell suspensions were centrifuged at 1500 rpm for 10 min. The pellets were resuspended in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% bovine serum, 0.1 mM bromodeoxyuridine, 1% penicillin–streptomycin, and 1.5 mM vitamin B12. Cell suspensions were filtered through a cell strainer and preplated for 30 min at 37 °C to remove fibroblasts. Cardiac myocytes (3.1×10^6) remaining in suspension were added to 35 mm plates coated with 1% gelatin and incubated overnight in the above medium at 37 °C in humidified air with 5% CO_2. Cultures were incubated overnight in serum-free DMEM and then maintained in serum-free DMEM containing 10 μg/ml insulin and 10 μg/ml transferrin at 37 °C in humidified air with 9% CO_2 for 3 days prior to exposure to experimental manipulations in serum-free DMEM.

Design and construction of lentiviral siRNA

The sequence of the siRNA specifically targeting the Cav1.2 gene was either purchased from Ambion (Silencer Pre-designed siRNA) siRNA ID # 199812 and 199811 or designed through siRNA Target Finder (Ambion, Austin, TX). The oligonucleotides were selected by Blast homology search among cDNA sequences of target genes from various species including mouse, rat and human. The oligo's designed were synthesized from IDT with a 5′ phosphate and PAGE purified. The oligo format is the following:

Sense Oligo: 5′ T-(19nt)-TTCAAGAGA)-(91nt)-TTTTTTC-3′ [Sense siRNA-Loop-Antisense siRNA-Stop]. Anti-sense oligo: Compliment of sense but with additional nucleotides at 5′ end generating an XhoI site.

Lentiviral vector cloning

The sense and anti-sense oligo's were resuspended in water at 60 pmol/μl concentration and annealed in annealing buffer: 100 nM K-acetate, 30 nM HEPES–KOH, pH 7.4, 2 mM Mg-acetate by incubating at 95 °C for 4 min, 70 °C for 10 min, then decreasing the temperature to 4 °C slowly (0.1 °C/min). The annealed oligo was subcloned into XhoI/HpaI site in pLentiLox 3.7 (pLL3.7) vector which encodes the CMV promoter driven eGFP (enhanced green fluorescent protein) marker as internal control. The resulting lentiviral siRNA vector was confirmed by restriction enzyme digestion with XbaI/NotI showing 60 bp band shift on a 2% agarose gel when compared to the parental vector pLL3.7. The constructed vector was also confirmed by DNA sequencing with FLAP primer (5′-CAGTGCAGGGGAAAGAATAGTAGAC-3′).

Lentivirus packaging

Packaging, purification, and titer determination of the lentivirus were performed as described previously [10,11]. The Recombinant viruses were produced by Ca phosphate transfection of HEK293T cells using standard protocols [12]. Briefly, 293T cells were cultured in Dulbecco's modified Eagles's medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 U/ml). At around 70% confluency in a 10 cm culture dish, the cells were co-transfected with the lentiviral vector (10 μg), and the lentiviral packaging vectors pRSV-REV (2 μg), pMDLg/RRE (5 μg), and the vesicular stomatitis virus G glycoprotein (VSVG) expression vector pMD2G (3 μg). The viruses were collected from the culture supernatant 48 h post-transfection, concentrated by ultra-centrifugation for 2 h at 25,000 rpm, and resuspended in phosphate-buffered saline (PBS). Titers were determined by infecting 293T cells with serial dilutions of concentrated lentivirus and counting eGFP positive cells after 48 h under fluorescent microscopy.

Lentiviral transduction in rat neonatal cardiomyocytes

At day 2–3 post-culture, the cells were incubated with lentivirus at various "multiplicity of infection" for 3, 4, and 5 days, respectively. Polybrene (10 μg/ml) was added to increase the efficiency of lentiviral transduction.

Cell sorting using fluorescence-activated cell sorting (FACS)

For sorting by means of FACS, rat neonatal cardiomyocytes were plated and the next day transfected using Lipofectamine 2000 with a combination of α_{1C} siRNA and cy3-labeled GAPDH siRNA [13]. After 48 h, the cells were harvested and FACS sorting of cy3 fluorescent cells was performed at the FACS core facility with a FACS-DIVA sorter. Cy3-positive cells with strong signals in the FL-1 channel were collected and plated on 10 cm dishes. One day after cell sorting, the cells were collected for experiments.

Isolation of RNA and real-time RT-PCR

Total RNA was extracted using the TRIZOL. With this method, we obtained minimal DNA in our RNA samples. RNA was quantified by spectrophotometry at 260 nM and the ratio of absorbance at 260 nm to that of 280 nm was >1.8 for all samples. Degradation of RNA was monitored by the observation of appropriate 28S to 18S rRNA ratios as determined by ethidium bromide staining of agarose gels. Aliquots of RNA were stored in RNase free water at −20 °C. For real-time RT-PCR, first the RNA samples were reverse transcribed using the Ambion reverse transcription Retroscript kit without heat denaturation. Real-time RT-PCR was performed using the Taqman Universal PCR Master Mix (Applied Biosystems) and Pre-designed and labeled primer/Taqman probe (Applied Biosystems) using 18S ribosomal RNA as internal control [14,15]. The reaction solution was assembled in a volume of 50 μl containing the Taqman universal PCR master mix, α_{1D} specific forward and reverse primers (final concentration 300 nM each), the TaqMan probe (200 nM) and the cDNA mixture. The quantity of the α_{1D} cDNA of interest in a certain cDNA mixture was normalized to that of 18S rRNA.

Protein extraction and Western blots

Cells were collected at 48, 72, and 96 h post-transfection. Total protein was prepared as previously described [16]. Briefly, rat neonatal cardiomyocytes were lysed in RIPA buffer (50 mM Tris–Cl, pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate, 1 mM PMSF) complete with protease inhibitors (in mmol/l: Leupeptin 0.1 and phenylmethylsulfony fluoride 0.3) and kept for 30 min on ice with vortexing every 5 min. The supernatant was collected following centrifugation at 14,000 rpm for 15 min at 4 °C. The protein concentration was determined in triplicate by a Bio-Rad D_C protein assay Kit. The same amount of membrane proteins (50 μg) were loaded for each lane of standard 4–12% SDS–polyacrylamide gels as previously described. After electrophoresis, proteins were transferred to a nitrocellulose membrane. Membranes were blocked in PBS containing 0.1% Tween and 5% non-fat milk. Membranes were incubated overnight at 4 °C in 1:200 primary anti-α_{1C} Ca channel antibody (sigma and Calbiochem). The immunoblots were devel-

oped with horseradish peroxidase-labeled goat anti-rabbit IgG (Chemicon) in PBS-Tween as a secondary antibody (1:2000) for 1 h, followed by detection by ECL (Amersham). The density of protein bands was quantified by NIH image software.

Electrophysiological recording of L-type Ca current native cells

Solutions. The composition of external solutions to record whole cell L-type Ca current, $I_{Ca\text{-}L}$ in (mM) was: NaCl 132, CsCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.8, NaH_2PO_4 0.6, 4-amino-pyridine 5, HEPES 10, dextrose 5, Na-pyruvate 5, pH 7.4. Patch electrodes were filled with control internal solution containing (in mM): CsCl 139.8, K-EGTA 10, $MgCl_2$ 4, $CaCl_2$ 0.062, Na_2-creatine phosphate 5, HEPES 10, Na_2 ATP 3.1, Na_2GTP 0.42, adjusted to pH 7.1 with KOH.

Electrophysiology. The whole cell patch-clamp technique was used [16]. To record L-type $I_{Ca\text{-}L}$, all K currents were blocked with intracellular and extracellular Cs^{2+} and 4-amino-pyridine. The fast Na and T-type Ca currents were blocked by a prepulse to −50 mV from a holding potential of −80 mV every 10 s. The junction potential was always compensated and was smaller than 5 mV. Membrane currents were recorded using Axopatch 200B patch-clamp amplifier (Axon Instruments).

Data analysis. Statistical comparisons were evaluated using either paired or unpaired Student's *t*-test, as appropriate. Data are presented as means ± SE. A value of $P < 0.05$ is considered significant.

Results

Chemically synthesized siRNA were unable to effectively silence Cav1.2

To establish a system for the characterization of Cav1.3 Ca channel in rat neonatal cardiomyocytes, chemically synthesized siRNA against the Cav1.2 gene were purchased from Ambion (siRNA #1

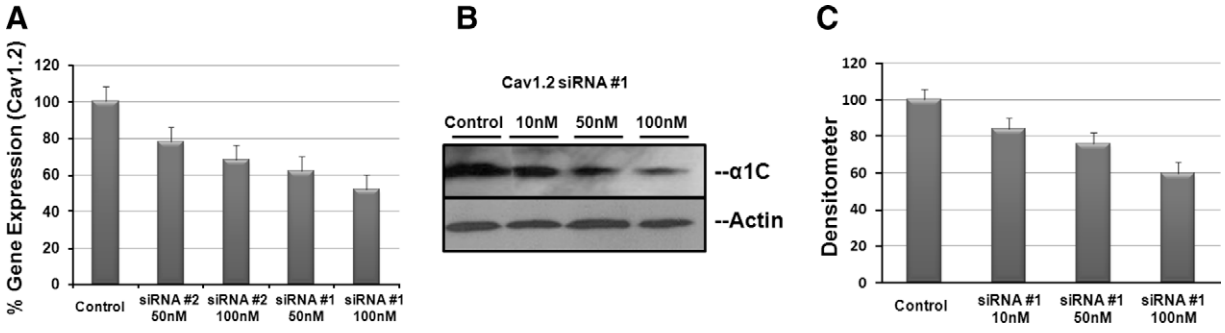

Fig. 1. Effect of Cav1.2 siRNA on Cav1.2 mRNA and protein. siRNA #1 and #2 purchased from Ambion were tested using lipofectamine as a transfection reagent on RNC. Fifty and 100 nM concentration were used from each of the above mentioned siRNA's. Forty-eight to 72 h post-transfection, RNA was extracted using TRIZOL reagent and subsequently reverse transcribed using Ambion retroscript kit. cDNA from control and silenced were assayed using real-time PCR using Taqman intron spanning primers (Applied Biosystem). siRNA #1 achieved the highest silencing effect reaching merely 50% (A). Similarly, proteins were extracted using RIPA buffer and resolved on 7.5% SDS–PAGE (B). The proteins were analyzed by densitometry (C) and the levels were comparable to the real-time PCR data shown in A.

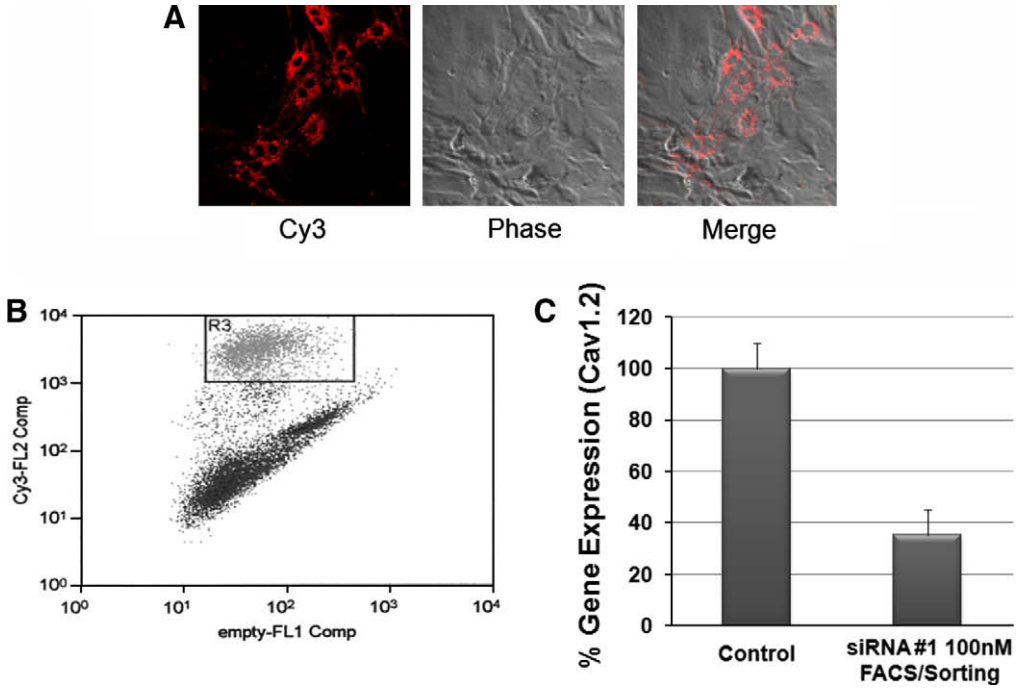

Fig. 2. Testing and optimizing transfection efficiency using cy3-labeled GAPDH siRNA. To measure the transfection efficiency, human GAPDH siRNA labeled with cy3 was co-transfected with siRNA #1 and immunofluorescent images were captured and analyzed for transfection efficiency. A shows 50–55% transfection efficiency. B shows the sorted cells co-transfected with cy3 labeled siRNA along with siRNA #1. Cells were sorted out using FACS, and tested using real-time PCR for the knockdown effect. C shows that the level of silencing reached 65% compared to control.

and siRNA#2) and were tested for efficacy. Lipid based reagent Lipofectamine 2000 (Invitrogen) was chosen as a choice for the delivery of the siRNA into the RNC. Different concentrations of siRNA #1 and #2 ranging from 10 to 100 nM were delivered at day 2–3 following the culture and plating of RNC. Forty-eight to 72 h later, the RNC were collected and analyzed using real-time PCR and Western blotting for the residual mRNA and protein content compared to scrambled siRNA controls. Fig. 1A shows the mRNA level for Cav1.2 using siRNA #1 and #2 at concentrations of 50 and 100 nM. Real-time PCR detected only limited silencing of Cav1.2 using siRNA#2 (up to 30%). In contrast siRNA#1 at 100 nM silenced approximately 50% of the Cav1.2 mRNA. Decrease in Cav1.2 protein (45%) was consistent with the decrease in the mRNA as shown in Fig. 1B and C.

Optimizing transfection efficiency by cotransfecting with human cy3-labeled GAPDH siRNA

The level of silencing achieved wih lipofectamine transfection as a delivery method for siRNA was low (50%) despite several attempts for optimization. We next used siRNA for GAPDH labeled with cy3 in the transfection experiments. Fig. 2A shows that the maximum achievable level of transfection was around 55% in the RNC. In order to collect only the enriched transfected cells, we cotransfected siRNA #1 along with human GAPDH cy3 labeled siRNA. The fluorescent assay cell sorter was used to sort out the fluorescent cells from the non-fluorescent cells (Fig. 2B). The isolated and transfected RNC were either replated or directly used to extract mRNA and tested for the level of silencing using real-time PCR. The reduction of Cav1.2 mRNA reached 65% with a residual 35% mRNA level remaining (Fig. 2C).

Lentivirus delivered shRNA construct of siRNA #1 efficiently transfected RNC

To achieve higher transfection levels, the lentivirus model was tested. Lentivirus shRNA was constructed from siRNA #1 as shown in Fig. 3A. Restriction sites were added to ends of the sense and anti-sense oligo's which contained a loop and a termination signal (Fig. 3A). The oligo's were inserted into pLL3.7 driven under a U6 promoter and contained GFP reporter under CMV promoter to detect the transfection efficiency simultaneously. The level of transfection achieved by the lentivirus construct was almost 100% as shown by immunoflourescent images in Fig. 3B. Next we sought to demonstrate the silencing of Cav1.2 gene. Fig. 3C, D, and E represent the mRNA and protein levels after silencing using different multiplicity of infection at day 3, 4, and 5. At day 4 and 5, more than 90% of Cav1.2 mRNA and more than 95% of the protein were silenced.

Residual L-type Ca current after Cav1.2 silencing

We examined functional consequences of the Cav1.2 silencing using the whole cell patch-clamp technique. Recording were obtained from GFP expressing RNC at days 4 and 5 as evidence for successful transfection. Fig. 4A and B show typical current record-

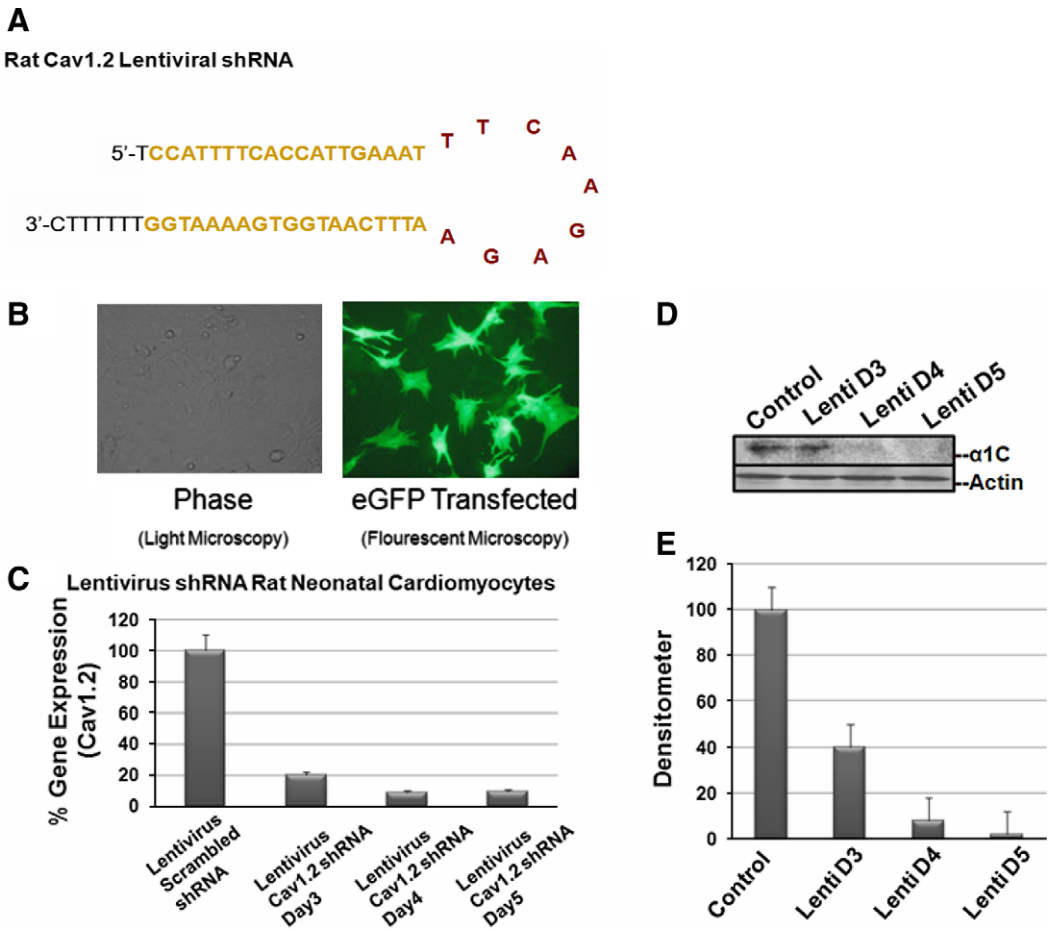

Fig. 3. Lentivirus construct and silencing effects in RNC. A shows the complete shRNA sequence of both sense and anti-sense complementary to each other along with the loop, restriction sites at both ends, and the terminal signal. B shows an immunofluorescent image for the reporter GFP expressed under a CMV promoter in RNC. The level of transfection approached the 100% mark. The level of mRNA after silencing is shown in C using real-time PCR. At day 4 and 5, more than 90% of the Cav1.2 mRNA was abolished. Similarly, D and E show the protein level using Western blotting and the corresponding densitometry, respectively.

ings from the control and the transfected cells. Fig. 4A shows a current–voltage relationship of $I_{Ca\text{-}L}$ density (7.1 ± 0.8 pA/pF at 0 mV) in control cells and following the silencing with the lentivirus/Cav1.2 construct ($I_{Ca\text{-}L}$ density was reduced to 2.5 ± 0.6 pA/pF at 0 mV). Fig. 4B shows representative $I_{Ca\text{-}L}$ traces elicited from a holding potential of −80 mV to a prepulse to −50 mV followed by voltage steps to 0 mV. The silenced current represents the residual Cav1.2 contribution (minimal) as well as Cav1.3 to the total $I_{Ca\text{-}L}$. Thus, we demonstrated a successful model for silencing Cav1.2 at both the molecular level (mRNA and protein) and at the functional level.

Discussion

This study is first to establish a model for efficient post-transcriptional silencing of a 240 kDa Ca channel protein (Cav1.2) that is ubiquitously and highly expressed throughout the heart. The results demonstrated a successful shRNA mediated selective silencing of Cav1.2 in primary cultured rat neonatal cardiomyocytes. Chemically synthesized siRNA had limited effects on the silencing of Cav1.2 Ca channel mostly due to difficulty in delivering the siRNA in primary cells. Using FACS sorting, we were able to increase the selection of transfected cells which achieved 65% silencing. When the lentivirus was used as a delivery method, 100% transfection efficiency and 92% silencing at the mRNA and protein level was achieved. To confirm these biochemical results, we functionally recorded the residual Cav1.2 current and showed that the total $I_{Ca\text{-}L}$ decreased to a 20% level representing the combined residual Cav1.2 (which is minimal post-silencing) and Cav1.3 (which is the main contributor in the silenced cells).

RNAi is a sequence specific post-transcriptional silencing of any gene of interest [17,18]. A limitation for the use of siRNA in primary cardiomyocytes is the low transfection efficiency that can be achieved with difficult delivery. Very few reports published the effective silencing of voltage-gated channels in primary cardiomyocytes [19,20]; none involving voltage gated L-type Ca channels. To achieve high efficiency of transfection, we generated a lentiviral vector carrying shRNA for Cav1.2. Driven under a U6 promoter, the shRNA is subsequently processed into siRNA in the cells and mediated the gene specific silencing.

Several reports show that the α_{1D} deletion causes sinus bradycardia and various degrees of AV-block [4,7,8]. This eludes to the critical role that Cav1.3 Ca channel plays in the diastolic depolarization of the SAN [7]. Cav1.3 Ca channel is expressed in the SAN, AVN and atria, but not in the ventricles of adult hearts [4]. We and others recently demonstrated that Cav1.3 Ca channel could play an important role in cardiac arrhythmias such as atrial fibrillation and heart block [16,21]. The challenge is that both Cav1.2 and Cav1.3 Ca channels contribute to the total $I_{Ca\text{-}L}$ [9] in the native cardiomyocytes, both are sensitive to the Ca channel blockers such as dihydropurydines and there is no biophysical method to functionally differentiate between Cav1.2 and Cav1.3 Ca channels. The use of transgenic mouse models has been limited due to embryonic lethality at E14.5 of the Cav1.2 knockout mouse [9].

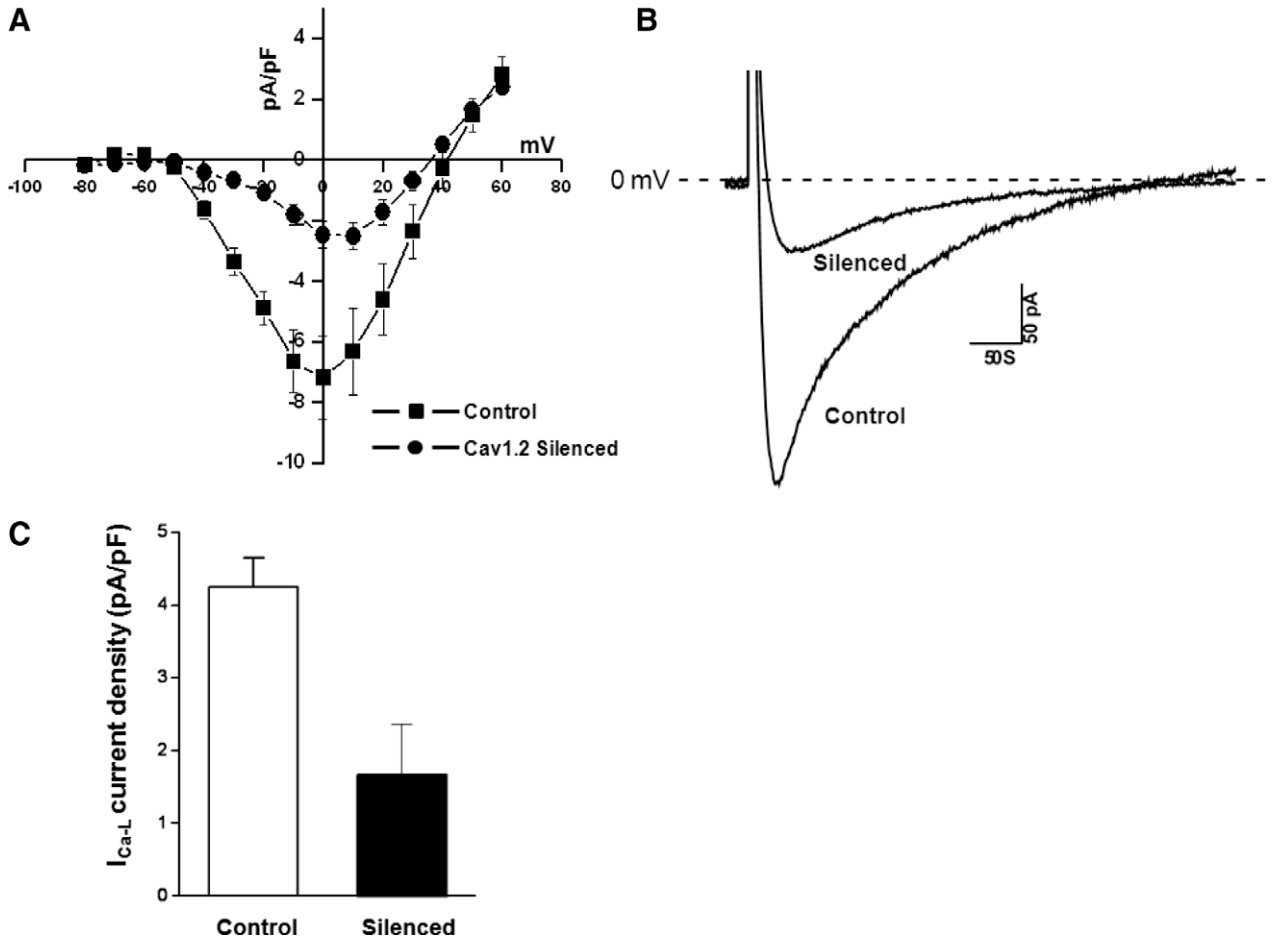

Fig. 4. Functional evidence of knockdown of Cav1.2 using the whole cell patch-clamp recordings. A shows a current–voltage relationship from control and silenced RNC. The current density decreased from from 7.1 pA/pF during control ($n = 8$) to 2.5 pA/pF in the silenced RNC ($n = 6$). B shows selected current traces from a control and a silenced cell. C shows the averaged data from the patch-clamp experiments. The residual current is a combination of Cav1.3 and the residual Cav1.2 after silencing.

Therefore, the study and characterization of post-natal Cav1.3 by Cav1.2 gene deletion is not possible.

The novel model reported in this study will allow for the investigation and characterization of the Cav1.3 Ca channels in physiological and pathological settings thereby elucidating the Cav1.3 role in cardiac arrhythmias such as atrial fibrillation.

Conflict of interest

No conflict to disclose.

Acknowledgments

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CHAPTER 3

Congenital Heart Block: Identification of Autoantibody Binding Site on the Extracellular Loop (Domain I, S5-S6) of α1D L-type Ca Channel

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Congenital heart block: Identification of autoantibody binding site on the extracellular loop (domain I, S5–S6) of α_{1D} L-type Ca channel

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ABSTRACT

Congenital heart block (CHB) is an autoimmune disease associated with autoantibodies against intracellular ribonucleoproteins SSB/La and SSA/Ro. The hallmark of CHB is complete atrioventricular block. We have recently established that anti-SSA/Ro -SSB/La autoantibodies inhibit α_{1D} L-type Ca current, $I_{Ca\text{-}L}$, and cross-react with the α_{1D} Ca channel protein. This study aims at identifying the possible binding sites on α_{1D} protein for autoantibodies from sera of mothers with CHB children. GST fusion proteins of the extracellular regions between the transmembrane segments (S5–S6) of each of the four α_{1D} Ca channel protein domains I–IV were prepared and tested for reactivity with sera from mothers with CHB children and controls using ELISA. Sera containing anti-Ro/La autoantibodies from 118 mothers with CHB children and from 15 mothers with anti-Ro/La autoantibodies but have healthy children, and from 28 healthy mothers without anti-Ro/La autoantibodies and healthy children were evaluated. Seventeen of 118 (14.4%) sera from mothers with CHB children reacted with the extracellular loop of domain I S5–S6 region (E1). In contrast, only 2 of 28 (7%) of sera from healthy mothers (−anti-Ro/La) and healthy children reacted with E1 loop and none (0 of 15) of sera from healthy mothers (+anti-Ro/La) and healthy children reacted with the E1 loop. Preincubation of E1 loop with the positive sera decreased the O.D reading establishing the specificity of the response. Electrophysiological characterization of the ELISA positive sera and purified IgG showed inhibition (44.1% and 49.8%, respectively) of the α_{1D} $I_{Ca\text{-}L}$ expressed in tsA201 cells. The inhibition was abolished when the sera were pre-incubated with E1 fusion protein. The results identified the extracellular loop of domain I S5–S6 of L-type Ca channel α_{1D} subunit as a target for autoantibodies from a subset of mothers with CHB children. This novel finding provides insights into the potential development of therapeutic peptides that could bind to the pathogenic antibodies and prevent CHB.

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1. Introduction

Autoimmune-associated congenital heart block (CHB) is a passively acquired autoimmune disease. Fetal injury is presumed to occur as a consequence of transplacental passage of maternal autoantibodies against the intracellular ribonucleoproteins 48-kDa SSB/La, 52-kDa SSA/Ro, and 60-kDa SSA/Ro [1]. The hallmark of CHB is characterized by irreversible atrioventricular (AV) block, not accompanied by any cardiac structural or anatomic abnormalities [1]. Sinus bradycardia has also been reported in animal models of CHB [2,3] and in the clinical settings [4,5]. Detection of CHB occurs at or after 16 weeks of gestation, when maternal antibodies effectively gain access to the fetal circulation. Because of the rarity and complex etiology of CHB, the incidence is not well established. A generally accepted mean incidence is 1:17,000 in the 1970s [6] and 1:11,000 in the latter decade [7,8]. However, this incidence dramatically increases to about 5% in Lupus patients and to 18% in subsequent pregnancies. This indicates that the incidence of CHB in the latter decades [7,9] was higher than previously reported likely due to more effective detection of CHB during pregnancy using fetal ultrasound and to the improved diagnostics. Current therapies include dexamethasone, plasmapheresis, sympathomimetics, and in-utero cardiac pacing. Unfortunately none have significantly altered mortality justifying the need for more basic and clinical investigations. CHB carries substantial morbidity and mortality approaching 30%, with 100% of affected children requiring lifelong pacemakers before entering adulthood [7,10].

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The association of anti-SSA/Ro -SSB/La autoantibodies with CHB is universally accepted. However, the mechanism by which these autoantibodies cause cardiac conduction abnormalities is not completely understood. Under physiologic conditions, the cognate antigens are normally intracellular and thus inaccessible to the circulating antibodies. Two plausible explanations can be considered: either the intracellular antigens SSA/Ro -SSB/La are trafficked to the cell surface or anti-SSA/Ro -SSB/La autoantibodies cross-react with a surface antigen on the sarcolemma of cardiomyocytes. A considerable amount of evidence has been proposed to explain the accessibility of the intracellular antigens and circulating antibodies. These include viral infection [11], ultraviolet light exposure [12] and apoptosis [13] which all lead to the translocation of SSA/Ro -SSB/La to the cell surface. The second possibility is that maternal antibodies react with cell surface components. To date, sarcolemmal proteins have been identified including the muscarinic receptor [14], laminin [15], the serotonin receptor 5-HT_4 [16], and the L-type Ca channels [2,17–19].

Cardiac L-type Ca channels are divided into α_{1C} and α_{1D} Ca channels. The α_{1D} Ca channel has been shown to localize exclusively to the supraventricular tissue including the conduction system [18,20,21] (SA and AV node) and has the biophysical properties that could account for its possible contribution to the diastolic depolarization and impulse generation in the SA node [18,20,21].

Accordingly, inhibition of the α_{1D} L-type Ca current ($I_{Ca\text{-}L}$) by anti-SSA/Ro -SSB/La antibodies from mothers with CHB children would be predicted to result in supraventricular electrocardiographic abnormalities. Indeed, knockout of the α_{1D} Ca channel in the mouse results in sinus bradycardia and AV block [18,20–24].

Ca channels have been identified as autoantigens and also as targets for autoantibodies in a number of diseases [25,26]. In this regard, we have recently established the direct interaction (inhibition) between the autoantibodies from mothers whose children have CHB and the α_{1D} Ca channel protein [18]. We proposed that the resulting inhibition of the α_{1D} $I_{Ca\text{-}L}$ by maternal autoantibodies may contribute to the electrocardiographic abnormalities seen in CHB [18,27]. The aim of the present study is to assess the possible antigenic sites in the α_{1D} Ca channel to maternal autoantibodies. Since antibodies, under physiological conditions, do not cross intact cellular membranes, we hypothesized that the anti-SSA/Ro -SSB/La autoantibodies cross-react with the extracellular region of the α_{1D} Ca channel protein.

2. Methods

2.1. Patients' sera

Sera were obtained from the U.S. based Research Registry for Neonatal Lupus established by the National Institute for Arthritis, Musculoskeletal and Skin Diseases (RRNL) previously described and characterized [7]. One hundred and sixty one sera were tested of which 118 were from mothers with anti-SSA/Ro -SSB/La antibodies and have children with CHB, 15 sera (with anti-SSA/Ro -SSB/La antibodies) from mothers whose children are healthy and did not have CHB and 28 from mothers (without anti-SSA/Ro -SSB/La antibodies) whose children are healthy and did not have CHB. The study was approved by the IRB and the subjects gave informed consent. IgG purification was performed as described previously [28].

2.2. GST fusion proteins

The Extracellular loops of I, II, III, IV Domains between S5–S6 (Fig. 1) were amplified from Human Cav1.3 pGFP- plasmid (kindly provided by Dr J. Striessnig, Innsbruck, Austria) using the following primers:

E1:
Forward: 5-CGCGGATCCATTGGAAAAATGCACAAAACA-3
Reverse: 5-CCGGAATTCCCATTCCCATCCTATCGCATC-3
E2:
Forward: 5-CGCGGATCCGGCAAGTTTAATTTTGATG-3
Reverse: 5-CCGGAATTCGATCATTCCTGAAGAGGATGG-3
E3:
Forward: 5-CGCGGATCCAAGGGGAAGTTCTATCGCTGT-3
Reverse: 5-CCGGAATTCCTCCACGCGGTGGTTGTAGAT-3
E4:
Forward: 5-CGCGGATCCTTTGGGAAAGTTGCCATGAG-3
Reverse: 5-CCGGAATTCAAAGTTGCTCCCACATGTAT-3

The E-Loops were then subcloned into an EcoR1/BamH1 sites in pGEX-6p1 vector (Amersham, Piscataway, NJ, USA). The sequence of all fusion proteins were verified by commercial sequencing (Genemed Synthesis, San Antonio, TX, USA). Recombinant vectors were screened for the presence of the E-Loops sizes of E1-264 bp, E2-162 bp, E3-270 bp, and E4-204 bp. Liquid cultures were induced by Isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.1–0.4 mM for 2 h, centrifuged and lysed using lysis buffer (2.5 mM ethylenediaminetetraacetic acid (EDTA) pH 7.4, 2% Triton X-100, 2% [v/v] with protease inhibitor cocktail III) and pulled down using 50% slurry of Glutathione Sepharose 4B. Samples were run on 15% bis-acrylamide gels and stained with coomassie blue and pictures taken using Kodak Imager 2000R (Carestream Health Molecular Imaging, New Haven, CT, USA).

2.3. ELISA

Enzyme-linked immunosorbent assays (ELISA) were performed as described previously [29] using purified GST fusion E-loop proteins (E1–E4) and recombinant SSA/Ro52 protein as control. Ten microgram per milliliter of each E-loops or SSA/Ro52 diluted in 1× PBS at a pH = 7.4 were incubated in 96-well ELISA plates overnight at 4 °C. The ELISA plates were blocked with 0.1% gelatin in PBS with 0.05% Tween, were incubated with sera diluted 1:10,000 in PBS with 0.05% Tween for 1 h followed by incubation with goat anti-human IgG alkaline phosphatase conjugate (Sigma, St. Louis, MO, USA), and were developed with disodium p-nitrophenyl phosphate in diethanolamine buffer. All samples were run in triplicates. Results are expressed as the optical absorbance at 405 nm minus that of the reagent blank. A result was considered positive if it was greater than 2 SD above the averaged value obtained for normal healthy donors.

In order to confirm the specificity of the positive sera in ELISA, an inhibition immunoassay was set up by titrating the positive sera pre-incubated with 10 μg/ml of the extracellular loops for 3 h at 37 °C. The samples were centrifuged at 14,000 rpm for 5 min and processed as in the ELISA procedure above. Concurrently, the extracellular loop S5–S6 of domain I was used in parallel to detect the optical density before and after the preincubation. All samples were done in triplicates.

2.4. Detection of extracellular fusion protein E1 using ELISA positive sera by Western blots

E1 GST fusion protein (5 μg) was resolved on a 15% SDS/PAGE. Blots were transferred, blocked with 5% milk and incubated with the ELISA positive sera (at 1:1000 dilution) identified in the ELISA at 4 °C overnight. After 5 washings with PBS-Tween, peroxidase conjugated secondary antibodies diluted 1:2000 in 1% milk in PBS were added for 1 h at room temperature. The blots were developed using ECL reagent (Amersham, Piscataway, NJ, USA).

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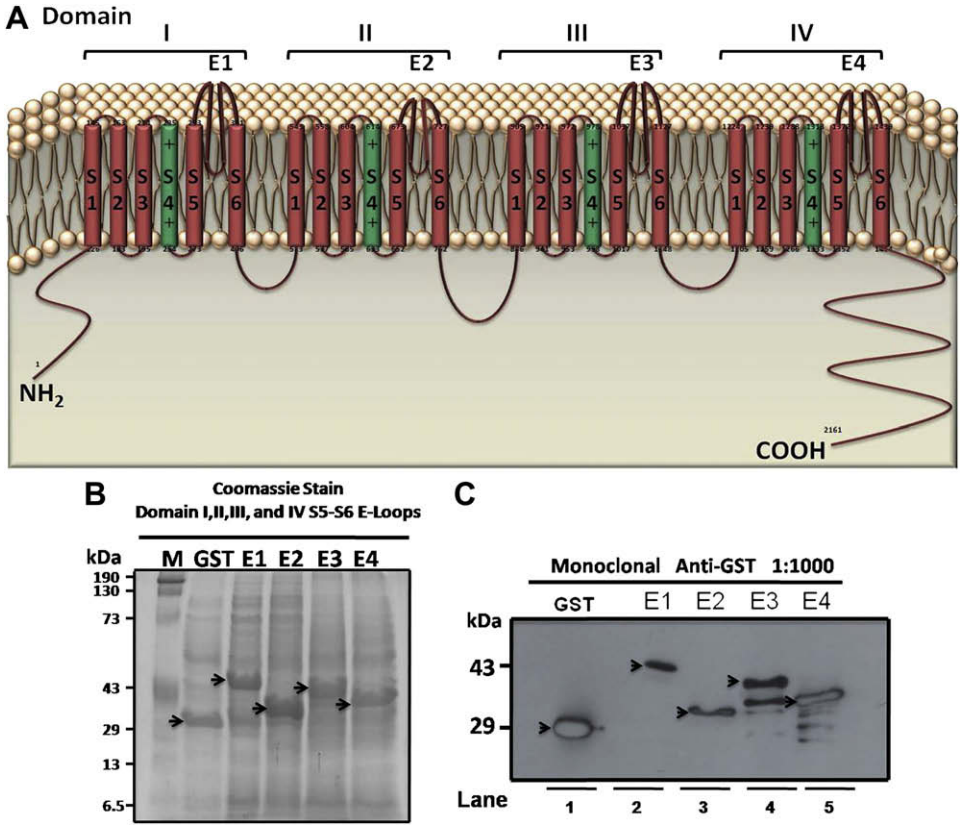

Fig. 1. Panel A: Schematic structure of L-type Ca channel α_{1D} subunit. α_1 subunit is organized in four domains, I–IV, each consisting of transmembrane segments S1–S6 connected with a small stretch of amino acids. The extracellular loop between transmembrane segments S5–S6, E-Loop, and is the ion conductance pore and selectivity filter. The 4 extracellular loops in each domain that were prepared and tested in this study are shown. Panel B: Coomassie stain of total extract from BL21 *Escherichia coli* following induction with 0.1 mM Isopropyl β-D-1-thiogalactopyranoside for 2 h, and the successful expression of the extracellular loops E1–E4 of representative domains I–IV S5–S6 segments. Panel C: Immunoblot of the extracellular proteins E1–E4 on 15% SDS-PAGE after pulldown using 50% slurry of Glutathione Sepharose 4B and probed with monoclonal anti-GST antibody at 1:1000 dilution.

2.5. Expression and electrophysiological recording of α_{1D} Ca current in tsA201 cells

tsA201 cells were grown in culture media consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml) as previously reported [18]. The cells were grown at 37 °C, 5% CO_2 and transiently transfected with 4 μg of a mix of α_{1D}, ß$_{2a}$, $\alpha_{2\delta}$, and lymphocyte surface antigen (CD8-a) cDNAs (in pCMV6b vector, kindly provided by Drs J. Striessnig, Innsbruck, Austria; S. Seino, Kobe, Japan) by the Ca phosphate method as described previously [30]. Cells expressing surface CD8-a fixed the beads and were visually distinguishable from non-transfected cells under the microscope. Whole-cell patch-clamp recording was performed with the Axopatch 200B (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA) at 48 h after transfection. The internal solution contained (in mmol/L): 135 CsCl, 4 $MgCl_2$, 4 ATP, 10 HEPES, 10 EGTA, and 1 EDTA, adjusted to pH 7.2 with tetraethylammonium hydroxide (TEAOH). The bath solution contained (in mmol/L): 135 choline chloride, 1 $MgCl_2$, 2 $CaCl_2$, and 10 HEPES, adjusted to pH 7.4 with TEAOH. Data were analyzed with pClamp version 9.0 (Axon Instruments). For α_{1D} $I_{Ca\text{-}L}$ current–voltage (I–V) relations, tsA201 cells were depolarized from a holding potential of −100 mV to test potentials between −80 and 60 mV with increments of 10 mV.

2.6. Statistical analysis

Statistical comparisons between groups were evaluated using unpaired student *t*-test, Unpaired *t*-test with Welch's correction, Mann Whitney test and one-way ANOVA as appropriate. Patch clamp data are expressed as mean ± SEM. *p* values less than 0.05 were considered significant.

3. Results

3.1. Epitope mapping in the molecular structure of α_{1D} L-type Ca channel: GST fusion proteins

Fig. 1A shows the schematic structure of the α_{1D} Ca channel subunit with the E-Loops in bold. The α_1 subunit of α_{1D} L-type Ca channel consists of 4 domains I–IV each with 6 transmembrane spanning segments referred to as S1–S6. Each domain has 3 extracellular loops between S1–S2, S3–S4, and S5–S6, with S5–S6 loop being the largest. The four S5–S6 loops (referred to as E-loops throughout) join together to form the pore of the channel and the selectivity filter. Successful expression of the E-loops is shown in Fig. 1B using total protein extract from transformed E-coli stained with coomassie blue and in Fig. 1C by Western blot using anti-GST antibody. Lanes1, 2, 3, 4 and 5 represent purified GST alone, E1, E2, E3, and E4 proteins respectively. All loops were tested in the ELISA experiments, however, since reactivity was only detected with E1 loops, further purification of E3 and E4 loops was not pursued.

3.2. Reactivity of extracellular loop E1 with patient sera using ELISA

Three sera groups were screened by ELISA for reactivity with the extracellular loops between S5–S6 (E-loops): Sera from 118 mothers whose children have CHB and are seropositive to SSA/Ro

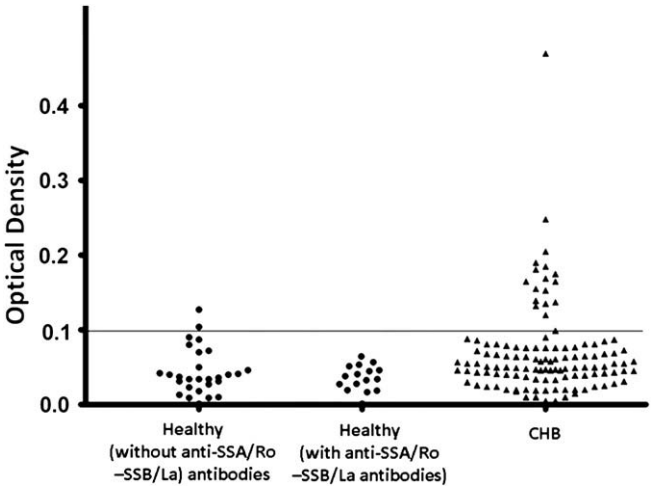

Fig. 2. ELISA results of 161 serum samples against the GST fusion proteins of the E1 loop of the α_{1D} L-type Ca channel. Seventeen of 118 (14.4%) samples from the CHB group were ELISA positive and above the O.D. cutoff point of 0.1 which represents the average for the healthy sera plus 2 standard deviation. In contrast, sera (with anti-SSA/Ro and SSB/La antibodies) from mothers with healthy children did not react (0/15) with the E1 loop, while in the sera (without anti-SSA/Ro and SSB/La antibodies) from mothers with healthy children, only 2 of 18 (7%) were ELISA positive.

-SSB/La antibodies in one hand and sera from 28 mothers whose children are healthy (no CHB) and are seronegative to SSA/Ro -SSB/La antibodies, and sera from 15 mothers whose children are healthy (no CHB) and are seropositive to SSA/Ro -SSB/La antibodies on the other hand. Seventeen of 118 (14.4%) CHB sera, 2 of 28 (7%) seronegative sera from mothers whose children are healthy and 0 of 15 (0%) seropositive sera from mothers with healthy children reacted with the E1 loop. The difference between the CHB and the other 2 control groups was statistically significant ($p < 0.05$; Unpaired t test $p = 0.0369$, Unpaired t-test with Welch's correction $p = 0.003$, and Mann Whitney test $p = 0.0088$). None (0%) of CHB samples or the healthy sera group reacted with GST protein alone, E2, E3, and E4. Fig. 2 shows the summary of the ELISA data with the horizontal line representing the cutoff of positive sera calculated by averaging the O.D. of the healthy sera plus two standard deviations. Although there were 2 positive sera from healthy controls, their O.D. value was low relative to the CHB positive sera.

3.3. *Specificity of ELISA positive sera using competition immunoassay*

A significant decrease of O.D was observed when ELISA positive sera (in Fig. 2) were pre-incubated with E1 protein, indicating the specificity of ELISA results. Fig. 3 shows the representative

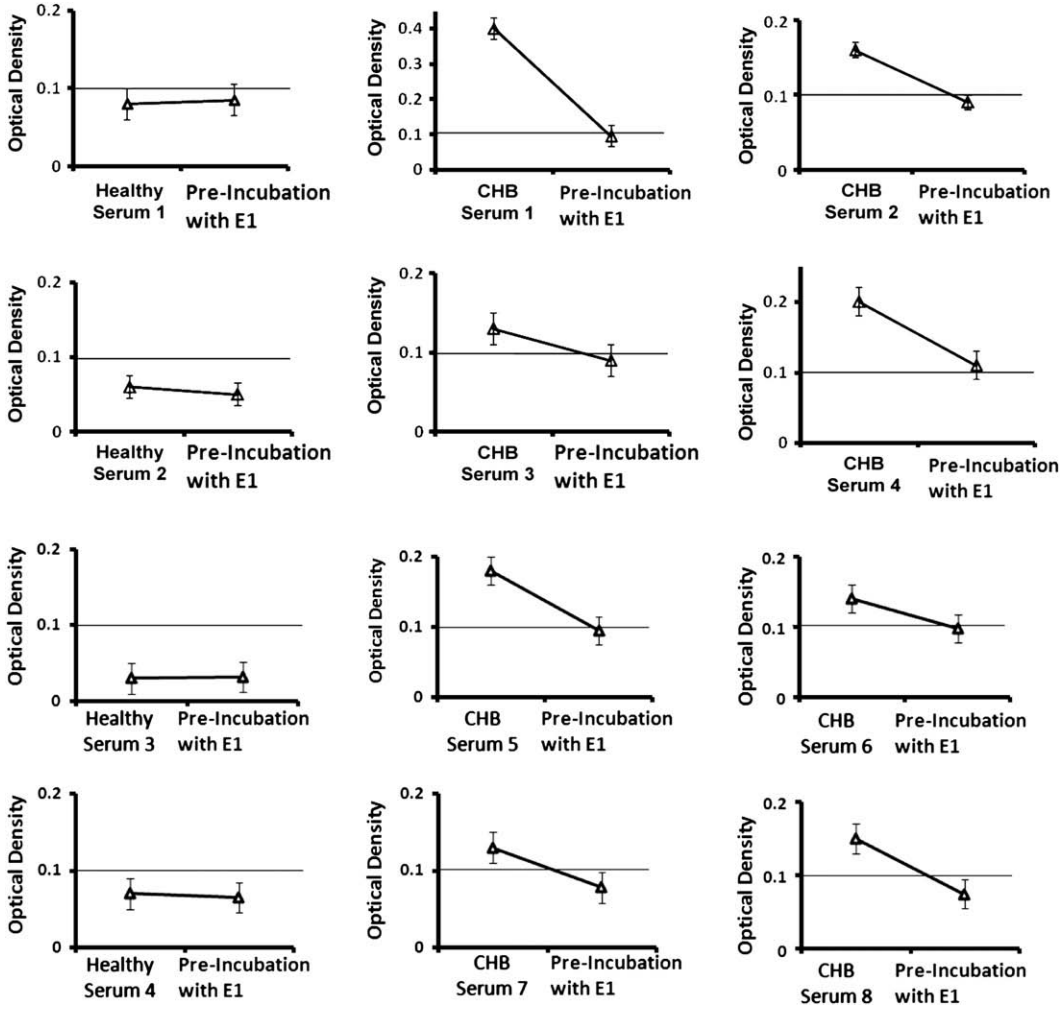

Fig. 3. Competitive ELISA assay using 4 healthy controls sera and 8 CHB samples with the highest O.D. out of the 17 ELISA positive sera in Fig. 2. Sera were incubated with 10 μg of E1 loop at 37 °C for 3 h, centrifuged and used as the probe in the ELISA experiment. The O.D. of each of the 8 CHB sera decreased by more than half to a value below the cutoff of 0.1 nm.

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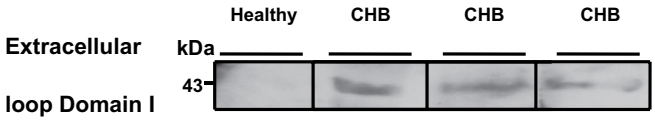

Fig. 4. Cross-reactivity of the ELISA positive sera with the E1 protein by Western blot. Five micrograms of the E1 protein was resolved on a 15% SDS-PAGE and probed using a healthy control serum and 3 ELISA positive sera with the highest O.D. from the congenital heart block (CHB).

O.D.'s from 4 control sera and 8 positive sera shown in Fig. 2, which after the preincubation resulted in an O.D. below the cutoff of 0.1 O.D. The inhibition immunoassay showed O.D. values that are 48–72% less than the original values in the ELISA experiments. In fact, all the CHB sera except CHB serum # 4 had an O.D. value below the cutoff of 0.1 after the inhibition immunoassay. Under the same conditions, the healthy sera (without anti-SSA/Ro and -SSB/La) showed very limited reactivity (2–8%). These results confirmed that the E1 loop is able to inhibit the reactivity of the anti-SSA/Ro and -SSB/La autoantibodies by 48–72% in the CHB sera group only.

3.4. Detection of reactivity of E1-loop with ELISA positive sera by Western blot

Further validation of the ELISA results was sought by Western blot. Three representative samples from the ELISA positive sera recognized the E1 GST fusion protein, whereas the ELISA negative sera did not react with the E1 loop (Fig. 4). The reactivity in the Western blot against the extracellular loop S5–S6 of domain I, further validates the ELISA results.

3.5. ELISA positive sera inhibited α_{1D} $I_{Ca\text{-}L}$ in tsA201 cells

We next tested whether the positive sera identified by ELISA can inhibit the α_{1D} Ca channel expressed in tsA201 cells. Mammalian tsA201 cells were used because α_{1D} $I_{Ca\text{-}L}$ cannot be separated biophysically or pharmacologically from α_{1C} $I_{Ca\text{-}L}$ in the native cardiac myocytes. ELISA positive sera inhibited the α_{1D} $I_{Ca\text{-}L}$ by 44.1% at −10 mV (control: -4.9 ± 1.1 pA/pF, $n = 7$ vs. -2.8 ± 0.6 pA/pF, $p < 0.05$, $n = 7$). Fig. 5A shows current–voltage relationships for α_{1D} $I_{Ca\text{-}L}$ density during control and with serum #1. Similarly, the application of purified IgG from serum #1 inhibited the α_{1D} $I_{Ca\text{-}L}$ by 49.8% at −10 mV (control: -5.49 ± 0.5 pA/pF, $n = 8$ vs. -2.75 ± 0.6 pA/pF, $p < 0.05$, $n = 8$) (Fig. 5B). Preincubation of the ELISA positive sera with 10 μg/ml E1 loop, prevented the inhibition of α_{1D} $I_{Ca\text{-}L}$ indicating the specificity of effect of ELISA positive sera on α_{1D} $I_{Ca\text{-}L}$, as shown in Fig. 5C. ELISA negative sera did not significantly affect $\alpha_{1D}I_{Ca\text{-}L}$ (control: -5.6 ± 1.3 pA/pF, $n = 6$ vs. healthy sera: -4.8 ± 1.4 pA/pF, $p =$ not significant (NS), $n = 6$); as shown in Fig. 5D. Table 1 shows the averaged data from 2 high O.D. ELISA positive sera and IgG vs. ELISA negative sera used in the patch clamp experiments.

4. Discussion

In this study, we expressed and purified GST fusion proteins corresponding to the extracellular loop S5–S6 of each of the four domains that form the pore of the Ca channel α_{1D} subunit and tested their reactivity with sera from mothers who have children with CHB. The results demonstrate that a fraction (14.4%) of sera containing anti-SSA/Ro and SSA/La autoantibodies from mothers whose children have CHB reacted specifically with the extracellular loop of S5–S6 of the first, but not the second, third or forth domain

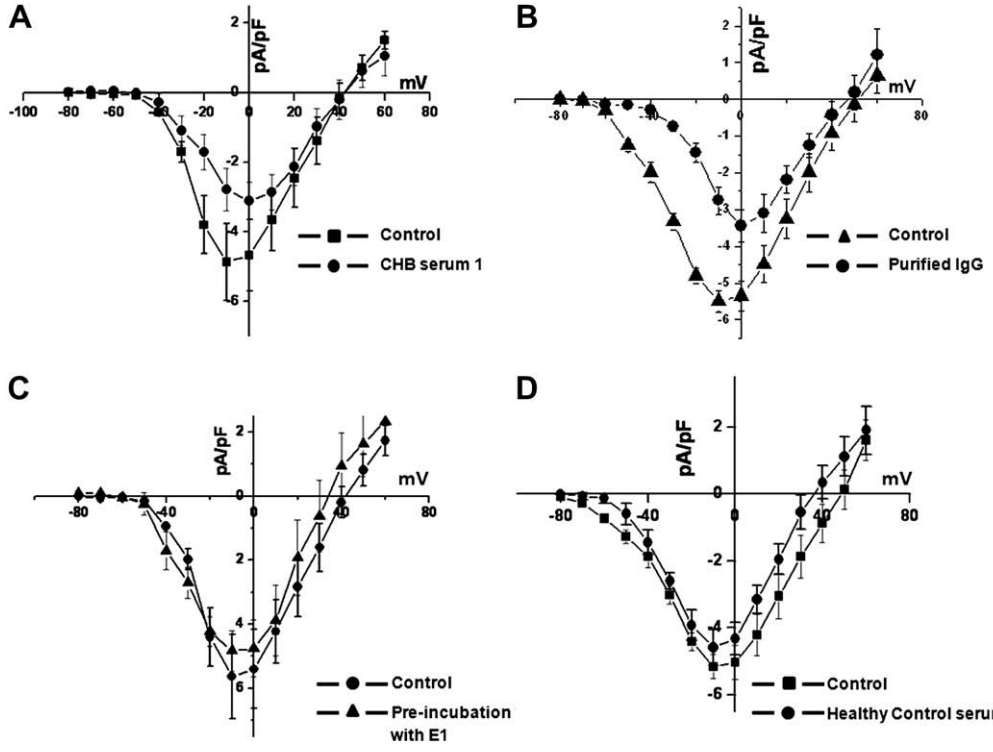

Fig. 5. A. Effect of ELISA positive sera on the α_{1D} $I_{Ca\text{-}L}$ expressed in tsA201 cells. α_{1D} $I_{Ca\text{-}L}$ was recorded using whole cell mode of the patch clamp technique with 2 mmol/L Ca as a charge carrier. Panel A shows the current–voltage relationships for the α_{1D} $I_{Ca\text{-}L}$ densities during control and in the presence of ELISA positive serum 1. Panel B shows the current–voltage relationships for the α_{1D} $I_{Ca\text{-}L}$ densities during control and purified IgG from serum 1. Panel C shows the current–voltage relationships of the α_{1D} $I_{Ca\text{-}L}$ densities before and after the application of pre-incubated serum 1 with E1 fusion protein. Panel D shows the current–voltage relationships for the α_{1D} $I_{Ca\text{-}L}$ densities before and after application of the healthy control serum.

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Table 1
Peak current densities for α_{1D} transfected tsA201 cells before and after the addition of respective sera.

	No antibody	With antibody
	Density pA/pF	Density pA/pF (*P*-value)
CHB serum 1 ($n = 7$)	-4.9 ± 1.1	-2.8 ± 0.6 ($p = 0.021$)
CHB serum 2 ($n = 6$)	-5.2 ± 0.4	-3.6 ± 0.4 ($p = 0.044$)
Purified IgG ($n = 8$)	-5.49 ± 0.5	-2.75 ± 0.6 ($p = 0.036$)
Healthy ELISA negative serum ($n = 6$)	-5.6 ± 1.3	-4.8 ± 1.4 ($p = 0.49$)

of the α_{1D} subunit as demonstrated by both ELISA and Western blots. Furthermore, the ELISA positive sera inhibited the expressed α_{1D} Ca current in tsA201 cells. The presence of anti-α_{1D} Ca channel antibodies in the sera of mothers with CHB children suggest that additional risk factors may contribute to the pathogenesis of CHB.

4.1. Relevance of α_{1D} L-type Ca channel to CHB

In previous publications, we and others established an active [2,28] and passive [3] animal model of CHB, reproduced the clinical complete AV block in isolated Langendorff perfused fetal hearts [2,17], and correlated these findings with maternal anti-SSA/Ro -SSB/La autoantibodies' specific inhibition and cross-reactivity with the L- and T-type Ca channel [18,27] without affecting other ion channels such as Na and K channels [17,27] indicating specificity for Ca channels. We showed that maternal antibodies' inhibition was higher for α_{1C} and α_{1D} Ca channels (from 40 to 60%) compared to α_{1H} T-type Ca channels (19%) [2,27]. This is consistent with the higher (70%) homology between the E1 loop of the α_{1D} Ca channel and that of the α_{1C} Ca channel compared with only 48% homology between E1 loop of the α_{1D} Ca channel and that of the α_{1H} T-type Ca channel. Based on these experimental findings, we proposed that L-type Ca channels play a major role in the pathogenesis of CHB.

In the cardiovascular system, voltage-gated L-type Ca channels are essential for the generation of normal cardiac rhythm, for induction of rhythm propagation through the SA node, AV node and for the excitation-contraction coupling of the atrial and ventricular muscles [31]. Cardiac L-type Ca channels are heteroligomeric complexes of up to four subunits: α_1, β, α_2/δ subunits. The α_1 subunit contains the voltage sensor domain, the selectivity filter, the ion-conducting pore, and the binding sites for the Ca channel blockers [31]. Interestingly, in the heart, the expression of α_{1D} L-type Ca channel is restricted to the SA node, AV node and atria, and is not expressed in the adult ventricles [18,20,21] consistent with a unique and discrete role in the supraventricular tissue. Moreover, genetic deletion of the α_{1D} Ca channel in the mouse, leads to various degrees of AV block and sinus bradycardia [21], phenotypes similar to those found in children with CHB. In this regard, we recently demonstrated that autoantibodies from mothers with CHB children inhibited the α_{1D} Ca current expressed in tsA201 cells by binding directly to the α_{1D} Ca channel protein [18]. However, the exact binding site (s) of these autoantibodies on the α_{1D} protein was unknown.

4.2. Pathogenic antibodies and sarcolemmal antigens

In this study, we show that a fraction of sera containing anti-SSA/Ro and SSA/La autoantibodies from mothers whose children have CHB reacted with the extracellular loop of S5–S6 of the first domain of α_{1D} Ca channel protein and inhibited α_{1D} Ca current.

Although only 17 of 118 (14.4%) sera from mothers with CHB children reacted with the E1 loop, the difference between CHB and control healthy group was statistically significant. This level of reactivity is similar to that reported by Kamel et al. [29] against a peptide corresponding to a portion of the extracellular loop of the sarcolemmal 5-HT_4 receptor. Specifically, they showed that of the 75 sera from mothers of children with CHB, 12 were reactive (16%) with the 5-HT_4 peptide. Takamori et al. [32] showed that 6 sera samples (20%) from 30 Lambert–Eaton Myasthenic syndrome patients reacted to synthetic peptides against Ca channels using immunoprecipitation, 5 of which were positive for antibodies to domain II S5–S6 linker peptide. It is not completely clear why the reactivity is infrequent in this study and the other studies cited above [29,32]. One plausible explanation for these observations is that the pathogenesis of CHB is multifactorial. In this regard, the low incidence of CHB and the universal association of anti-SSA/Ro -SSB/La antibodies with CHB suggest that maternal autoantibodies are necessary but not sufficient to cause the disease. The fact that no reactivity (0/15) with seropositive sera from mothers with healthy children was detected to the E1 loop in the ELISA experiment points to the potential involvement of other factors such us fetal, maternal and environment factors individually or in combination which may be necessary for the full expression of the disease.

4.2.1. Proposed pathogenesis of CHB

Considerable progress has been made in understanding the pathogenesis by which anti-SSA/Ro and SSB/La autoantibodies mediate CHB [33–35]. One critical challenge is that SSA/Ro -SSB/La autoantigens are intracellular proteins and are inaccessible to the circulating autoantibodies under physiological conditions. Apoptosis is one proposed mechanism for the SSA/Ro -SSB/La antigen translocation to the surface of cardiomyocytes where they are accessible to maternal anti-SSA/Ro -SSB/La autoantibodies to initiate an inflammatory response and subsequent activation of cardiac fibroblasts [36]. Although apoptosis in the normal conduction tissue has been shown to occur [36], the percentage of myocytes undergoing this physiological deletion during embryogenesis remains minimal albeit apoptotic cells are normally cleared rapidly thus limiting detection. Others have also proposed that abnormal intracellular Ca homeostasis followed by cell death is another plausible mechanism in the pathogenesis of CHB [37]. Here, we propose that circulating maternal autoantibodies bind to and react with an extracellular portion of L-type Ca channels which are known to play an essential role in the electrogenesis and conduction at the SA and AV node of the human fetal heart [20]. These autoantibodies bind to the extracellular region of Ca channels and inhibit the vital entry of Ca needed for SA and AV nodes action potential genesis, conduction and excitation-coupling of the fetal myocytes during development. We speculate that this chronic inhibition may lead to internalization of the channel thereby decreasing the channel density at the cell surface which in turn may lead to the abnormal intracellular Ca levels and to the subsequent cell death. Cell death *per se* can lead directly to fibrosis and calcification of the conduction system and/or to the surface expression of the autoantigens SSA/Ro and SSB/La which can then be accessible to their cognate antibodies triggering an inflammatory reaction, fibrosis and calcification of the conduction system.

4.3. Therapeutic significance

The identification of the necessary or essential factors is only part of the challenge in defining the pathology of CHB. The potential novel therapeutic development in identifying the epitope(s) that could bind to the pathogenic autoantibodies is obvious. In this study, preincubating E1 fusion protein with ELISA positive patients' sera abolished the sera's inhibition of α_{1D} Ca current in tsA201 cells. These results may lead to the development of short peptides that could serve as a decoy to the maternal autoantibodies in the fetal circulation thereby preventing the interaction with the Ca channel in the fetal cardiac cells and subsequently prevent the development of CHB.

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5. Study limitations

The inability to detect reactivity to S5–S6 loops of domain II, III, or IV as well as the low cross-reactivity to S5–S6 loop of domain I could be due to the possibility that the small fusion proteins are unable to assume the antigenic conformation required for recognition by the autoantibodies as occurs in native cardiac cells. Plating of fusion proteins to the ELISA plates might thus be a limiting factor. The peptides could attach at different angles and expose different epitopes. We recognize that the complexity of the etiology and pathogenesis of CHB makes it challenging and difficult to account for all the clinical aspects of CHB by the "Ca channel hypothesis" or "the apoptosis hypothesis" and that further studies are warranted.

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CHAPTER 4

Rescue and Worsening of Congenital Heart Block Associated Electrocardiographic Abnormalities in Two Experimental Transgenic Mice

Rescue and Worsening of Congenital Heart Block-Associated Electrocardiographic Abnormalities in Two Experimental Transgenic Mice

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Abstract

Rationale: Congenital heart block (CHB) is a passively acquired autoimmune disease due to the transfer of maternal autoantibodies anti-SSA/Ro –SSB/La to the fetus resulting in atrioventricular (AV) block and sinus bradycardia. We previously established a murine model for CHB where pups born to immunized mothers demonstrated electrocardiographic abnormalities similar to those seen in CHB and demonstrated inhibition of L-type Ca channels (LTCC) by maternal antibodies. **Objective:** Here, we hypothesize that overexpression of LTCC should rescue, whereas knockout of LTCC should worsen the electrocardiographic abnormalities in mice. **Methods and Results:** Transgenic (TG) mice overexpressing and missing (knockout) LTCC were immunized with SSA/Ro and SSB/La antigens. Pups born to immunized WT mothers had significantly greater sinus bradycardia and AV block compared to pups from nonimmunized WT. TG pups overexpressing LTCC and born to immunized mothers had significantly less sinus bradycardia and AV block compared to their non-TG littermates and to pups born to immunized WT mothers. All LTCC knockout pups born to immunized mothers had sinus bradycardia, advanced degree of AV block and decreased fetal parity. No sinus bradycardia or AV block were manifested in pups from control nonimmunized WT mothers. **Conclusions:** Cardiac specific overexpression of LTCC significantly reduced the incidence of AV block and sinus bradycardia in pups exposed to anti-SSA/Ro - SSB/La autoantibodies; whereas exposure of LTCC knockout pups to these autoantibodies significantly worsened the electrocardiographic abnormalities. These findings support the hypothesis that maternal antibodies inhibit LTCC and contribute to the development of CHB. Altogether, the results are relevant to the development of novel therapies for CHB.

Key Words

Autoantibody, immunization, Calcium channel, neonatal lupus.

Introduction

Congenital heart block (CHB) detected at or before birth in a structurally normal heart is associated with autoantibodies against the intracellular ribonucleoproteins SSA/Ro 52 kDa, SSA/Ro 60 kDa, and SSB/La 48 kDa [1]. The source of the autoantibodies is mothers with rheumatic diseases such as Systemic Lupus Erythrematosus (SLE) or Sjogren Syndrome (SS) or from asymptomatic mothers harboring the autoantibodies [1]. The autoantibodies gain access to the fetal circulation by crossing the placenta and cause injury to different organs such as the heart, liver, and skin [2]. CHB is usually detected between 16 to 24 weeks of gestation, with varying degrees of atrioventricular (AV) block and sinus bradycardia [3-6]. Complete AV block or third degree AV block is irreversible resulting in 30% mortality with 100% of the infants requiring lifelong pacemakers [7].

The autoantigens SSA/Ro and SSB/La are intracellular and are inaccessible to the circulating autoantibodies suggesting that the autoantibodies cross react with a protein (s) on the sarcolemma of cardiomyocytes [8-11]. A considerable amount of evidence explains the accessibility of the intracellular antigens to the circulating antibodies. These include viral infection [12], ultraviolet light exposure [13] and apoptosis, [14] all of which lead to the translocation of SSA/Ro -SSB/La to the cell surface. The second possibility is that maternal antibodies react with cell surface components. Sarcolemmal proteins have been identified including the muscarinic receptor [15], laminin [8], the serotonin receptor, 5-HT_4 [9], and the L-type Ca channels (LTCC) [10, 11, 16, 17].
Two subtypes of cardiac LTCC are encoded by Cav1.2 and Cav1.3 pore-forming subunits [18-20]. Cav1.2 is ubiquitously expressed in the heart and mediates cardiac excitation-contraction coupling. Cav1.3 on the other hand is highly expressed in the sinoatrial (SAN) and AV node (AVN) [21, 22]. Cav1.2 Ca current (I_{Ca-L}) activates at –40 to –30 mV and accounts for the electrogenesis of the action potential at the AVN, whereas Cav1.3 I_{Ca-L} activates between –60 to –40 mV, at a range corresponding to the diastolic depolarization in the SAN [23]. Interestingly, the knockout mice for Cav1.3 exhibit sinus bradycardia and AV block; a phenotype similar to that seen in the clinical CHB [21, 22, 24-26].

A pathological interaction between LTCC and anti-SSA/Ro and –SSB/La autoantibodies is well documented [10, 11, 27-32]. IgG from mothers with CHB children inhibited I_{Ca-L} and this inhibition could account for the AV block and sinus bradycardia seen in CHB [11, 27-33]. Impulse generation in the SAN and conduction through the AVN critically depends on I_{Ca-L} [10, 21, 22].

A murine model for CHB has been established by the immunization of WT female mice with recombinant SSA/Ro or SSB/La proteins [16, 34]. Electrocardiographic abnormalities included sinus bradycardia and various degrees of AV block (I°, II°, and III°, with the latter being quite rare) [16, 34].

In this study, two transgenic (TG) mouse models were utilized: cardiac specific overexpression of Cav1.2 LTCC and knockout of Cav1.3 LTCC. In the overexpressed Cav1.2 LTCC, the Cav1.2 mRNA was increased by 2.8 fold, the protein level was increased by 2.7 fold, and I_{Ca-L} density increased by 1.5 fold compared to WT littermates [35, 36]. We hypothesize that the increase in the Cav1.2 I_{Ca-L} density should minimize the inhibition caused by the autoantibodies and subsequently reduce or prevent the manifestation of the electrocardiographic abnormalities seen in the pups of immunized mice. Although the knockout of Cav1.3 *per se* results in sinus bradycardia and AV block, not all pups are affected [21, 22, 25, 26, 37]. We further hypothesize that because Cav1.3 knockout mice already exhibit cardiac phenotypes of CHB, immunization should worsen the electrocardiographic abnormalities in the pups. To test the above hypotheses, we immunized female WT and two TG mouse models, Cav1.2 and Cav1.3 with recombinant

SSA/Ro52, SSA/Ro60 and SSB/La48. After mating and the establishment of an immune response, electrocardiographic recordings were assessed in the pups.

Methods

Animal Protocol: All experiments were performed in accordance with the IACUC at the VA New York Harbor Healthcare System and conform to the NIH guidelines (NIH Publication 86-23).

Preparation of recombinant antigens: Recombinant full length human SSA/Ro52, SSA/Ro60, and SSB/La48 were prepared as previously described [34].

Immunization: FVB and C57BL/6 mice were selected in this study because of the established FVB mice that overexpress Cav1.2 LTCC [35], and C57BL/6 mice because of the established C57BL/6 Cav1.3 LTCC knockout mice [26]. For details see supplementary methods.

Groups of mice and their genotype: Six groups of mice were utilized as shown in the table below:

GROUPS (Mothers)	***GENOTYPE (Pups)***
Control Wild Type Nonimmunized (FVB)	WT
Wild Type Immunized (FVB)	WT
Cav1.2 Overexpression Nonimmunized (FVB)	TG, NTG
Cav1.2 Overexpression Immunized (FVB)	TG, NTG
Cav1.3 KO Nonimmunized (C57BL/6)	KO, Heterozygous, NTG
Cav1.3 KO Immunized (C57BL/6)	KO, Heterozygous, NTG

WT: Wild Type; TG: Transgenic; NTG: Non-Transgenic; KO: Knockout.

Enzyme-Linked Immunosorbent Assay (ELISA): The recombinant proteins were coated overnight, washed, blocked with 3% BSA/PBST and probed in diluted serum followed by anti-mouse IgG conjugated to alkaline phosphatase. An O.D. value was considered positive if it was > 2 SD above the value obtained for controls. For details see supplementary methods.

Electrocardiographic recordings: One to three days after delivery, electrocardiograms were recorded [33, 34]. Recordings were analyzed for heart rate, PR interval, and conduction abnormalities. For details see supplementary methods.

Data Analysis: Statistical comparisons were evaluated using either unpaired Student's t-test or ANOVA. Data are presented as means ± SE. A value of $P < 0.05$ is considered significant.

Results

Establishment of autoantibody responses

The active model CHB was established by immunizing female WT FVB, C57BL/6, and the two TG lines (Cav1.2 and Cav1.3) with a combination of SSA/Ro52, SSA/Ro60, and SSB/La48 antigens. A total of 5 control WT nonimmunized FVB mothers, 8 WT immunized FVB mothers, 5 nonimmunized Cav1.2 overexpression mothers, 30 immunized Cav1.2 overexpression mothers, 8 nonimmunized Cav1.3 knockout C57BL/6 mothers, and 29 immunized Cav1.3 knockout mothers gave birth to a total of 520 pups. To ensure that the antibodies were generated in the mothers and crossed the placenta to the pups, mothers were bled and the sera obtained were tested separately for reactivity to each of the recombinant antigens by ELISA.

Both the immunized mothers and the pups had high levels of antibodies reactive with SSA/Ro52, SSA/Ro60, and SSB/La48 confirming the generation and transfer of the antibodies from the mother to the pups. All immunized groups (mothers and pups) had high titers of antibodies. Specifically, the immunized Cav1.2 overexpression (n=30 mothers; n=199 pups) had an O.D. ranging from 0.825-1.643 and the immunized Cav1.3 KO (n=29 mothers; n=101 pups) had an O.D between 0.734-1.790 (See Supplementary Table 1). In contrast, no detectable antibody levels were present in the mothers (n=5) and pups (n=48) of control nonimmunized WT (O.D. <0.095) and in the mothers (n=8) and pups (n=57) of control nonimmunized Cav1.3 (O.D. < 0.077, Supplementary table 1). The antibody titer was followed every 10 days following the primary injection of the mothers to ensure that the titer remained high throughout the experimental period. Within 10 days from the first injection, the titer was high (0.85-0.98) and remained high (1.15-1.75) with continuous boosters given every 3 weeks.

Electrocardiographic parameters of pups from the immunized WT mice

Electrocardiographic abnormalities in WT pups born to immunized mothers include sinus bradycardia and varying degrees of AV block [32-34]. In this study, we immunized WT mice to serve as a control for the two TG mice to detect improvement or worsening of conduction abnormalities. Table 1 and supplementary table 2 summarize the electrocardiogram parameters in the WT groups. The WT nonimmunized FVB (n=48), had no electrocardiographic abnormalities (neither sinus bradycardia nor AV block). In the WT immunized FVB, eight mothers gave birth to 76 pups of which 19.7% had sinus bradycardia and 25 % had AV block (10 out of 76 had I° AV block, 6 had II° AV block, and 3 had III° AV block).

Electrocardiographic parameters of pups from the immunized mice overexpressing Cav1.2 L-type Ca channel

Table 1 summarizes the electrocardiographic measurements for the Cav1.2 overexpression. In the immunized Cav1.2 overexpression, 30 mothers gave birth to 90 TG pups with overexpressed Cav1.2 and 109 NTG pups. The Cav1.2 overexpression TG pups had significantly fewer abnormalities [10% ($p<0.05$) sinus bradycardia and 8.8% ($p<0.05$) AV block] compared to the NTG littermates and pups from immunized WT mothers. Of these, 7 had I° AV block, 1 had II° AV block, and 0 had III° AV block. Of the 109 NTG pups, 22% had sinus bradycardia and 27.5% had AV block with 24 pups exhibiting I° AV block, 4 pups II° AV block, and 2 pups III° AV block.

Figure 1 (and supplementary table 2) summarizes the heart rates and PR intervals. TG pups from the immunized Cav1.2 overexpression mothers showed significantly fewer abnormalities than pups from the immunized WT mothers. The average heart rate and PR interval of TG pups from the immunized Cav1.2 overexpression was 315±36 bpm (n=90) and 48±10 ms (n=90) respectively. The average heart rate and PR interval of WT pups from the immunized WT mothers was 282±32 bpm (n=76) and 64±14 ms (n=76) respectively. Interestingly, NTG littermate pups (n=109) born to the same immunized Cav1.2 overexpression mother were not protected and showed sinus bradycardia (268±21 bpm) and prolonged PR interval (71±12 ms).

Figure 2 shows representative electrocardiograms from WT pups and from Cav1.2 overexpression pups. Panel A represents a control ECG from a pup born to a nonimmunized WT mother with a normal sinus rate of 454 bpm and a PR interval of 46 ms. Panel B shows electrocardiographic abnormalities in the pups as a result of immunization of WT mothers, including II° AV block (upper trace of panel B) and III° AV block with complete AV dissociation (lower trace of panel B). Panel C shows the fewer electrocardiographic abnormalities in TG pups from immunized mothers overexpressing Cav1.2: normal ECG (upper trace of panel C) and sinus bradycardia (296 bpm) with I° AV block (PR interval of 70 ms, lower trace of panel C).

Electrocardiographic parameters of pups from the immunized Cav1.3 L-type Ca channel knockout mice

Table 2 summarizes the data obtained from the Cav1.3 knockout mice (C57BL/6). The 8 nonimmunized Cav1.3 knockout mice gave birth to 57 pups of which 9 were knockouts, 28 heterozygous, and 20 NTG littermates. No electrocardiographic abnormalities were found in NTG pups (n=20) from the nonimmunized Cav1.3 knockout mothers. Heterozygous pups (n=28) from the nonimmunized Cav1.3 knockout mothers had 3.6% bradycardia and 3.6% AV block (one I° AV block). However, the knockout pups (n=9) from the nonimmunized Cav1.3 knockout mothers had 100% sinus bradycardia and 100% AV block (8 I°AV block, 1 II° AV block, and 0 III° AV block).

The 29 immunized Cav1.3 knockout mothers gave birth to a total of 101 pups of which 16 were knockout pups, 52 were heterozygous pups, and 33 NTG pup littermates (Table 2). Sinus bradycardia occurred at a rate of 18.2% in NTG pups, 34.6% in heterozygous pups, and 100% in knockout pups. Eighteen percent of NTG pups had AV block which included 5 I° AV block, 1 II° AV block, and 0 III° AV block. The heterozygous pups had 42.3% AV block, which included 16 I° AV block, 5 II° AV block, and 1 III° AV block. The knockout pups on the other hand had 100% AV block with 9 I° AV block, 4 II° AV block, and 3 III° AV block. Overall, pups from the immunized Cav1.3 knockout mothers had the most severe degrees of AV block, the highest of all groups with 3 out of 16 pups (19%) presenting III° AV block.

Figure 3 (and supplementary table 2) summarizes the average heart rates and PR intervals. The Cav1.3 knockout pups from the immunized Cav1.3 knockout mothers had severe sinus bradycardia (232±30 bpm; n=16), and prolonged PR intervals (112±21 ms, n=16) compared to the Cav1.3 knockout pups from the nonimmunized Cav1.3 knockout mothers (sinus bradycardia was 265±33 bpm and PR-intervals were 93±15 ms, n=9).

Figure 4 shows representative electrocardiograms from pups born to the immunized Cav1.3 knockout mothers. Panel A shows a control electrocardiogram with a heart rate of 442 bpm and a PR interval of 48 ms from a NTG pup born to nonimmunized Cav1.3 knockout mother. Panel B shows sinus bradycardia (266 bpm) with I° AVB (PR interval 96 ms) from a Cav1.3 knockout pup born to nonimmunized Cav1.3 knockout mother. Panel C shows worsening degrees of AV block (III°) from a Cav1.3 knockout pup born to immunized Cav1.3 knockout mother.

Fetal Parity

The fetal parity was significantly lower ($p<0.05$ using ANOVA) in the immunized Cav1.3 knockout mothers (Figure 5). Immunized Cav1.3 knockout mothers had the lowest fetal parity (3.2±0.8 litters per pregnancy) likely due to *in utero* death related to bradycardia and AV block. Furthermore, in 18 out of 31 births, dead pups were delivered by the immunized Cav1.3 knockout

mothers. However, the fetal parity in the nonimmunized and immunized WT and/or Cav1.2 overexpression mothers ranged between 5.83±1.2 and 6.3±1.6 litters per pregnancy (p=NS).

Discussion

In this study, overexpression of Cav1.2 L-type Ca channel in mice significantly reduced electrocardiographic abnormalities and the knockout of Cav1.3 L-type Ca channel significantly worsened the electrocardiographic abnormalities in pups born to immunized mothers. Together, these findings support the hypothesis that L-type Ca channels play an important role in the pathogenesis of CHB.

Immunization

A main requirement in establishing an immune response is the demonstration of high antibody response in the mothers and the placental transfer of the antibody to the pups. All immunized mice developed a high titer of antibodies for each of the recombinant antigens SSA/Ro52, SSA/Ro60, and SSB/La48 (supplementary table 1). The placental transfer of the antibodies from the mothers to the pups was demonstrated (supplementary table 1).

Immunization and overexpression of Cav1.2 L-type Ca channel

Ca channels are functionally inhibited by maternal autoantibodies, and these antibodies interact directly with the Ca channel [11, 16, 29, 30]. We hypothesize that if inhibition of the Ca current is critical in cardiac conduction disorders found in CHB patients, then upregulation of the Ca current should rescue or reverse the electrocardiographic abnormalities seen in CHB. The cardiac specific overexpression of the Cav1.2 α_{1C} subunit of the L-type Ca channel was used. Detailed molecular, hemodynamic and electrophysiological characteristics of these TG mice are reported elsewhere [35]. Briefly, these TG mice had no overt phenotype by observation, and litter sizes and pup survivals were similar to non-TG littermates. Cav1.2 α_{1C} transcript was increased by 2.8 fold without any changes in the accessory subunits, $\alpha 2/\delta$ and β subunits of L-type Ca channel. Similarly, the density of $I_{Ca\text{-}L}$ increased by 44% to 52% in cardiac myocytes from the TG mice. This % increase is ideal since maternal autoantibodies inhibit $I_{Ca\text{-}L}$ within the same range, 40-60% [10, 11]. Therefore, we postulated that immunization of TG mice overexpressing Cav1.2 should give birth to pups with no or fewer electrocardiographic abnormalities. Indeed, a lesser degree of sinus bradycardia (10% in TG pups from in immunized mothers overexpressing Cav1.2 vs. 22% in NTG littermates vs. 19.7% in pups from WT immunized mothers), and fewer AV conduction abnormalities (8.8% in TG pups from mothers overexpressing Cav1.2 vs. 25% in pups from WT immunized mothers vs. 27.5% in NTG littermates) were observed in TG pups from immunized mothers overexpressing Cav1.2. These data support the concept that maternal antibodies inhibit the Cav1.2 Ca channel, and consequently conduction abnormalities occur in the heart, as expressed in the electrocardiogram.

Immunization and knockout of Cav1.3 L-type Ca channel

Biochemical and functional evidences show that the Cav1.3 Ca channel is expressed in the human fetal heart and that anti-SSA/Ro –SSB/La autoantibodies from mothers of children with CHB inhibit Cav1.3 $I_{Ca\text{-}L}$ [10]. The autoantibodies also recognize the Cav1.3 Ca channel protein (Western blot)[10] and specifically bind to the extracellular loop between transmembrane region between S5-S6 in domain I [27]. These data along with the unique localization of the Cav1.3 in the conductive tissue established the Cav1.3 Ca channel as a novel target in the pathogenesis of CHB. We propose that together with the knockout of Cav1.3, the inhibition of the Cav1.2 $I_{Ca\text{-}L}$ by anti-SSA/Ro –SSB/La autoantibodies could account for the worsening of the electrocardiographic abnormalities seen in the Cav1.3 knockout pups.

Further evidence comes from the fact that pups from the Cav1.3 knockout mothers have similar electrocardiographic abnormalities even in the absence of immunization [22, 25, 26, 37]. Pups from nonimmunized Cav1.3 knockout mothers showed sinus bradycardia and I°/II° AV block while the

heterozygous had minimal abnormalities (3.6% sinus bradycardia and only I° AV block); no electrocardiographic abnormalities were seen in the NTG.

Immunized Cav1.3 knockout mothers had decreased parity with 3.2 litters per pregnancy and only 31 deliveries in 39 pregnancies. This low parity could be due to in-utero death because of the complete AV block or bradycardia. Of the pups that survived, 100% had sinus bradycardia and I°/II°/III° AV block. Heterozygous pups had 34.6% sinus bradycardia and 42.3% AV block. NTG pups had 18.2% sinus bradycardia and 18% AV block. It is important to mention the high incidence of III° AV block seen in the pups from immunized Cav1.3 knockout mothers. The unique localization and the higher expression of Cav1.3 Ca channel in the supraventricular conductive tissue compared to Cav1.2 Ca channel may explain why complete AV block occurred in 19% of pups (3 out of 16 pups) in the Cav1.3 knockout model. In addition, Cav1.3 knockout mice are more responsive to the anti-SSA/Ro –SSB/La antibodies likely because of the genetic deletion of the Cav1.3 together with the inhibition of the remaining Cav1.2 Ca channel.
These data support the hypothesis that further inhibition of L-type Ca channels by maternal antibodies generated by the immunization worsens the electrocardiographic abnormalities already seen in the pups from the Cav1.3 knockout mothers. Furthermore, the immunized Cav1.3 knockout mice provide a novel animal model of CHB where therapeutic drugs could be tested. Previous models [34, 38] showed the incidence of III° AV block to be very low (0% III° AV block, and 5.8% III° AV block from the group immunized with 52 Ro β and 1.8% III° AV block in the 52 Ro α immunized group).

Possible Mechanisms of CHB

Based on the facts that there is no convincing evidence that maternal antibodies can cross the sarcolemma of a normal cardiac myocyte, essentially two major categories of mechanisms for CHB can be identified. The first is abnormal surface expression of intracellular SSA/Ro and SSB/La antigens. Substantial experimental evidence has been proposed to account for the translocation of Ro and La to the cell surface, including viral infection [12], UV light and IFNγ treatment [39]. In human fetal cardiac myocytes in culture, induction of apoptosis results in surface translocation of SSA/Ro and SSB/La antigen and their recognition by antibodies at the cell surface [40]. Maternal antibodies inhibit the normal clearance of the apoptotic cells by macrophages leading to inflammation and thus the fibrotic changes in the conduction system [14]. The other possible mechanism is a cross-reactivity of maternal antibodies with targets other than SSA/Ro and SSB/La antigens. Maternal antibodies were reported to cross-react with laminin and with human cardiac myosin heavy chain at a critical stage during fetal cardiac development [8, 41]. Similarly, cross reactivity with the serotonin receptor 5-HT_4 has also been shown [9].

The role of L-type Ca channels in the pathophysiology of CHB has been supported by several studies [10, 11, 16, 17, 28-31, 33] . IgG from mothers with CHB children induced: 1) complete AV block in the Langendorff perfused human fetal [16] and rat [11] heart 2) suppressed AV node action potentials and inhibited $I_{Ca\text{-}L}$ [11]. Since conduction in the AV node is dependent on $I_{Ca\text{-}L}$, AV block should be expected from interventions that reduce $I_{Ca\text{-}L}$. Data imply an interaction between autoantibodies and Ca channel proteins that results in perturbation of sarcolemmal Ca fluxes. IgG from mothers with CHB children, but not control IgG, cross-reacted with the native [29] and the expressed [10, 27, 29] Cav1.2 and Cav1.3 L-type Ca channels by immunoprecipitation and Western blot. Furthermore, the failure of maternal IgG to affect the fast cardiac Na [11] and K [11] channels indicates specificity to Ca channels.

In summary, the animal models used in this study provide evidence for a pathogenic interaction of anti-SSA/Ro and/or anti-SSB/La antibodies with the L-type Ca channel in the development of

CHB. We propose that the maternal autoantibodies against SSA/Ro52, SSA/Ro60 and SSB/La48 are causally related to the abnormalities in the developing conduction system and that chronic exposure of Ca channels to these maternal autoantibodies during pregnancy leads to Ca channel inhibition, intracellular Ca dysregulation[42], cell death, and finally fibrosis and calcification of the SA and AV nodes as demonstrated in biopsies from infants with CHB [2, 3, 43-45]. An overexpression of the Cav1.2 rescued the electrocardiographic abnormalities whereas a deletion of the Cav1.3 exacerbated the abnormalities. The finding that TG pups from Cav1.2 overexpression mothers had reduced conduction abnormalities following immunization with SSA/Ro and SSB/La, points to the importance of finding a suitable Ca channel agonist that could help infants diagnosed with CHB and ameliorate the severity of conduction abnormalities.

Therefore, this study strongly suggests that autoantibodies interaction with Ca channels contribute to the development of CHB. However, the range and frequency of conduction abnormalities suggest that additional factors may also promote expression of CHB.

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Conflict:

No Conflict to disclose

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TABLES

Table 1. Electrocardiographic parameters of pups from the nonimmunized and immunized Cav1.2 L-type Ca channel overexpression mice

	Immunized Mothers (n)	Pregnancy (n)	Deliveries (n)	Total Pups (n)	Genotype (n)	Bradycardia n (%)	Abnormality			AVB (%)
							I° AVB (n)	2° AVB (n)	3° AVB (n)	
WT Nonimmunized FVB	5	8	8	48	WT (n=48)	0 (0%)	0	0	0	0
WT Immunized FVB	8	12	12	76	WT (n=76)	15 (19.7%)	10	6	3	25
Cav1.2 Overexpression Nonimmunized	5	7	7	39	TG (n=15) ★	0 (0%)	0	0	0	0
					NTG (n=24)	0 (0%)	0	0	0	0
Cav1.2 Overexpression Immunized	30	36	36	199	TG (n=90)	9 (10%)	7	1	0	8.8
					NTG (n=109) ★	24 (22%)	24	4	2	27.5

n, number of animals; WT, wild type; TG, transgenic; NTG, nontransgenic. The AV block percentage was counted as 100% only when all pups had at least I°, II°, or III° degree AV block. Connection bars compare sinus bradycardia and AV block between TG pups from immunized Cav1.2 overexpression mothers with the NTG littermates and WT immunized. ★ Denotes statistical significance $p<0.05$.

Table 2. Electrocardiographic parameters of the pups from nonimmunized and immunized Cav1.3 L-type Ca channel knockout mice

	Immunized Mothers (n)	Pregnancy (n)	Deliveries (n)	Total Pups(n)	Genotype (n)	Bradycardia n (%)	Abnormality			AVB (%)
							I° AVB (n)	2° AVB (n)	3° AVB (n)	
Cav1.3 KO C57BL/6	8	13	12	57	KO (n=9)	9 (100)	8	1	0	100
Nonimmunized					Hetero (n=28)	1 (3.6)	1	0	0	3.6
					NTG (n=20)	★ 0 (0)	0	0	0	0
Cav1.3 KO C57BL/6	29	39	31	101	KO (n=16)	16 (100)	9	4	3	100
Immunized					Hetero (n=52)	18 (34.6)	16	5	1	42.3
					NTG (n=33)	6 (18)	5	1	0	18

n, number of animals; WT, wild type; KO, knockout. The AV block percentage was counted as 100% only when all pups had at least I°, II°, or III° degree AV block. Highlighted Rectangle denotes advanced degrees of AV block. Connection bar compares sinus bradycardia and AV block between KO pups from the nonimmunized and immunized Cav1.3 mothers. ★ Denotes statistical significance $p<0.05$.

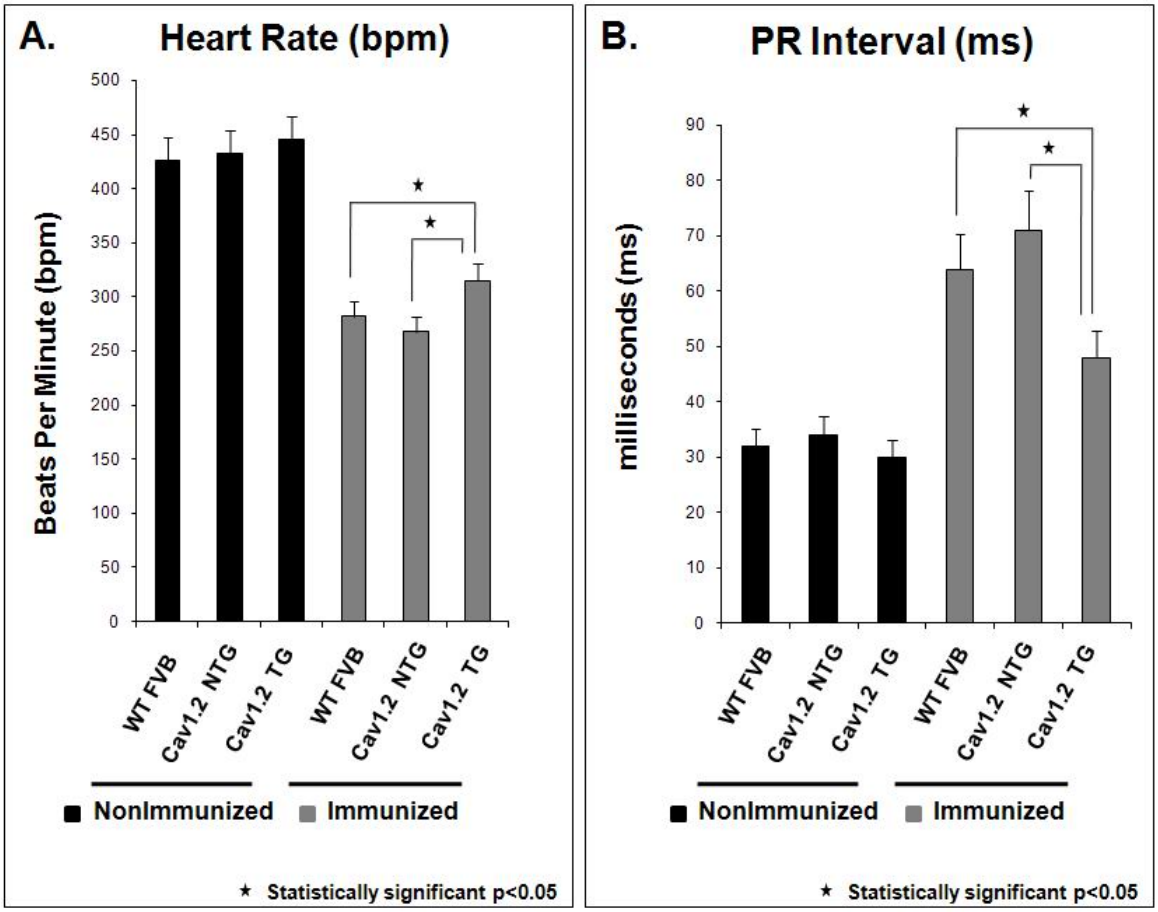

Figure 1. Heart rate and PR interval in WT FVB and Cav1.2 overexpression groups.
Panel A shows the heart rate, beats per minute (bpm) in the wild type (WT) FVB and Cav1.2 transgenic mice (TG). The average heart rate of pups from the immunized transgenic (TG) Cav1.2 overexpression was 315±36 bpm (n=90) compared to immunized WT FVB with an average heart rate of 282±32 bpm (n=76). Non-Transgenic (NTG) littermate pups born to the same immunized Cav1.2 overexpression showed sinus bradycardia (268±21 bpm, n=109). Pups from the nonimmunized WT FVB and the nonimmunized Cav1.2 overexpression had similar heart rates (426±41 bpm, n=48 and 445±21 bpm, n=15 respectively).
Panel B represents the PR interval in the WT FVB and Cav1.2 TG. The average PR interval in the pups from the nonimmunized WT FVB and the nonimmunized Cav1.2 overexpression was similar 32±8 ms (n=48) and 30±7 ms (n=15), respectively. The pups from the immunized WT FVB had a prolonged mean PR interval of 64±14 ms (n=76). The NTG immunized littermates in the Cav1.2 overexpression were similarly not protected and showed an increase in PR interval to 71±12 ms (n=109). The average PR interval of pups from the immunized TG Cav1.2 overexpression improved to 48±10 ms (n=90, $p<0.05$ compared to the immunized WT FVB and immunized NTG littermates).

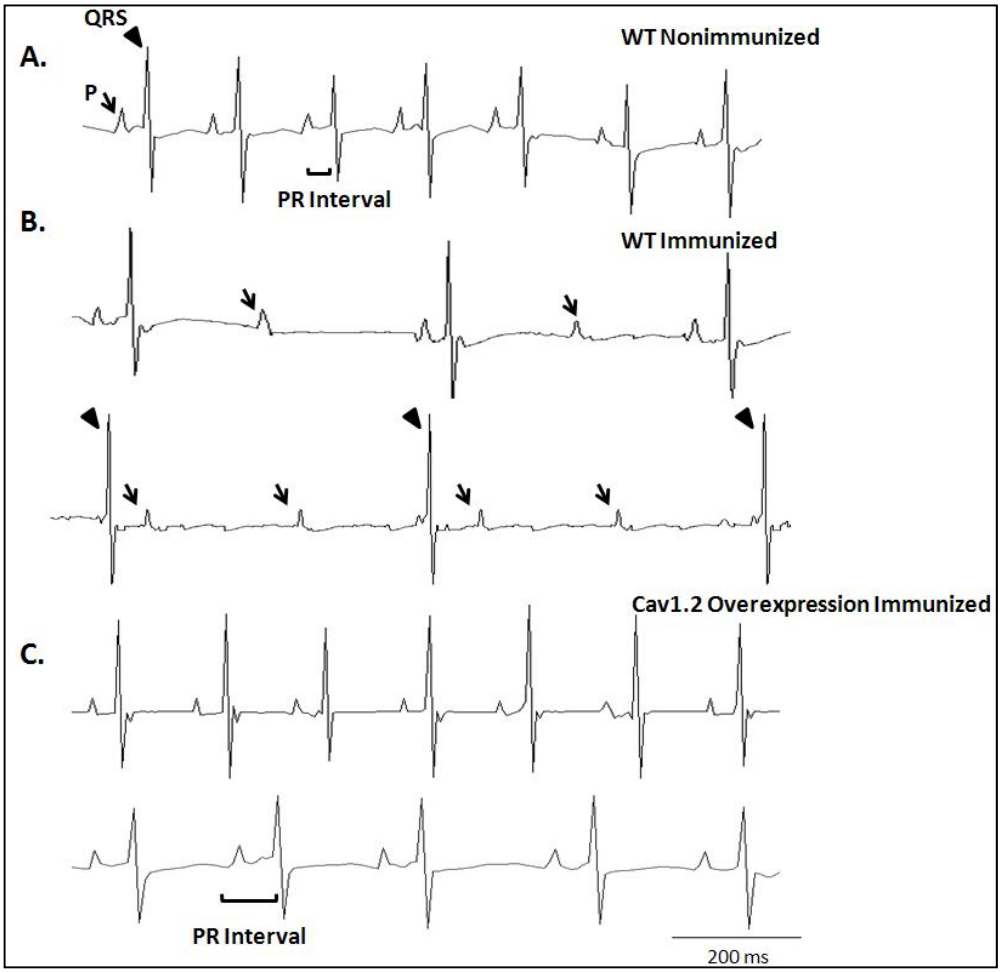

Figure 2. Electrocardiogram traces from the FVB immunized and nonimmunized groups within 1 to 3 days of birth. *Panel A* represents a control ECG from a pup born to a nonimmunized WT mother with a normal sinus rhythm of 454 and a PR interval of 42 ms. The ECG showed a normal PR interval followed by a QRS complex. Panel *B* shows increased electrocardiographic abnormalities in the pups as the result of immunization of WT mothers [II° AV block (upper trace of panel B) and III° AV block with complete AV dissociation (lower trace of panel B)]. *Panel C* shows the fewer electrocardiographic abnormalities in the TG pups from the immunized mice overexpressing Cav1.2 L-type Ca channel as illustrated by a normal sinus rhythm, 438 bpm and normal PR interval, 44 ms (upper trace of panel C), and sinus bradycardia (296 bpm) with I° AV block (PR interval 70 ms). The arrow indicates the P wave and the arrowhead indicates the QRS complex.

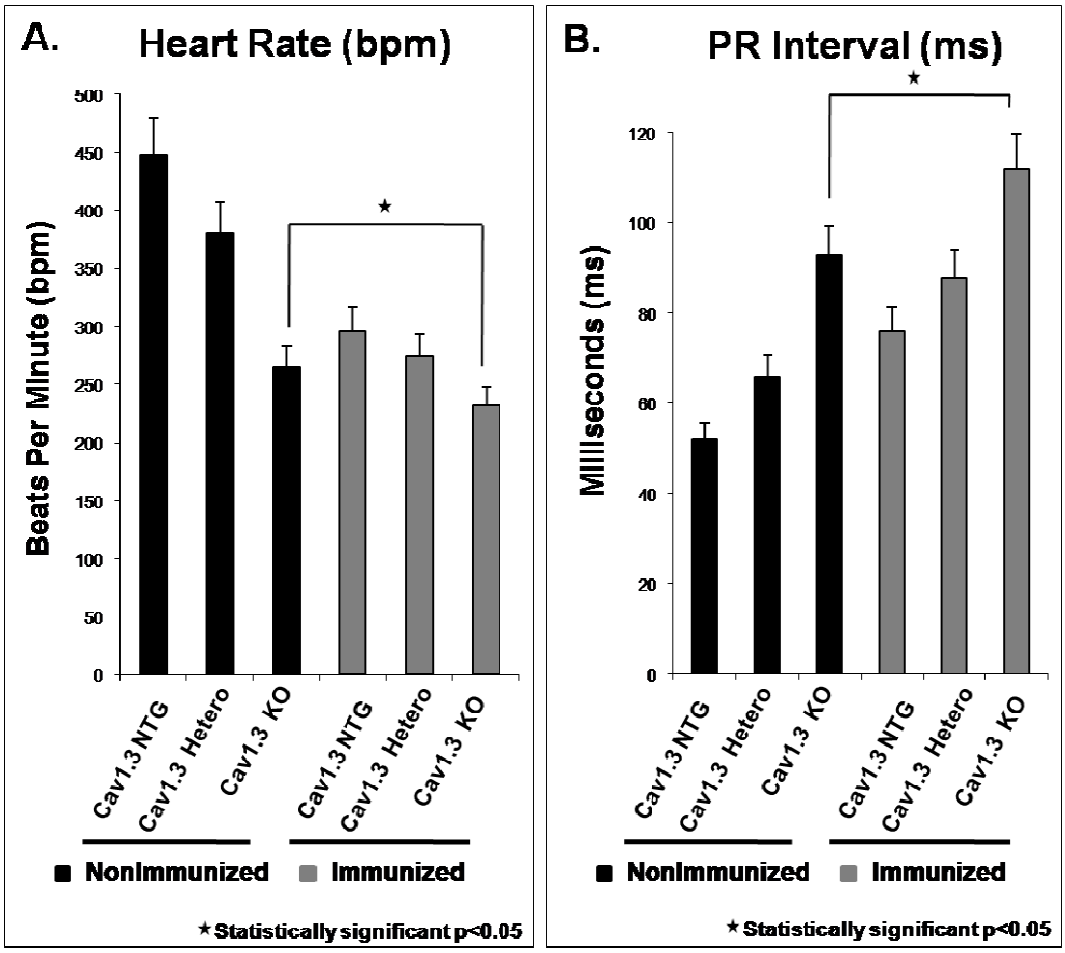

Figure 3. Heart rate and PR interval in Cav1.3 knockout groups.
Panel A: The Cav1.3 knockout pups had significantly slower average heart rates 265±33 bpm (n=9) vs. the non-transgenic (NTG) littermates 448±56 bpm (n=20). The KO pups from the immunized Cav1.3 knockout mothers had severe sinus bradycardia with an average heart rate of 232±30 bpm (n=16). *Panel B:* PR interval (ms) in the Cav1.3 knockout pups. The NTG pups had a PR interval of 52±6 ms and the Cav1.3 knockout pups from nonimmunized mothers had a PR interval of 93±15 (n=9). The pups from the heterozygous immunized mothers had a mean PR interval of 88±16 ms. The KO pups from the Cav1.3 knockout immunized mothers had a very prolonged average PR interval of 112±21 ms (n=16).

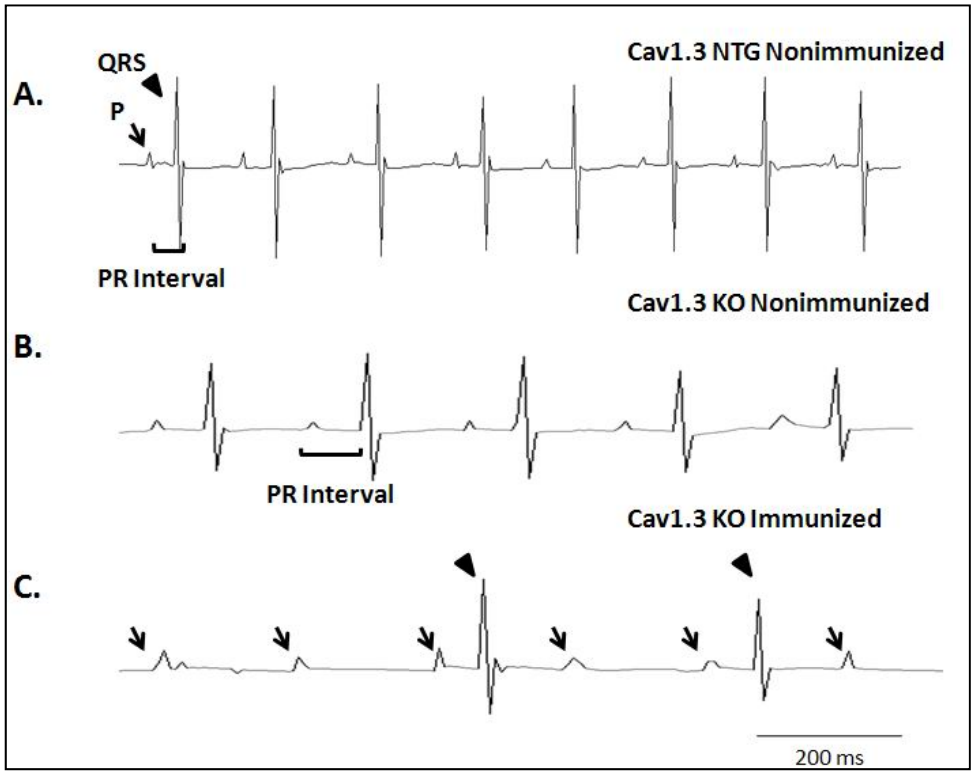

Figure 4. Electrocardiogram traces from the C57BL/6 Cav1.3 immunized and nonimmunized groups within 1 to 3 days of birth. *Panel A* shows a control ECG with sinus rhythm (442 bpm) from a pup born to nonimmunized Cav1.3 NTG.
Panel B shows sinus bradycardia (266 bpm) with I° AV block (PR interval of 96 ms) from a KO pup born to nonimmunized Cav1.3 knockout mother.
Panel C shows worsening of AV conduction abnormalities and development of III° AV block in a KO pup following immunization of Cav1.3 knockout mothers. The arrow indicates the P wave and the arrowhead indicates the QRS complex.

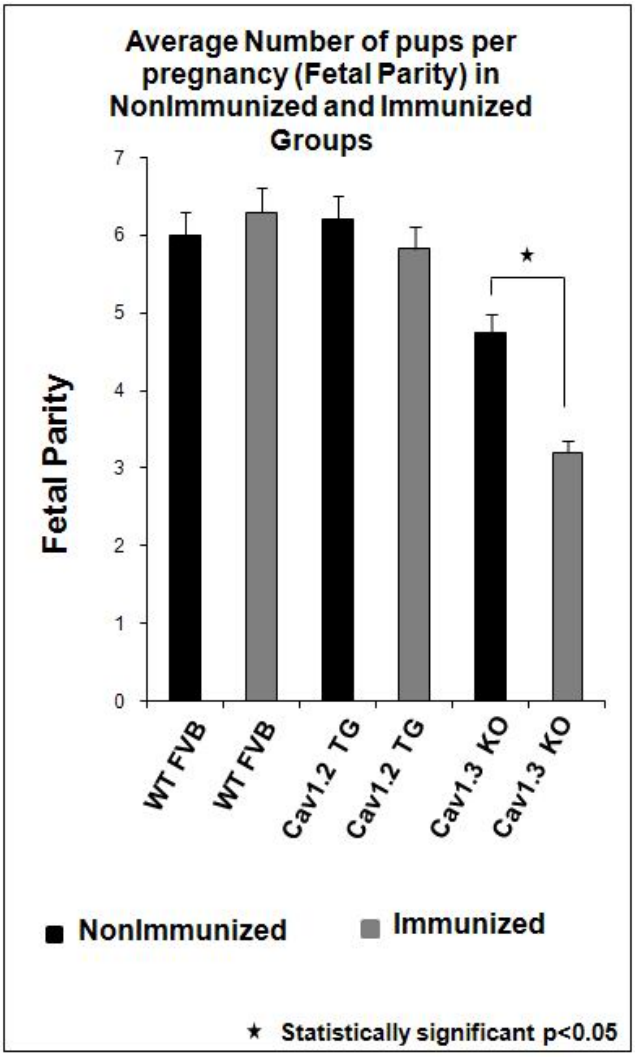

Figure 5. Fetal parity/average number of litters per pregnancy in the Cav1.2 and Cav1.3 immunized and nonimmunized groups. The fetal parity in the nonimmunized and immunized WT and/or Cav1.2 mothers ranged between 5.83±1.2 and 6.3±1.6 pups per pregnancy (p = not significant). However, immunized Cav1.3 knockout mothers had the lowest fetal parity (3.2±0.8 pups per pregnancy) and was much lower compared to the nonimmunized Cav1.3 knockout mothers ($p<0.05$).

DISCUSSION

The data in this thesis showed that 1) α_{1D} Ca channel is expressed in human fetal heart (Chapter 1); 2) positive IgG from mothers of children with CHB inhibits α_{1D} $I_{Ca\text{-}L}$; (Chapter 1) 3) positive IgG from mothers of children with CHB inhibits α_{1D} $I_{Ca\text{-}L}$ in native cardiomyocytes following silencing of Cav1.2 Ca channel (Chapter 2); 4) the extra-cellular loop of domain I S5-S6 of L-type Ca channel α_{1D} subunit as a target for autoantibodies from a subset of mothers with CHB children (Chapter 3); 5) the animal models used in this study provided evidence for a pathogenic interaction of anti-SSA/Ro and/or anti-SSB/La antibodies with the L-type Ca channel (Chapter 4) and 6) overexpression of Cav1.2 ameliorated the severity of electrocardiographic abnormalities which points to the need to develop new therapeutic agents that will specifically target cardiac Ca channels (Chapter 4); specifically an ideal Ca channel agonist that can be used in pregnant patients with signs of a child with CHB to rescue IgG inhibition of α_{1D} $I_{Ca\text{-}L}$.

Maternal anti-SSA/Ro –SSB/La and CHB

The association of anti-SSA/Ro -SSB/La autoantibodies with CHB is universally accepted. However, the mechanism by which these autoantibodies cause cardiac conduction abnormalities is not completely understood. Several studies have demonstrated the reactivity of the anti-SSA/Ro -SSB/La autoantibodies with fetal cardiac tissue (Deng et al., 1987; Taylor et al., 1986). Using immunoblotting, fetal heart tissue was shown to contain the cognate antigens SSA/Ro and SSB/La. The causal autoantibodies have also been shown in the fetal circulation. The fetal concentrations of total IgG are marginally detectable in the first trimester (<100 mg/dl) and remain low until 17 weeks when they increase to 400 mg/dl by 24 weeks and to 800 mg/dl by 32

weeks gestation (Miranda et al., 1998). Therefore, the logical next step is whether the autoantibodies are binding to their cognate antigen (SSA/Ro and SSB/La which are intracellular ribonucleoproteins) or are cross reacting with a surface protein on the sarcolemma of cardiomyocytes. Normally, IgG can not cross a plasma membrane. Alternatively, if the antibody can not cross a cell membrane, then the possibility is either that the cognate antigens translocate to the cell surface or the autoantibodies cross react with another independent surface protein. The apoptosis hypothesis supports the latter observation, whereas the cross reactivity is supported by the Ca channel and the 5HT-4 hypotheses. At the present time there is little evidence that fetal genetic differences play a role, however, they will be discussed to cover all possible aspects of CHB.

Apoptosis Hypothesis

Apoptosis is a selective process of cell deletion during embryogenesis that has been shown to play a role in the morphogenesis of the SA and AV nodes (James, 1970). In this regard, healthy cardiomyocytes rendered apoptotic either by treatment with staurosporine or plating on pHEMA plates resulted in surface translocation of the SSA/Ro and SSB/La antigens. However, apoptosis does not trigger inflammation which is a necessary component in CHB since the final sequelae are fibrosis and calcification of the SA and AV nodes. Apoptosis can not explain the extent of damage seen in the conduction system, since cardiomyocytes undergoing this physiological process are minor. Finally, the electrophysiological abnormalities caused by the autoantibodies on perfused hearts and isolated cardiomyocytes can not be explained by apoptosis and translocation of the cognate antigens to the surface. Nevertheless, Buyon et al., showed that apoptosis causes the translocation of the SSA/Ro and SSB/La antigens to the cell surface which

allows the binding of the autoantibodies. They further show that upon antigen/autoantibody binding, the apoptotic cells are not cleared by neighbouring macrophages and/or cardiomyocytes. This triggers inflammation, fibrosis and calcification.

The autoantibodies have been shown on the surface of fetal cardiomyocytes. Other possible mechanisms may explain the accessibility of the intracellular antigens and circulating antibodies. These include viral infection (Baboonian et al., 1989), ultraviolet light exposure (LeFeber et al., 1984), and IFNγ which all lead to the translocation of SSA/Ro -SSB/La to the cell surface. However, none of these have been shown in the heart.

The second possibility is that maternal antibodies cross react with cell surface components. To date, many surface proteins have been found including the muscarinic receptor (Bacman et al., 1994), laminin (Horsfall et al., 1996), the serotonin receptor $5HT_4$ (Eftekhari et al., 2000), and the L-type Ca channels (Boutjdir et al., 1997; Boutjdir et al., 1998; Garcia et al., 1994; Qu et al., 2005a). The $5HT_4$ and L-type Ca channel hypotheses will be discussed further due to the limited work on the other proteins.

$5HT_4$ (Serotonin) Hypothesis

The $5HT_4$ serotonin receptor is a G-protein coupled receptor which is found in the atria but not the ventricles (Kaumann and Sanders, 1994; Kaumann et al., 1991). Endogenous serotonin exerts positive chronotropic and inotropic effects on the atria due to stimulation of Gs/adenylyl cyclase/cAMP/PKA pathway that phosphorylates the L-type Ca channels (Kaumann et al., 1991; Ouadid et al., 1992). The unique localization of the serotonin receptor as well as the effect on the Ca channel could explain the electrocardiographic picture in CHB. In this regard, Eftekhari et al.,

showed that 17 out of 26 (65%) reacted with the $5HT_4$ receptor using ELISA. However a reassessment by the Research Registry for Neonatal Lupus showed that of the 75 sera from mothers of children with CHB, only 12 were reactive (16%) with the $5HT_4$ peptide (Kamel et al., 2005).

Ca Channel Hypothesis

Data from our lab (Boutjdir et al., 1997; Boutjdir et al., 1998; Qu et al., 2005a; Qu et al., 2001; Xiao et al., 2001a) and from others (Garcia et al., 1994) showed that maternal autoantibodies inhibited sarcolemmal $I_{Ca\text{-}L}$. Although the homology between the Cav1.2 or Cav1.3 channel with SSA/Ro or SSB/La do not exceed 4 amino acids when aligned, the 3D representation reveal several epitopes in the extracellular loop especially the one in the pore-forming α1 subunit of the channel. These epitopes could underlie the potential interaction between the autoantibodies and the L-type Ca channel. Indirect immunostaining showed that positive IgG reacted with surface components on the sarcolemma of human fetal cardiomyocytes (Qu et al., 2001). Biochemical experiments showed that the maternal antibodies directly cross react with both the Cav1.2 and Cav1.3 channel proteins (Qu et al., 2005a; Qu et al., 2001). Functional experiments showed that maternal antibodies inhibit individually expressed Cav1.2 and Cav1.3 Ca channels in tsA201 cells (Qu et al., 2005a; Qu et al., 2001). Similarly, using native myocytes, SA and AV nodal cells, we demonstrated that both anti-SSA/Ro and –SSB/La autoantibodies from mothers with CHB children inhibited the total L-type Ca current (Boutjdir et al., 1998).

In fact, Ca channels have been identified as autoantigens and also targets for cross reactivity of autoantibodies in a number of diseases such as Amyotrophic Lateral Sclerosis (AML) and Lambert-Eaton syndrome (LES) (Lennon et al., 1995; Smith et al., 1992; Takamori, 2004).

Consequences of inhibition of Cav1.2 and Cav1.3 Ca channels by maternal antibodies

Sinus Bradycardia and AV Block

There are 3 types of currents that contribute to the impulse generation at the SA node: 1) the delayed rectifier K current, I_k 2) the hyperpolarization activated inward current, I_f 3) the Ca currents, $I_{Ca\text{-}L}$ and $I_{Ca\text{-}T}$. The ionic basis for the spontaneous activity of the AV node is quite similar to the SA node except that it lacks the inward rectifier I_k. The Ca currents make a major contribution to the SA and AV nodal electrogenesis and an inhibition of $I_{Ca\text{-}L}$ is expected to result in sinus bradycardia and AV block. The data from our lab and this thesis show that maternal IgG inhibit the $I_{Ca\text{-}L}$ without affecting the I_k and I_f. $I_{Ca\text{-}T}$ was inhibited to a lesser extent than the $I_{Ca\text{-}L,}$ possibly due to homology between the Ca channel families. Interstingly, the Cav1.3 knockout mice exhibit sinus bradycardia and AV block suggesting that the Cav1.3 Ca channel might play a major role in these tissues. In fact, the unique biophysical properties of Cav1.3 (activating at more negative potentials compared to Cav1.2) and the high level of expression within the SA and AV node establishes Cav1.3 as a plausible target for in CHB. Therefore, it is the Cav1.3 Ca channel that makes the major contribution to the initiation of diastolic depolarization at the SA and AV node which pushes the potential from -60-50 mV to -40-30 mv in order to activate the Cav1.2 Ca channel that carries the major Ca current in.

One interesting aspect is the permanent and irreversible AV block even after the disappearance of the maternal antibodies from the fetal circulation and how blockade of the L-type Ca channels translates to permanent conduction abnormalities. A possible mechanism is that the binding of the antibodies to L-type Ca channels on the surface exposes the Fc portion of the antibody for binding by the C1q component of the complement system initiating the complement cascade leading to lysis and death of the target cell. Several lymphocytes can also bind to the Fc region of

the antibody which triggers the release of perforins and degredative enzymes that lyse the cell. In either event, this leads to the decrease in surface Ca channels with decrease in Ca entry eventually leading to cell death triggering inflammation, and eventually fibrosis and calcification of the conduction system.

Genetic factors

The relative rarity of CHB has posed a challenge to the study of genetic factors involved. The fact that the reccurrence rates are low and that twins can be discordant for the disease, suugests that a genetic factor is most likely responsible for susceptibility in CHB. Due to the low incidence, it has been difficult to demonstrate any HLA associations in infants with CHB.

MHC and non-MHC associations with CHB

The SSA/Ro and SSB/La antibodies have been for decades associated with CHB, and the risk is estimated to be 1-2% in mothers with these antibodies. Since the risk for a subsequent affected pregnancy is only 20%, this indicates that tere are further factors involved in susceptibility other than antibody specificity. There are a number of reports of dizygotic twins discordant for the disease, which also indicates that there may be a genetic fetal factor involved (Buyon et al., 1998; Lee et al., 1987; Solomon et al., 2003). However, there are also reports of monozygotic twins discordance (Cooley et al., 1997), indicating that not only fetal geneticsare responsible for induction of the disease. This infers that there maybe multiple genes involved in susceptibility and that the sum of these susceptibility genes in each individual determines the outcome of the disease. A certain threshold of susceptibility must be exceeded for the disease to be expressed in

each individual. There are no data on what the discordance rates for CHB are for monozygotic and dizygotic twins at this time because the numbers of cases are too few.

Association of mothers with CHB children and MHC genes have been described, particularly associations with haplotype B8/DR3 (Arnaiz-Villena et al., 1989; Colombo et al., 1999; Julkunen et al., 1995; Lee et al., 1983; Watson et al., 1992), but these are believed to be related to the presence of SSA/Ro and SSB/La and not to CHB. The same genetic associations are not found in the children born with CHB(Siren et al., 1999a). There have been some suggested HLA class I and II alleles which predispose a child to CHB, but these studies are too small and few to draw any conclusions (Siren et al., 1999b).

Non-HLA genes have also been suggested to play a role in CHB predisposition and susceptibility to disease. Clancy et al. demonstrated an association of CHB with cytokine polymorphisms as there was an increased frequency of TGFβ and TNFα polymorphism in 40 CHB children compared to healthy controls (Clancy et al., 2003). In fetal CHB hearts, protein expression of TGFβ and mRNA expression of TNFα was demonstrated in septal region. In sum, even though significant effort has been tried into the genetic aspects of CHB, a causal genetic aspect has not been identified to date.

Heart failure

It is interesting that around 10% of children with CHB not only exhibit conduction abnormalities but also mechanical effects leading to congestive heart failure likely due to inhibition of the ventricular L-type Ca channels. This injury could be the result of the insult during fetal/infancy period or as a result of pacemaker placement during neonatal period. Cav1.2 is the major channel involved in the EC coupling. However, Cav1.3, which is absent in the adult ventricle, is

expressed in the ventricles during fetal life. It has been suggested that the presence of the Cav1.3 Ca channel in the ventricles during fetal life is needed to aid in the EC coupling since fetal cardiommyocytes lacks the normal calcium induced calcium release (CICR) found in the adult heart. CICR plays a critical role in cardiac contractile function as it is major source of Ca. During early phase of systole in adult hearts, membrane depolarization opens L-type Ca channels and results in influx of Ca. The relatively small amount of Ca entering via this route is insufficient to initiate contraction, however, it activates Ryanodine receptor 2 (RyR2) in the sarcoplasmic reticulum (SR) triggering the release of Ca from the intracellular store required for EC coupling. However, there are striking morphological and functional differences between the mature and developing heart SR. In contrast to the adult heart, the SR is sparse and less functional during fetal life. It has been proposed that as a consequence of the underdeveloped SR, the sarcolemmal Ca channels play a major role for Ca delivery to the contractile proteins in the fetal heart. Therefore, it might be necessary to have both Cav1.2 and Cav1.3 during fetal periods to compensate for the lack of SR in order to trigger the contractile function of the myocardium. In this regard, blockade of the L-type Ca channels in the fetal heart by maternal antibodies will diminish the contractile force and worsen cardiac function eventually leading to congestive heart failure.

Vulnerability of the fetal heart vs the mothers heart

One of the most intriguing aspects of CHB is that it is an injury unique to the developing heart, since complete block has never been reported in the maternal heart despite the presence identical circulating antibodies (Behan et al., 1989; Dorner et al., 1992; O'Neill et al., 1993). Several explanations have been put forward. For example, the L-type Ca channel density is lower in the

fetal than adult heart and as such, maternal antibodies adversely affect the fetal heart. $I_{Ca\text{-}L}$ current density is 2 to 4 folds lower in human fetal than in the adult heart (Qu and Boutjdir, 2001). Normalized L-type Ca channel mRNA abundance significantly increased from 1 at 8-week gestation to 4 at neonate stages, and further increased to 5 at adult stage. Additionally, the sarcoplasmic reticulum is less abundant in the fetal heart (Hoerter and Vassort, 1982; Maylie, 1982) and the fetal myocytes rely mainly on the extracellular Ca entering through the L-type Ca channels for their Ca needs. Both functional current density and message levels for Ca channels increased in parallel with development, thus, supporting the hypothesis that fewer Ca channel density in the fetus heart and exposure of these channels to the same level of antibodies would be expected to adversely affect the fetal heart and may account for its vulnerability to CHB.

SIGNIFICANCE

It is clear that autoantibodies towards SSA/Ro and SSB/La are strongly if not universally present in congenital heart block at or before birth. This thesis helped in identifying the pathophysiological role of these autoantibodies and possible cross reactivity with the cardiac L-type Ca channels. Identification of the extracellular loop of domain I S5-S6 of L-type Ca channel Cav1.3 subunit as a target for autoantibodies from mothers with CHB children will provide novel insights into the development of therapeutic peptides that could bind to the pathogenic antibodies, thereby, preventing the initiation and progression of CHB. The finding that Cav1.2 overexpression mice reduced the conduction abnormalities following immunization with SSA/Ro and SSB/LA, points to the importance of finding a suitable Ca channel agonist that could help infants diagnosed with CHB and ameliorate the severity of conduction abnormalities. Further efforts should be directed towards these 2 potential therapeutic approaches which could reverse the clinical picture in CHB.

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