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Congenital Heart Block Maternal Sera Autoantibodies Target an Extracellular Epitope on the α_{1G} T-Type Calcium Channel in Human Fetal Hearts

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Abstract

Background: Congenital heart block (CHB) is a transplacentally acquired autoimmune disease associated with anti-Ro/SSA and anti-La/SSB maternal autoantibodies and is characterized primarily by atrioventricular (AV) block of the fetal heart. This study aims to investigate whether the T-type calcium channel subunit α_{1G} may be a fetal target of maternal sera autoantibodies in CHB.

Methodology/Principal Findings: We demonstrate differential mRNA expression of the T-type calcium channel *CACNA1G* (α_{1G} gene) in the AV junction of human fetal hearts compared to the apex (18–22.6 weeks gestation). Using human fetal hearts (20–22 wks gestation), our immunoprecipitation (IP), Western blot analysis and immunofluorescence (IF) staining results, taken together, demonstrate accessibility of the α_{1G} epitope on the surfaces of cardiomyocytes as well as reactivity of maternal serum from CHB affected pregnancies to the α_{1G} protein. By ELISA we demonstrated maternal sera reactivity to α_{1G} was significantly higher in CHB maternal sera compared to controls, and reactivity was epitope mapped to a peptide designated as p305 (corresponding to aa305–319 of the extracellular loop linking transmembrane segments S5–S6 in α_{1G} repeat I). Maternal sera from CHB affected pregnancies also reacted more weakly to the homologous region (7/15 amino acids conserved) of the α_{1H} channel. Electrophysiology experiments with single-cell patch-clamp also demonstrated effects of CHB maternal sera on T-type current in mouse sinoatrial node (SAN) cells.

Conclusions/Significance: Taken together, these results indicate that CHB maternal sera antibodies readily target an extracellular epitope of α_{1G} T-type calcium channels in human fetal cardiomyocytes. CHB maternal sera also show reactivity for α_{1H} suggesting that autoantibodies can target multiple fetal targets.

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Introduction

Congenital heart block (CHB) is a passively acquired autoimmune disease that occurs in pregnancies of rheumatic mothers, but also in healthy mothers, and has been associated with maternal anti-Ro/SSA and anti-La/SSB antibodies. The disease is characterized by atrioventricular (AV) block, which can be detected in the developing fetus between 16–25 weeks gestation [1,2]. In the absence of congenital structural abnormalities in the offspring, maternal autoantibodies are usually present, and it is generally accepted that maternal antibodies cross the placenta and induce fetal injury in the AV node. More generalized effects on the heart, associated with anti-Ro/SSA and anti-La/SSB, have also been suggested in the past decade such as sinus bradycardia, myocardial inflammation, QTc prolongation, endocardial fibroelastosis and dilated cardiomyopathy [3–9]. In most studies, untreated autoimmune CHB has been associated with high fetal/neonatal mortality rates (14%–34%) [4,10–16].

Understanding the pathology of CHB, and predicting outcome in pregnancies, have been complicated by low incidence and recurrence rates. In a population of women with anti-Ro/SSA and anti-La/SSB autoantibodies, the incidence of CHB is approximately 1–2% [17], yet the recurrence rate in these mothers is approximately 18% [1,18,19], despite persisting antibodies [20], indicating that additional factor(s) contribute to the fetal susceptibility of CHB.

A link between maternal anti-Ro52 antibodies and CHB is supported by a number of studies [20–31], whereas maternal sera reactivity to the La protein tends to be associated with dermatologic neonatal lupus erythematosus (NLE) [32]. Nevertheless, both anti-Ro60 and anti-La autoantibodies have been suggested to amplify the immune insult occurring in fetal hearts after development of CHB [33].

CHB pathology reports have shown that the disease is associated with deposition of IgG and with the presence of inflammatory cells in the AV node of fetal hearts, as well as AV node fibrosis and calcification [34–36]. Effects on the sinoatrial (SA) node of the fetal heart have also been reported, with sinus bradycardia demonstrated in patients [3,37,38], as well as *in vitro* [39] and *in vivo* animal models [40,41]. Mothers are not affected by AV block, which could be due to developmental expression of the target in the AV node, or unique vulnerability of the fetal heart.

Maternal-fetal antibody transfer as the event precipitating CHB was initially proposed in 1977 [42]. A single protein target for these antibodies, however, has not emerged. Instead, maternal sera reactivity to several proteins has been demonstrated in previous studies, including the serotoninergic 5-HT4 receptor [43,44], and two voltage-dependent L-type calcium channel subunits: α-1C/Cav1.2 (α_{1C}), and α-1D/Cav1.3 (α_{1D}) [39,45,46]. Sera from mothers with CHB affected pregnancies (CHB^+ sera) were shown by patch clamp to affect currents mediated by recombinantly expressed α_{1C} in *Xenopus* oocytes [47], and by α_{1D} in a transformed embryonal human kidney cell line (tsA201) [48]. Contribution of L-type channels to CHB was also demonstrated in a mouse model where α_{1C} transgenic and α_{1D} knock out mice were immunized with Ro52, Ro60 and La [49], but these channels did not account for all of the effects of CHB. Results from the *Xenopus* system also indicated that currents through the recombinant voltage-dependent T-type calcium channel subunit α-1H/Cav3.2 (α_{1H}) were decreased in the presence of CHB^+ serum [47]. Since multiple targets of CHB maternal autoantibodies have been identified, and the spectrum of symptoms in affected offspring are not limited to AV block alone, it remains possible that the specific pathogenic autoantibody in anti-Ro/La pregnancies recognizes an epitope that is at least partially common to several ion channels and receptors.

The voltage-dependent T-type calcium channel subunit α-1G/Cav3.1 (α_{1G}) is a developmentally regulated channel that is thought to participate along with α-1H/Cav3.1 (α_{1H}) regulating cardiac conduction through the AV-node [50,51]. It is known that α_{1G} is highly expressed in the compact node (CN) of the AV axis in human hearts [52], and homozygous transgenic mice lacking α_{1G} exhibit first-degree AV block and bradycardia [53] – a phenotype consistent with extant reproducible rodent models of CHB [29,54–56].

The suggestion of a role for α_{1G} in cardiac development, and the comparable electrophysiology of the α_{1G} knock out and CHB mouse models, led us to hypothesize that α_{1G} might represent an additional cross-reactive target of maternal serum antibodies in CHB. In the present work, we found that α_{1G} (mRNA and protein) is expressed in human fetal hearts and that CHB-affected maternal sera contains antibodies reacting to the α_{1G} protein. Accessibility of the epitope by serum antibodies is further demonstrated with confocal microscopy and α_{1G} affinity-purified serum. Antibody reactivity of maternal sera maps to peptide sequences found in the extracellular S5–S6 portion of repeat I of α_{1G} (aa305–319), and a similar sequence is found in the α_{1H} T-type channel subunit, but not in the L-type channel subunits α_{1C} or α_{1D}. Single-cell patch-clamp studies demonstrated that CHB serum irreversibly decreases T-type calcium channel current in mouse SAN cells. In summary, our results support the conclusion that maternal autoantibodies in CHB pregnancies bind to the T-type calcium channel α_{1G}, and can decrease T-type currents. These antibodies may also bind to the α_{1H} channel, but binding to the native protein remains to be verified in human fetal hearts. These results provide further support for the contention that more than one target may be involved in the complex pathology of the disorder.

Results

Ion channel blockers demonstrate preferential inhibition of newborn heart AV conduction likely conferred by α_{1G}

Since CHB affects the fetal heart, but not the maternal heart, we wanted to identify ion channels that may be preferentially affected by autoantibodies in fetal hearts. To accomplish this objective we applied selective calcium channel blockers and pacemaker current (I_f) blockers to newborn and adult rabbit Langendorff hearts and measured Wenckebach cycle length (WBCL) by examining the effects of decremental atrial pacing on the P-R interval and AV-conduction block. We have used this method previously to demonstrate that WBCL prolongation occurs with perfusion of sera from mothers of children with CHB, but not in the presence of sera from mothers of unaffected children [57]. We have also used this method previously to establish a greater sensitivity to AV block in newborn versus adult rabbit Langendorff hearts upon perfusion of sera from mothers of children with CHB (RM Hamilton, unpublished observations). Once baseline WBCL was established, hearts were perfused with either L-type calcium channel blockers (Diltiazem or Verapamil), the pacemaker current (I_f) blocker (ZD7288), or, a relatively selective T-type calcium channel blocker (Mibefradil) and 15 minutes after equilibration, WBCL was recorded every five minutes for 15 minutes (Figure 1A–D). The ion channel blocker concentration range was optimized in a pilot study to cause at least double prolongation of WBCL in neonatal rabbit hearts. Baseline WBCLs did not differ significantly in any of the treatment groups (data not shown). Although both newborn and adult hearts are affected by channel blockers, there was no significant difference in WBCL between newborn and adult hearts when they were perfused with L-type calcium channel blockers (Diltiazem, Figure 1A; or Verapamil, Figure 1B), or with the I_f blocker ZD7288 (Figure 1C). However, newborn hearts were more sensitive to T-type calcium channel blockade (Mibefradil, Figure 1D) compared to adults. Moreover, the percent increase of WBCL in newborn compared to adult hearts during atrial pacing was only increased ($p<0.001$) with Mibefradil. In order to evaluate whether Mibefradil-induced AV Wenckebach block resulted from blockade of α_{1G} or α_{1H}, we also measured WBCL during perfusion with increasing concentrations of nickel chloride ($NiCl_2$), because α_{1G} and α_{1H} have different sensitivities to Ni^{2+} block ($IC_{50} = 13$ μM for α_{1H} and $IC_{50} = 250$ μM for α_{1G}) [58]. Since WBCL was unaffected by Ni^+ up to concentrations of 300 μM (Figure 1E) in 5/6 hearts, we conclude that α_{1G} is

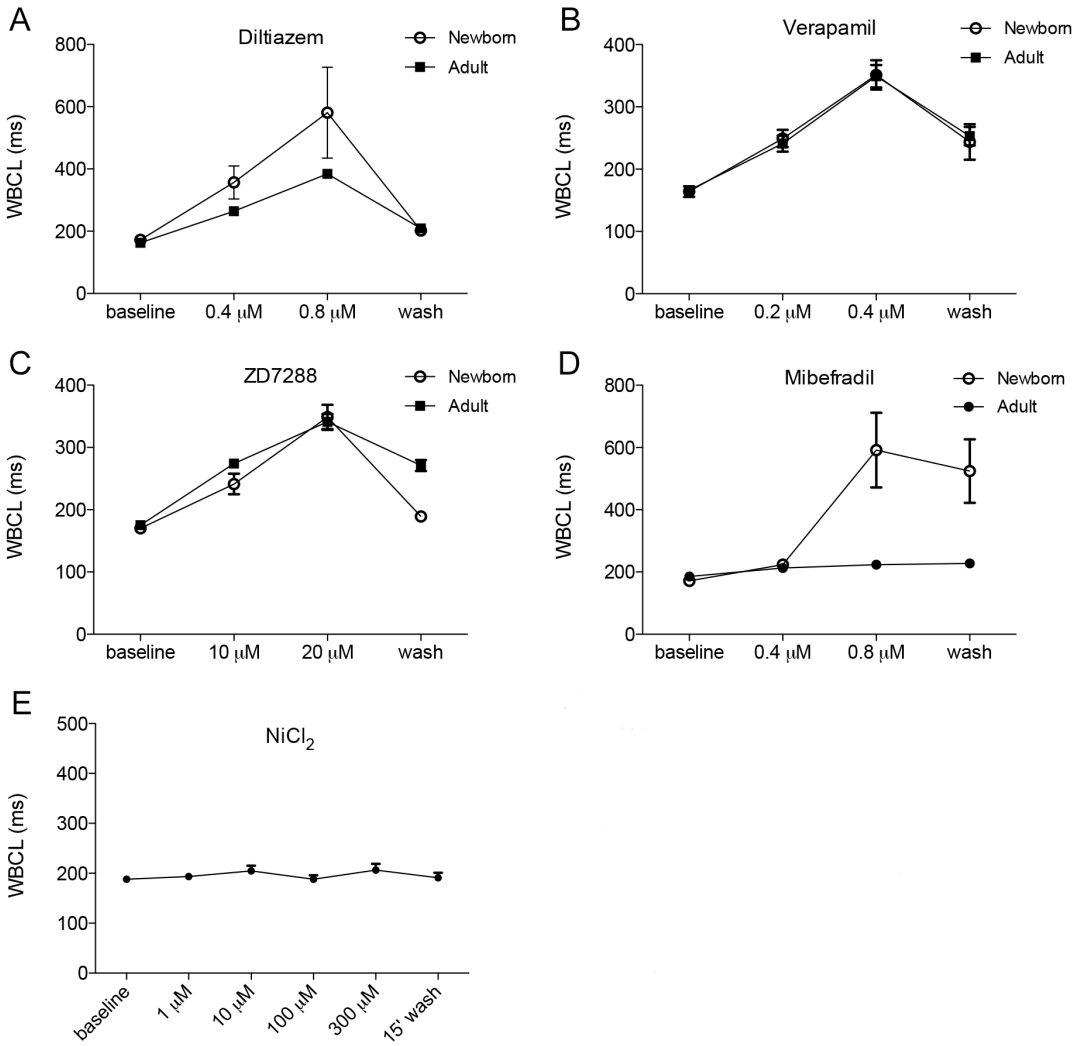

Figure 1. Ion channel blocker effects on Wenckebach cycle length in atrial paced newborn and adult rabbit hearts. Wenckebach cycle length (WBCL) was recorded during perfusion with calcium channel and I_f channel blockers in newborn and adult rabbit hearts (WBCL in milliseconds). Equivalent effects were observed in adult and newborn hearts treated with L-type calcium channel blockers (**A**) Diltiazem and (**B**) Verapamil and with the I_f blocker ZD7288 (**C**) ($p = ns$ repeated measures ANOVA). The T-type calcium channel blocker Mibefradil (**D**) demonstrated significant preferential AV block in the newborn hearts as compared to the adult hearts ($p<0.05$). Values shown are mean ± SEM, newborn ($n = 7$) and adult ($n = 8$). Diltiazem showed a trend toward a difference in sensitivity of adult and newborn hearts, however there was great variability in results between experiments. (**E**) Effects of the α_{1H} T-type calcium channel blocker $NiCl_2$ were also investigated on WBCL in newborn Langendorff rabbit hearts. WBCL measurements do not differ from each other significantly with mean ± SEM (repeated measures ANOVA $p = ns$). One heart out of 6 that appeared to be an outlier showed some variability in response to $NiCl_2$, and was excluded from statistical analysis.
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primarily responsible for the Mibefradil effects on WBCL prolongation in newborn vs. adult hearts.

α_{1G} expression in the AV node of human fetal hearts

Since the expression of α_{1G} in the AV node of the human fetal heart is a prerequisite for any potential involvement of this protein in the AV block characteristic of CHB, we performed anatomical dissection, as previously described [59], and immunohistochemistry (IHC) studies to demonstrate presence of α_{1G} in the AV node. Sections from a 21-week human fetal heart with Masson's trichrome stain demonstrates morphology of the AVJ region, clearly showing the AV node and AV bundle, which coincides with appropriate anatomical regions identified by collagen (green) and myocardium (light pink) (Figure 2A). The NF-160 antibody (green), which identifies cardiac conducting tissue [60], clearly shows the AV node and AV bundle (Figure 2B). We observed dense staining with α_{1G} antibodies (red) in the region of the AV node and AV bundle with more diffuse staining in surrounding regions corresponding to ventricular myocardium identified as regions without NF-160 staining (Figure 2B). Due to lack of suitable antibodies, we were not able to perform IHC to investigate expression in the AV node of the other T-type channel subunit expressed in the heart, α_{1H}.

α_{1G} gene expression is enriched vs. α_{1H} in the atrioventricular junction

To further explore the expression of α_{1G} channels in fetal human hearts, we anatomically dissected [59] atrioventricular junction (AVJ) and the ventricular apex from three fetuses between 18–22.6 weeks gestation (18 weeks, 18.6 weeks, 22.6 weeks),

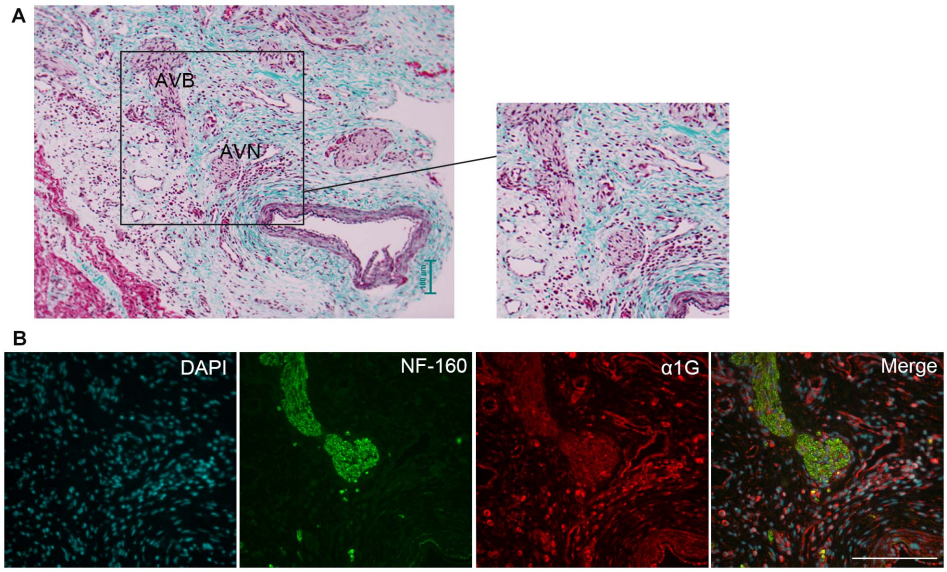

Figure 2. T-type calcium channel α_{1G} is expressed in the AV node of human fetal hearts. (**A**) Masson's trichrome staining of 21-week human fetal heart demonstrates morphology of the AVJ region with light pink staining of the spindle-like cells in the AV node (AVN) and the AV bundle (AVB), green staining corresponding to collagen fibres. (**B**) α_{1G} staining (Red) is present in AVJ and AV bundle regions which are distinguishable by positive NF-160 staining (Green). Scale bars represent 100 μm (A) and 150 μm (B).
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coinciding with the time point when CHB is diagnosed *in utero*. Real time PCR measurements revealed that the expression of the α_{1G} genes (*CACNA1G*), normalized to the level of expression in the apex, is higher at 18 weeks compared to later time points (Figure 3A). Although the availability of only a single sample per gestational week does not permit us to assess the reproducibility of these expression patterns, pooling of the results supports the conclusion that the relative expression of *CACNA1G* is greater ($p<0.05$) in the AVJ compared to the ventricular apex (Figure 3B). For comparison, we also measured the mRNA expression levels of the α_{1H} gene, *CACNA1H*, and could not detect higher expression in the AVJ versus the ventricular apex, suggesting low expression of α_{1H} in the AVJ.

Maternal sera autoantibodies from CHB-affected pregnancies are immunoreactive to the α_{1G} protein in human fetal hearts

In order to investigate reactivity of CHB maternal sera to α_{1G}, we performed Western blot and immunoprecipitation experiments with lysates from fetal heart ventricle. We first confirmed presence of α_{1G} protein in human fetal hearts by Western blot with lysates from a 20.4-week-old human fetal heart. A commercial α_{1G}/Cav3.1 antibody (specific for aa1–22 of α_{1G}) identified a band ~180–200 kDa in size (Figure 3C, lane 1) which was eliminated by pre-incubation with a peptide corresponding to aa1–22 of rat α_{1G}. Blotting with anti-Ro/La positive CHB$^+$ maternal sera (titer >100 IU for both anti-Ro and anti-La) also identified a similar band (~180–200 kDa, Figure 3C, lane 3) that was eliminated by pre-incubation with peptide p305 (aa305–319 of α_{1G}, see below). Serum obtained from a mother positive for anti-Ro/La autoantibodies (anti-Ro titer = 60 IU and anti-La titer >100 IU), but with an unaffected fetus, did not yield a band with the expected molecular weight of α_{1G} (Figure 3C, lane 5, no bands). We also show in Figure 3C (lane 6) that a commercial antibody to α_{1H} revealed a band, but the predicted molecular weight was much smaller than α_{1G} (180–200 kDa). Note further that proper loading in all the wells was ensured using control anti-calnexin (CNX) antibody (Figure 3C, bottom panel).

The presence of α_{1G} in human fetal heart lysates, and verification of immune reactivity by CHB maternal sera, was next assessed via immunoprecipitation (IP) experiments. IP was performed with the commercial α_{1G}/Cav3.1 antibody (specific for aa1–22 of α_{1G}) on lysates prepared from three dissected regions of human fetal hearts (AVJ, ventricle, apex). Immunoblots of IP samples from the AVJ, ventricle and apex, yielded α_{1G} bands when probed with commercial α_{1G}/Cav3.1 antibody and maternal serum from an anti-Ro/La positive pregnancy with CHB outcome (CHB serum, titer >100 IU for both anti-Ro/La), but not with maternal serum from an anti-Ro/La negative healthy pregnancy (Normal serum) (Figure 3D). These blots were subsequently stripped and re-probed with a second commercial α_{1G}/Cav3.1 antibody recognizing a different α_{1G} epitope (specific for aa6–50), demonstrating specificity of the α_{1G} IP. We were unable to find appropriate conditions, or antibodies, that yielded evidence for the presence of α_{1H} protein levels with IP; and, although Western blot did demonstrate a band for α_{1H}, the size of this band differed from that recognized by CHB$^+$ maternal sera.

Accessibility of α_{1G} T-type calcium channel epitope on cardiomyocytes

Because our RT-PCR and Western Blotting studies showed convincingly the expression of α_{1G} in the ventricles, we elected to explore whether α_{1G} affinity-purified CHB sera identified surface targets in the myocardium using immunofluorescence and confocal microscopy. Although CHB is primarily characterized by atrioventricular block affecting the AV node, effects of anti-Ro/La autoantibodies have also been suggested on the whole heart [3–9,61], thus expression of targets in regions such as the ventricle are also of interest. Immunofluorescence co-staining of human fetal heart (21.6 weeks) with anti-α_{1G} (Red) and with anti-cTnT

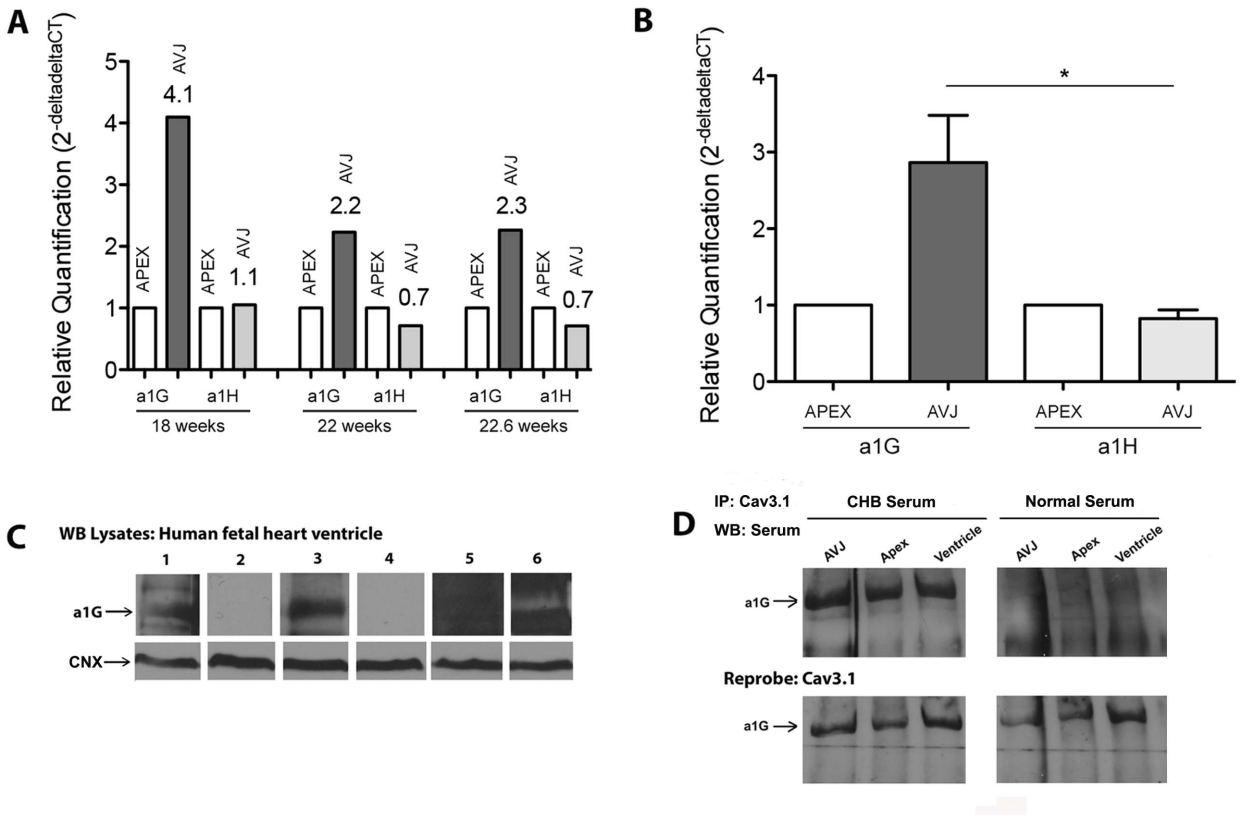

Figure 3. Expression analysis, and CHB maternal sera immune reactivity towards α_{1G}. (**A**) Although both α_{1G} and α_{1H} are expressed in human fetal hearts, real time PCR demonstrates that transcripts from *CACNA1G* (α_{1G}) are 2.2- to 4-fold higher in the AVJ than in the apex tissue (between 18–22.6 weeks gestation), whereas *CACNA1H* expression levels are between 0.7- and 1.1-fold in the AVJ compared to the apex. (**B**) Combining the data from 3 hearts (18.0 weeks-22.6 weeks), shows that *CACNA1G* expression in the AVJ is significantly higher than *CACNA1H* in the AVJ ($p<0.05$). (**C**) Western blot with human fetal heart lysate (20.4 weeks) demonstrates α_{1G} expression by a Cav3.1 commercial antibody (lane 1), that is blocked by the peptide immunogen of this antibody (aa1–22 of rat α_{1G}) (lane 2). CHB sera (anti-Ro and anti-La titer >100 IU) also binds to α_{1G} (lane 3); this reactivity is blocked by the α_{1G} p305 peptide (lane 4). Sera from mothers with anti-Ro/La antibodies (anti-Ro = 60 IU and anti-La titer >100 IU) giving birth to normal babies do not have immune reactivity to the α_{1G} protein (lane 5), and a commercial α_{1H} antibody confirms that the band seen is not α_{1H}. (**D**) α_{1G} isolated from human fetal heart (AV junction, ventricle, and apex) was immunoprecipitated with a Cav3.1 antibody, and the immunoblot was probed with human sera. Immunoblot was subsequently stripped and re-probed with a second Cav3.1 antibody towards a different epitope, demonstrating presence and specificity of the IP towards α_{1G}. Note that CHB sera is defined as anti-Ro/La positive sera from pregnancies affected by CHB. Normal sera are from pregnancies with a healthy outcome and not affected by CHB.
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antibodies (Green) exhibited preferential staining of α_{1G} localized predominantly to the cell surface, with some perinuclear staining observed by confocal microscopy (Figure 4A). Differential intereference contrast (DIC) microscopy images of cardiomyocytes used for staining experiments demonstrate typical morphology of ventricular cardiomyocytes (Figure 4A, right panel). Maternal CHB sera (titer >100 IU for both anti-Ro and anti-La), that had been affinity-purified towards the α_{1G} peptide p305 (aa305–319, see below), preferentially stained surface regions of the ventricular cardiomyocytes (yellow, Figure 4B, right panel). Interestingly, some surface regions only showed α_{1G} staining with the commercial antibody, and not co-staining with the affinity-purified sera, suggesting that the α_{1G} epitope recognized by the maternal sera may be masked (or even absent) in some membrane areas.

CHB maternal sera profile screening of α_{1G} peptides

In order to better understand the patterns of antibody reactivity to α_{1G} described above, we undertook to delineate the α_{1G} epitope(s) recognized by CHB maternal sera. The α_{1G} protein is comprised of 24 transmembrane segments organized into four homologous domains (termed repeats I–IV); consequently, the protein has many extracellular (12) and intracellular sequences (13) [62]. The first 4 transmembrane segments of each internal repeat (S1–S4) form the voltage-sensors, whereas the S5–S6, and the intervening extracellular loop, form the pore [62].

Given that CHB-derived antibodies stained surface-expressed α_{1G} in cardiomyocytes, it seemed reasonable to conclude that this sera recognizes extracellular epitopes of the α_{1G} protein. Accordingly, a PEPscreen (Sigma) custom peptide library consisting of 15aa-long overlapping peptides was generated that encompassed selected regions of the α_{1G} protein [aa130–380 (Figure 5A, B)]; II [aa774–963 (Figure 5C, D)]; and, III [aa1308–1536 (Figure 5E, F)] corresponding to the largest extracellular loops formed between S5 and S6 of repeat domains I, II and III. Sera from three mothers with CHB affected pregnancies, and three patients with pregnancies unaffected by CHB (denoted CHB^+ and CHB^-, respectively), were subsequently screened for reactivity to the peptide library (see Figure 5 for representative data). We identified two peptides in repeat I, zero peptides in repeat II, and six peptides in repeat III with levels of reactivity that exceeded background (Figure 5, A–C); no reactivity was observed with

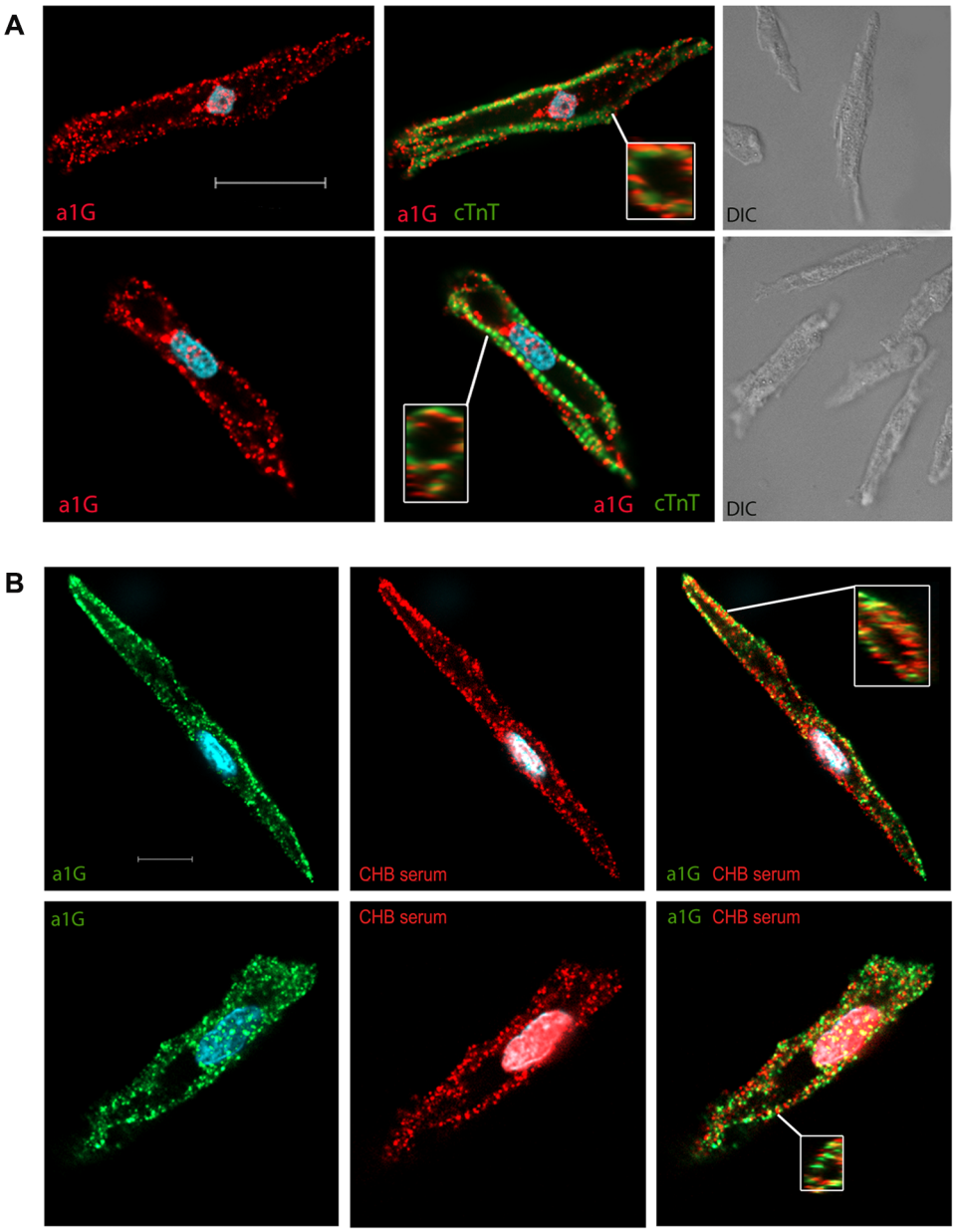

Figure 4. Affinity-purified maternal CHB serum antibodies co-localize with α_{1G}. (**A**) Cardiomyocytes from ventricle of human fetal hearts, gestational week 20.6, were dissociated, immunofluorescence stained, and visualized with confocal microscopy. Cardiomyocytes are stained for Cav3.1 (α_{1G}) (Red), for the cardiomyocyte marker anti-Troponin-T (Green), and nuclei visualized with DAPI (Blue). Secondary antibodies alone gave no stain. Inset image demonstrates expression on the surface in a cross section image. Images shown are representative of various cardiomyocyte samples; similar staining patterns were obtained in four replicate experiments. Secondary antibodies, anti-rabbit-IgG-Cy3 (Red) and anti-mouse-IgG-Alexa488 (green) alone give no stain (data not shown). Note that DAPI stains the nuclei of cardiomyocytes and of other cells present in the samples, such as fibroblasts. Scale bar represents 12 μm. (**B**) Dissociated and non-permeabilized cardiomyocytes from ventricle of 20.6 week human fetal hearts, immunofluoresence stained for α_{1G} affinity-purified serum (red), and α_{1G} (green). Co-localization of staining (yellow) was assessed with confocal microscopy. Scale bar represents 12 μm.
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CHB^- sera (Figure 5, D–F). The magnitude of response of CHB^+ sera differed between peptides based on repeat I and repeat III. For example, all the peptides derived from repeat I exceeded background by ~80–100%, whereas the most reactive repeat III peptide read out was ~20% above background (compare Figure 5A and Figure 5C). We therefore chose to continue our epitope mapping to the region of α_{1G} repeat I encompassed by the two highly reactive peptides. These peptides, denoted p305 (aa305–319, Figure 5A, light grey bar) and p315 (aa315–325, Figure 5A, dark grey bar), are comprised of sequences predicted to reside between S5 and S6 in the third extracellular loop of repeat I.

Epitope mapping of the repeat I peptides was refined in enzyme-linked immunosorbent assay (ELISA) experiments that utilized four overlapping 15aa peptides, and one 20aa peptide, with sequences corresponding to this protein region, as follows: p300 (aa300–314); p305 (aa305–319); p310 (aa310–324); p315 (aa315–329); and, p305/310 peptide (aa305–324). Assay param-

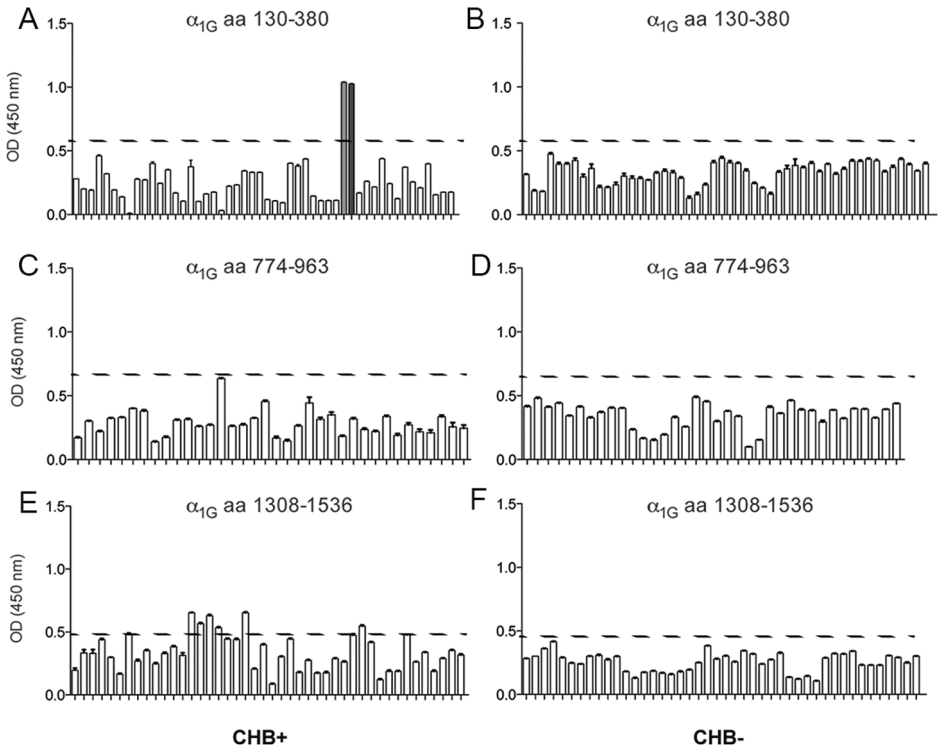

Figure 5. Maternal sera antibody profile screening of α_{1G} peptides. Maternal sera from one CHB$^+$ pregnancy (**A, C, E**) and one CHB$^-$ pregnancy (**B, D, F**) were tested in ELISA against 15aa long overlapping peptides derived from extracellular regions of α_{1G}: T-type aa130–380 (**A, B**); aa774–963 (**C, D**); and, aa1308–1536 (**E, F**). Reactivity above threshold (HC: Average+3×St. Dev; set at 0.59, 0.64, 0.48 respectively for A/B, C/D, and E/F) was observed in the CHB$^+$ but not CHB$^-$ serum to α_{1G} peptides p305 (aa305–319, light grey bar) and p315 (aa315–323, dark grey bar) was observed in the CHB$^+$ but not the CHB$^-$ serum. Above threshold reactivity was also observed among peptides in the region spanning aa1308–1536.
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eters were developed using healthy control sera as a negative control, with the positive control provided by serum from a patient clinically identified as anti-Ro/La positive and confirmed as immunoreactive to α_{1G} by immunoblotting (Figure 3C, lane 1). Using these controls, a reproducible ELISA screen for each peptide was established, and the reactivity of small cohorts of maternal CHB$^+$ and CHB$^-$ serum samples to each peptide was investigated (Figure 6A–E).

Maternal sera reactivity from CHB$^+$ or CHB$^-$ pregnancies was not different towards peptides p300 and p315 (Figure 6A and Figure 6D, respectively), which were also the slowest-reacting peptides (read at 2 h 30 min). However, CHB$^+$ sera were far more reactive to p305, p310, and p305/310 than CHB$^-$ sera (Figure 6B, C, E) with the greatest reactivity (Figure 6E, $p<0.05$) seen for the p305/310 peptide. Moreover, pre-incubation of the CHB$^+$ maternal sera with p305 peptide reduced reactivity on ELISA, indicating specificity (Figure 6F). These results support the conclusion that p305 and p305/310 contained the predominant epitope(s) of α_{1G} recognized by CHB$^+$ maternal sera.

Protein structure and identity in S5–S6 extracellular loop regions in T- and L-type calcium channels

Inspection of the patterns of the CHB$^+$ maternal sera reactivity to the peptides revealed that the sequence NTTCVNWNQY, herein referred to as the 'core sequence', was common to all the reactive peptides (Figure 7A). An alignment of the α_{1G} and α_{1H} proteins revealed that α_{1H} contains a similar peptide sequence in the S5–S6 loop in repeat I with sequence identity for NWNQY, as well as nearby amino acids, with the conserved Cys residue being implicated previously in channel gating activation, inactivation, and deactivation [63].

By contrast, alignments of α_{1G} with the L-type calcium channel α_{1C} and α_{1D} sequences did not uncover evidence of sequence similarity (data not shown). It is important to note that examination of the peptide sequences of the Ro52, Ro60 and La autoantigens, despite their association with CHB, do not have comparable sequences in α_{1G}, α_{1H}, α_{1C} or α_{1D}. However, only 2% of Ro/La positive pregnancies are affected by CHB, supporting the conclusion that these antibodies are not specific to the CHB outcome alone.

To evaluate whether the core sequence has the potential to form a protruding epitope for antibody binding, we employed the ElliPro Epitope Modeling Analysis Tool (http://tools.immuneepitope.org) which predicts linear and discontinuous antibody epitopes based on a protein antigen's 3D structure [64]. We attempted to avoid bias towards the core sequence by submitting the entire sequence of the S5–S6 extracellular loop of α_{1G} repeat I (aa235–370, UniProt) to this server for epitope prediction. Six potential linear epitopes were identified; one of these had the sequence SSSNTTCVNWNQYY, which obviously encompasses the core sequence, and is nearly identical in sequence to p305 (see Figure 8A). We also submitted the same region to the Kolaskar & Tongaonker Antigenicity prediction

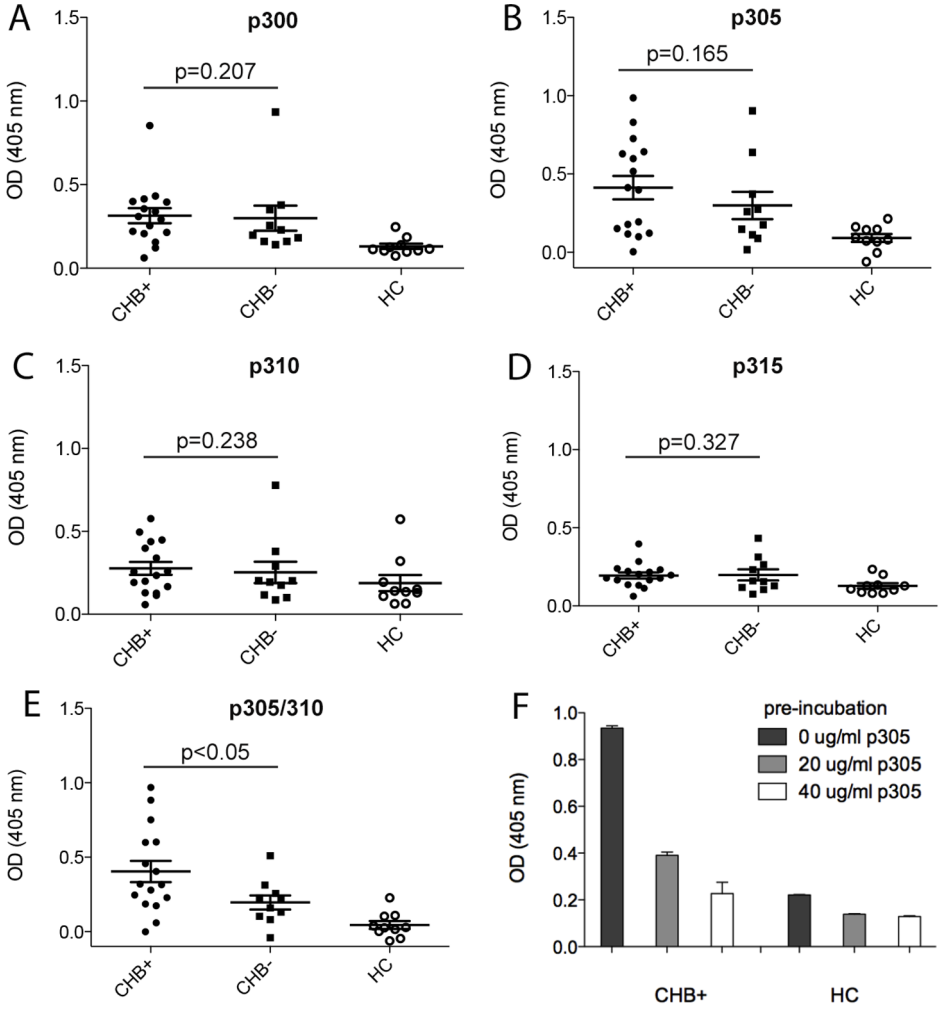

Figure 6. Reactivity in CHB pregnancies is specific for the p305 peptide of α_{1G} and can be blocked. Four overlapping 15aa peptides, and one 20aa peptide were selected to further characterize maternal sera reactivity to α_{1G} in mothers with CHB pregnancies (CHB^+) compared to mothers with unaffected pregnancies (CHB^-). Reactivity to (**A**) peptide p300 (aa300–314), (**B**) p305 (aa305–319), (**C**) p310 (aa310–324), (**D**) p315 (aa315–329) demonstrate that sera from mothers with pregnancies affected by CHB (CHB^+), have significantly higher p305 antibody levels compared to unaffected (CHB^-) pregnancies ($p<0.05$). Although p305 (aa305–319) had the highest reactivity, a longer peptide combining p305 and p310 designated p305/310 (aa305–24) demonstrated a significant difference between CHB^+ and CHB^- maternal serum (**E**). Pre-incubation of one CHB^+ maternal sera, and one healthy control (HC) sera with increasing concentration (0, 20, 40 μg/ml) of peptide p305 (aa305–319) demonstrates that the reactivity of sera is specific for this peptide and can be blocked (**F**). Error bars indicate mean ± SE.
doi:10.1371/journal.pone.0072668.g006

and found that the eight amino acids NTTCVNWN, present in our ''core sequence'', were predicted to be an antigenic peptide based on a method predicting antigenicity from the expression of certain hydrophobic amino acids present on the surface of a molecule [65].

CHB^+ Maternal sera are immunoreactive to peptides derived from the S5–S6 extracellular loops of α_{1G} and α_{1H} repeat I

Based on the results above, we hypothesized that CHB^+ maternal sera might react with the S5–S6 segment in repeat I of α_{1H}. A peptide corresponding to the aligned region of α_{1H} (p330, aa330–343, Figure 7B) was accordingly utilized to screen a group of 23 CHB^+ maternal sera, and the reactivity profile compared to that of p305. The pattern of reactivity obtained with each peptide was qualitatively similar (Figure 7B), and the levels of α_{1H} reactivity observed among the sera were significantly correlated to those of α_{1G} (Spearman correlation, $r = 0.89$, $p = 0.0001$). The parallel reactivity of CHB^+ sera towards p305 and p330 led us to hypothesize that p330 might also provide a means to distinguish between CHB^+ and CHB^- sera. Screening of maternal sera with p305 (α_{1G}) and with p330 (α_{1H}) indicated a tendency for the CHB^+ and CHB^- groups to have differential reactivity for the peptides, although these results were of marginal significance ($p = 0.075$ and 0.184 for p305/α_{1G} and p330/α_{1H}, respectively). Each peptide was nevertheless capable of distinguishing between the CHB^+ and healthy control (HC) groups ($p<0.05$ for both peptides, Figure 7C). These results support the conclusion that CHB^+ sera can also react with α_{1H}, as well as α_{1G}, consistent with previous studies [66]. This conclusion is consistent with our RT-PCR and Western blot studies (Figure 3) establishing expression of α_{1H} in human fetal hearts.

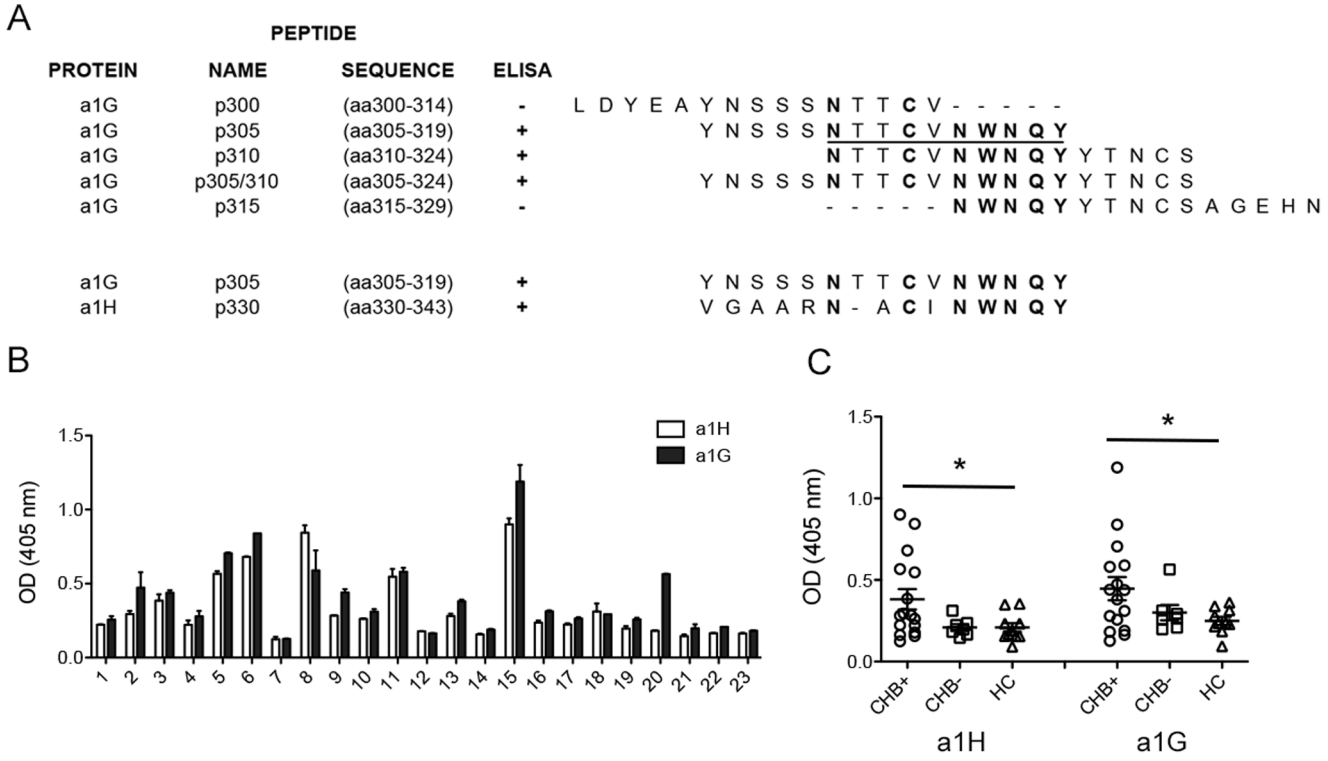

Figure 7. Sequence identity and maternal sera reactivity for α_{1G} and α_{1H} T-type calcium channel peptides. (**A**) Alignment of peptides derived from human α_{1G} and α_{1H} S5–S6 extracellular loop I sequences. The 'core sequence' of interest with 10 amino acids in common among the α_{1G} peptides is underlined, and the seven residues that are identical in the α_{1G} and α_{1H} sequences are indicated in bold type. Note that this group is present in the sequence of each immunoreactive peptide, whereas the non-reactive peptides lack two or more of these amino acid residues. Sequences shown correspond to human CAC1G (α_{1G}) and human CAC1H (α_{1H}) (UniProt accession numbers O43497 and 095180, respectively). (**B**) Peptides derived from the aligned loop regions of α_{1G} and α_{1H} exhibit a similar pattern of CHB^+/CHB^- reactivity. CHB maternal sera ELISA reactivity (OD 405 nm) was plotted with interleaved bars for comparison. Error bars represent Mean ± SE. (**C**) Reactivity of CHB^+ maternal sera was observed to be significantly higher than HC sera, where $p<0.05$ for both α_{1G} p305 (aa305–319) and α_{1H} p330 (aa330–343), but did not reach significance for comparison with CHB^- sera ($p=0.075$ and $p=0.184$ for α_{1G} and α_{1H}, respectively).
doi:10.1371/journal.pone.0072668.g007

Electrophysiological recordings of T-type current in SAN cells

To evaluate a functional effect of CHB^+ maternal serum on T-type Ca^{2+} current, we isolated sinoatrial node (SAN) cells from mice. Single-cell patch-clamp experiments were performed on adult mouse heart SAN cells to isolate Ca^{2+} currents since protein analysis demonstrated expression of α_{1G} in both neonatal and adult mouse (Figure 8A). As expected from previous reports [67,68], the Ca^{2+} current began activating at voltages negative to −50 mV with the current-voltage relationship showing clear evidence for the presence of two components when holding at −80 mV, which became one component at holding potentials of −40 mV. Previous studies have established that the first component activated at the more negative potentials (between −60 mV and −40 mV) is a signature of T-type calcium currents in SAN pacemaker myocytes [69]. Importantly, the application of CHB^+ serum irreversibly and preferentially decreased ($p=0.0313$) Ca^{2+} current at voltages between −60 and −20 mV, precisely where T-type calcium currents are differentially activated (Figure 8B, D). At −50 mV, the CHB^+ serum reduced the Ca^{2+} current from -1.23 ± 0.34 pA/pF to -0.48 ± 0.15 pA/pF ($n=6$) while no significant change ($p=0.8772$) was observed when normal serum was added (baseline: -1.32 ± 0.90 pA/pF, control serum: -1.22 ± 0.81 pA/pF; (Figure 8C, E; $n=6$). CHB^+ serum also decreased ($p=0.0438$) current at −40 mV from -2.25 ± 0.53 pA/pF to -1.32 ± 0.24 pA/pF ($n=6$). At more positive potentials (i.e. above −20 mV) the relative effects of the serum were far less (and the changes did not reach significance) because L-type Ca^{2+} currents are the dominant currents at these voltages [69].

Discussion

Anti-Ro/SSA and anti-La/SSB autoantibodies have been associated with CHB for decades, but the precise targets for these autoantibodies remain incompletely understood. For example, maternal sera from CHB pregnancies were reported to affect both L- and T-type currents ($I_{Ca,T}$) [47]. Arguments can be made for the involvement of both of these Ca^{2+} currents in CHB. Indeed, mice lacking α_{1D} ($Cav1.3^{-/-}$) or α_{1G} ($Cav3.1^{-/-}$) display heart block [53,70] as well as altered Wenckebach cycle lengths (WBCL). On the other hand, mice lacking the α_{1H} T-type calcium channel ($Cav3.2^{-/-}$) have shown no ECG alterations [71]. These latter findings led us to hypothesize that the α_{1G} T-type calcium channel might be a target of CHB autoantibodies.

Consistent with our hypothesis, IHC staining demonstrated that α_{1G} is expressed in the AV node and AV bundle of the fetal human heart (Figure 2). In addition, our immunofluorescence and confocal results demonstrate that α_{1G} is expressed on the surface of ventricular cardiomyocytes (Figure 4). Protein (Western Blotting and IPs) and mRNA expression results (Figure 3) further support

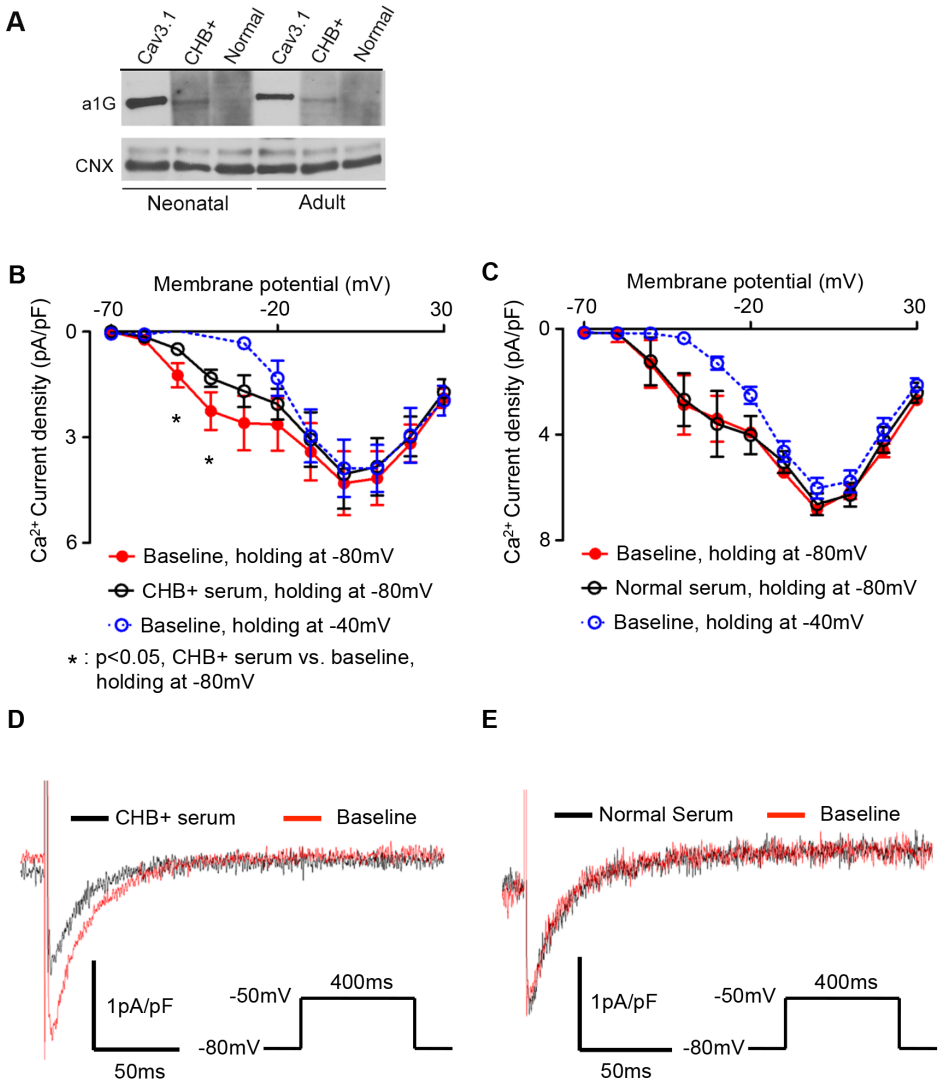

Figure 8. Effect of maternal sera on calcium current in mouse sinoatrial node (SAN) cells. (**A**) CHB maternal serum antibodies, but not healthy control serum, show reactivity to mouse α_{1G} in Western blot. Commercial α_{1G} (Cav3.1) antibody demonstrates the α_{1G} positive band and calnexin (CNX) antibody probing shows equal loading of mouse heart lysate. (**B**) Calcium current density-voltage curve with CHB serum, (**C**) Calcium current density-voltage curve with normal serum, (**D**) representative Calcium current traces recording at −50 mV from holding at −80 mV with CHB serum, (**E**) representative Calcium current traces recording at −50 mV from holding at −80 mV with normal serum. CHB serum is defined as an anti-Ro′La positive serum from a pregnancy affected by third-degree AVB or CHB. Normal serum was obtained from a mother with a pregnancy unaffected by CHB, resulting in a healthy outcome.
doi:10.1371/journal.pone.0072668.g008

the presence of α_{1G} in the fetal myocardium (AVJ, ventricle). However, the expression of α_{1G} in the fetal heart does not establish a functional role. In this regard, previous studies in rabbit sinoatrial cells have established that T-type Ca^{2+} current is blocked by Mibefradil, a T-type calcium channel blocker at low concentrations [72,73]. Consistent with these functional effects on T-type current, we found that Mibefradil strongly affected the WBCL in newborn, but not adult, rabbit hearts. On the other hand, the T-type calcium channel blocker $NiCl_2$, had no effect on WBCLs in newborn rabbit hearts at concentrations up to 300 μM. These pharmacological observations support the conclusion that the α_{1G} (Cav3.1)-dependent T-type Ca^{2+} current, rather than the α_{1H}-dependent T-type Ca^{2+} current, is the dominant current in the AV node in newborns since the Ni^{+} sensitivity of α_{1H} channels (IC_{50} = 13 μM) is much lower than α_{1G} channels (IC_{50} = 250 μM). This contention is consistent with results showing that mice lacking α_{1G} channels ($Cav3.1^{-/-}$) have decreased T-type currents ($I_{Ca,T}$) in both sinoatrial (SAN) and AV nodal (AVN) cardiomyocytes, as well as slowed AV conduction [53], while mice lacking α_{1H} channels ($Cav3.2^{-/-}$) show no such abnormalities [67]. Further, the differential WBCL response in adult and newborn rabbits matches observations in mouse models showing that α_{1G} and α_{1H} are differentially regulated during development, with expression reductions occurring with aging [74], a trend that we observe in our quantitative PCR studies (decrease in α_{1G} and slight decrease in α_{1H}, Figure 3A). However, reports differ on developmental expression of α_{1G} and α_{1H} [75]. Human studies have demonstrated expression of both α_{1G} and α_{1H} channel transcripts in adult human heart [51,76], and α_{1G} protein in adult heart sinus node [77], but ours is the first report of α_{1G} protein expression in the AV node of human fetal hearts. A limitation of our mRNA measurements, however, was sample numbers of human fetal

hearts (one per time point). Clearly, further investigation of the temporal expression of α_{1G} in human fetal hearts will be necessary in order to understand developmental expression. We nevertheless demonstrate expression of α_{1G} protein in the AV node of 21 week-old fetal hearts, indicating that α_{1G} is present during the time period when CHB usually occurs in the fetus.

Involvement of α_{1G} in CHB is supported by a number of our present observations. Firstly, CHB^+ sera recognized α_{1G} protein from human fetal hearts in Western blot. Further, the reactivity of CHB^+ sera was epitope mapped to the α_{1G} p305 peptide (aa305–319), where we have identified an essential "core" signature sequence. However, although our ELISA screen identified clear patterns of reactivity/binding consistent with the need for a core sequence of 10 amino acids for recognition, a significant difference in reactivity of CHB^+ vs. CHB^- sera was observed only for the 305/310 peptide, with considerable overlap between the two sera types. These observations suggest that maternal antibody reactivity to an additional target(s) may be involved in the disease.

Additional evidence for functional involvement of T-type currents in CHB was obtained by applying our CHB^+ sera to adult mouse SAN cardiomyocytes, cells previously demonstrated to exhibit relatively high, and therefore easily measured, T-type Ca^{2+} currents [78]. Moreover, the T-type Ca^{2+} current in the adult mouse heart is primarily comprised of α_{1G}-dependent channels [53]. Consistent with these previous studies, we observed expression of α_{1G} (Cav3.1) in the neonatal and adult mouse heart. CHB^+ sera also recognized a protein with the same molecular weight in neonates and in adults, and was able to preferentially block Ca^{2+} currents activating at more negative voltages (from -60 to -30 mV) – known to correspond with activation of T-type channels at a more negative voltage [78]. Additionally, this block by CHB^+ sera was not reversed on washout, consistent with high affinity binding, and no block was seen when CHB^- sera was added.

While collectively our results firmly support the conclusion that α_{1G} channels play a role in CHB in newborns, we cannot rule out the possible involvement of other channels. Indeed, maternal sera from CHB pregnancies have been shown to effect both L-type Ca^{2+} currents and α_{1H}-based T-type Ca^{2+} currents [47]. Our peptide mapping studies of α_{1G} identified a short sequence located in the S5–S6 extracellular (P-loop) segment in repeat I as a likely potential antigenic epitope for CHB^+ sera. Sequence alignment revealed that the other cardiac T-type calcium channel subunit, α_{1H}, has a sequence with high similarity with the α_{1G} channel protein in this region, which also was recognized by CHB^+ sera. Potential involvement of α_{1H}-dependent T-type calcium channels in CHB is further supported by our observation that α_{1H} mRNA is expressed in fetal human hearts. Surface expression of α_{1H} in human fetal cardiomyocytes however, has not been demonstrated in this study. Accessibility of the α_{1G} extracellular epitope on ventricular cardiomyocytes was demonstrated by CHB maternal sera antibodies affinity-purified towards the p305 α_{1G} peptide surface staining non-permeabilized ventricular cardiomyocytes (Figure 4). After permeabilization, and staining for α_{1G}, co-staining could be seen with a portion of α_{1G} present on the surface. Further studies are needed to determine the accessibility of the α_{1H} epitope for antibody binding in human fetal hearts and its functional significance. CHB^+ sera has also been shown previously to interact with a recombinant protein derived from the S5–S6 extracellular region in repeat I of the L-type calcium channel alpha subunit, α_{1D} [48]. Although sequence alignments did not reveal a similar "core sequence", it seems possible that the S5–S6 extracellular loop region in repeat I of both T-type and L-type calcium channels may have a conserved structure or, alternatively, this region may have a strong propensity for promoting immune responses, consistent with identification of this region of α_{1G} protein by ElliPro Epitope Prediction and the Kolaskar & Tongaonker Antigenicity as having a high likelihood of antigenicity. The possibility of multiple protein targeting in CHB requires further investigation, and characterization of sera antibody specificity with respect to anti-Ro52, anti-Ro60 and anti-La will be necessary to determine the fine specificity of the antibodies cross-reacting with these channels. In this regard, we wish to note that expansion beyond CHB of the clinical spectrum for mothers with anti-Ro/SSA and anti-La/SSB antibodies [3–9,61] might be tied to the existence of a common epitope on several protein targets.

In summary, the data presented here implicate α_{1G} as one specific target of CHB maternal sera antibodies, and suggest the potential for involvement of α_{1H}, although we note that further functional studies with human cardiomyocytes are necessary to determine this conclusively. Investigations into specific channel isoforms and modifications, that may impact antibody binding to the native human proteins, will be important in understanding which targets bound by antibodies are important for development of the human disease.

Materials and Methods

Patients

The study included sera from 28 women who were followed at the Hospital for Sick Children, Toronto, Canada. Seventeen of the samples were from mothers with anti-Ro/La autoantibodies and CHB affected pregnancies with AVB III (CHB^+). Ten samples were from mothers with anti-Ro/La antibodies who were at risk for CHB, and seen at the rheumatology clinic, but with normal fetal heart rate (CHB^-) outcome, evaluated by fetal echocardiography (ECG) during pregnancy, and 10 anti-Ro/La negative samples were used as healthy controls (HC). The Hospital for Sick Children Research Institute Research Ethics Board (REB) approved the study under protocol 0019970143 and written consent was obtained from mothers for use of the sera.

Human fetal heart tissue

Human fetal hearts (17- to 23-week gestation) were obtained through the Heart Centre Biobank Program from elective terminations of normal pregnancies. The use of human fetal tissue was approved by the Hospital for Sick Children Research Institute REB under protocol 1000029263. Written consent was obtained in the clinic, and portions of fetal hearts from fetal terminations are sent under a material transfer agreement to undergo banking within the Heart Centre Biobank Registry program under REB protocol number 1000011232.

Fetal hearts were dissected by an experienced cardiologist following an established method [59]. In brief, hearts are immobilized in a dissecting dish, right ventricle uppermost, which is slit open to visualize the tricuspid inlet below the interatrial septum. The AV junction is identified using anatomical landmarks of the septal cusp of the tricuspid valve inferiorly, the coronary sinus posteriorly, the tendon of Todaro superiorly. Tissue was either dissociated for immunofluoresence, flash frozen for immunoprecipitation experiments, or quickly submerged in RNA stabilizing reagents for immediate RNA preparation.

Preparation and perfusion of the Langendorff rabbit CHB model

The Animal Care Committee at the Hospital for Sick Children has approved the use of neonatal and adult rabbits as well as the experimental design of this study under protocol number 3250.

The animals were housed in the Animal Care Facility at the Hospital for Sick Children and maintained in accordance with hospital regulations and CCAC guidelines. Rabbit hearts were prepared for perfusion as previously described [57]. Briefly, following loss of the pain reflex, the rabbit sternum was incised and removed. The beating heart was dissected, and perfused with fresh oxygenated modified Krebs-Henseleit solution. The flow rate was constant at 9 ml/min for neonates and 36 ml/min for adults, the temperature was kept at 37°C and the left ventricle was left unloaded. The sinus node at the level of the superior vena cava-right atrial junction was excised until junctional rhythm was obtained, following which the hearts were allowed to equilibrate for 15 min.

Electrophysiologic recording for WBCL analysis

In order to compare calcium channel blocker effects on newborn versus mature AV nodal conduction, the rabbit Langendorff heart model was modified by atrial pacing the preparation at sequentially shorter cycle lengths until the AV nodal Wenckebach cycle length (WBCL) was identified, as described previously [57]. At baseline and 15 minutes following equilibration of each concentration of channel blocker, the atrium was paced at incrementally faster rates to determine WBCL. Finally, the hearts were perfused with fresh modified Krebs-Henseleit solution for 15 min and paced to determine WBCL in the washout state. The WBCL was defined as the slowest atrial paced rate with any failure of AV conduction within 8 consecutive seconds of pacing.

Calcium channel blockers

Prior to the comparative study, we assessed the effect of each blocker on WBCL prolongation in neonatal rabbit Langendorff preparations. We chose the concentration of each drug to cause at least double prolongation of WBCL in newborn rabbits. Adults (n = 7) and newborn (n = 8) hearts were exposed to two concentrations of either I_f blocker (ZD7288; 10, 20 μM), $I_{Ca\text{-}L}$ blockers (Verapamil; 0.2, 0.4 μM. Diltiazem; 0.4, 0.8 μM) or $I_{Ca\text{-}T}$ blocker (Mibefradil; 0.4,0.8 μM). Increasing concentrations of a single drug were added to the perfusate in a sequential cumulative pattern. Newborn hearts were also assessed for WBCL prolongation upon exposure to a specific blocker of the T-type calcium channel α_{1H} subunit (Nickel Chloride; 1, 10, 100 and 300 μM).

Histology and Immunohistochemistry

AVJ was dissected and fixed in 4% paraformaldehyde. After fixation, AVJ tissue was preserved as paraffin-embedded blocks. Slides were made from the blocks and underwent Masson's trichrome stain and were immunolabelled for α_{1G} and NF-160. The slides were dewaxed, rehydrated and rinsed 2×5 min in double distilled water. The slides were then incubated with Target Retrieval Solution (DAKO Canada) at 95°C for 40 min and then room temperature 20 min. After rinsing 2× with PBS, sections were blocked with blocking buffer (PBS with 3% fish gelatin, 2 mg/ml BSA and 2% Tween-20) for one hour, and incubated with rabbit-anti-α_{1G} (Novus Biologicals Canada) at a dilution of 1:400 and mouse anti-NF-160 (1:500, US Biological) for 2 hours. After rinsing 3× with PBS, slides were then incubated in the dark with donkey-anti-rabbit Cy3 (1:1000, Jackson Immunolabs) and donkey anti-mouse Alexa488 (1:1000, Invitrogen) for 40 min, DAPI was then added in the last 5 min, slides were washed 3×5 min in PBS and mounted with a coverglass. Slides were examined using a Zeiss Epifluorescence microscope.

Real time PCR

Total RNA was prepared according to the protocol *RNeasy Fibrous Tissue Mini Kit* (Qiagen). Homogenization was performed with a Tissue Tearor. RNA concentration and purity was determined and purified total RNA was converted to cDNA with Superscript III reverse transcriptase (Invitrogen). Power SYBR Green PCR Master Mix (Applied Biosystems) was used to perform relative quantification of target sequences *CACNA1G* (forward primer: CAAACTTGTGGCCTTTGGTT, reverse primer: GGTGGACTCCTGGTCACAGT) and *CACNA1H* (forward primer: ATAACCAACCCAAGTCGCTG, reverse primer: CAGGAGCATGAAAAGAAGGC) compared to the housekeeping gene *GAPDH* (forward primer: CCTGTTCGACAGTCAGCCGCATC, reverse primer: GGTGACCAGGCGCCCAATACG). Assay was optimized for the cDNA amount as well as PCR conditions to validate the primers and ascertain equal efficiency of primers. Samples were prepared in triplicates and subjected to default conditions for the 7000 Sequence Detection System by ABI (annealing at 60°C for 1 minute).

Immunoprecipitation, Western blot

Fresh tissue from 20–22 week-old fetal human hearts were homogenized in NP40 lysis buffer (1% NP40, 0.15M NaCl, 0.01M Na_3PO_4 pH 7.2, 2.5 mM $Na_4O_7P_2$, 2 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF) with proteinase inhibitor cocktail (Roche, Penzberg, Germany). Homogenized lysates were prepared at room temperature, incubated for 30 min and then centrifuged at 12,000 rpm for 15 min. Protein concentration was determined with the Bradford protein assay (Bio-Rad, Hercules, CA, USA) and 1 mg of total protein lysates were used per immunoprecipitation sample. Immunoprecipitation of α_{1G} protein was achieved with Protein G Sepharose beads and 2 μg of α_{1G} antibody (Cav3.1 antibody, Alamone Labs). Samples were incubated at 70°C for 5 min in Laemmli loading buffer. Samples were loaded onto 10% Tris-glycine sodium dodecyl sulfate polyacrylamide gels electrophoresed at 120 V for 2.5 h, and transferred overnight at room temperature (20 V) to nitrocellulose membranes. Membranes were blocked with 5% milk in Tris-buffered saline/0.05% Tween-20 (TBS-T), then probed with either human sera (1:100), anti-Cav3.1 antibody (1:200 dilution, Santa Cruz for Western blot probing after IP, 1:100 dilution, Sigma for western probing), or anti-Cav3.2 antibody (1:200 dilution, Sigma) for 2 h at room temperature. For pre-incubation to demonstrate specificity of commercial antibody for α_{1G}, a peptide corresponding to aa1–22 of rat α_{1G} was added at the recommended concentration of 1:1 with the antibody prior to addition to the blot. Membranes were washed between each step 3×15 min with TBS-T. Membranes were incubated 1 h with anti-human IgG-HRP (1:10,000 dilution, Jackson Immunolabs) or goat anti-rabbit IgG-HRP (1:5,000 dilution Jackson Immunolabs). Anti-calnexin (CNX) antibody was used as loading control for Western blot. Membranes were developed with ECL substrate (Santa Cruz Biotechnology) and film exposed for visualization of bands. Anti-Ro and anti-La titers given for maternal sera used in immunoassays are from routine clinical screening based on an ELISA using recombinant human anti-Ro60-kD and anti-Ro52-kD, and anti-La 48-kD protein (Phadia GmbH, Freiburg, Germany).

Immunofluorescence

Dissected regions of fetal heart tissue was washed in Hank's solution (HBSS) and incubated with gentle shaking overnight in Hank's solution supplemented with 5 mM BDM and 1 mg/ml collagenase. Heart pieces were minced and incubated in fresh mincing solution (HBSS with 10 mM taurine, 0.1 mM EGTA, 10 mM BDM, 1 mg/ml BSA, 1 mg/ml collagenase) gently

spinning with a small magnet at 37°C to harvest cells for 5 min. This procedure was repeated 3 times. Cells were washed then fixed with 2% paraformaldehyde for 10 min, shaking slowly for subsequent immunofluoresence labeling.

For detection of α_{1G} T-type calcium channel, rabbit anti-human Cav3.1 antibody was used at a dilution of 1:500 (Novus Biologicals Canada), and for detection of cardiomyocyte markers mouse anti-human cardiac Troponin-T was used at a dilution of 1:500 (Thermo Scientific). Cells were resuspended in blocking buffer (PBS with 3% fish gelatin, 2 mg/ml BSA, 0.05% Tween-20) at room temperature for 1 h. Primary antibodies were added overnight at 4°C, shaking gently. After 2×15 min washes in PBS, cells were incubated in the dark for 4 h at 4°C with secondary antibodies: donkey anti-rabbit-IgG-Cy3 (1:5,000 Jackson ImmunoResearch) and donkey anti-mouse-IgG-Alexa488 (1:1,000 Green Invitrogen). DAPI stain was used to visualize nucleus (0.1 μg/ml). Cells were washed 2×15 min in PBS and mounted on glass for confocal microscopy (60× magnification).

For the surface staining with affinity-purified sera, non-permeabilized cells were blocked (PBS with 3% fish gelatin, 2 mg/ml BSA) at room temperature for 1 h before incubation with Cav3.1 antibody. Affinity purified sera was diluted in blocking buffer (without detergent) 1:2 (as already diluted from elution/neutralization buffers) for 2 h at room temperature. Cardiomyocytes were washed and secondary antibody (rabbit anti-human-IgG-Cy3 at 1:1,000, Jackson Immunolabs) was added, and cells were incubated for 1 h at room temperature in the dark. Subsequent staining for the α_{1G} T-type calcium channel followed the procedure described above, except that the secondary antibody used was donkey anti-rabbit-IgG-Dylight488 (1:1,000 Jackson ImmunoResearch).

Affinity Purification of Antibodies from Maternal Sera Specific for the p305 Peptide

Serum was affinity purified towards the α_{1G} p305 peptide (aa305–319 of α_{1G}) for use in immunofluorescence experiments as follows: A streptavidin resin filled column was equilibrated and washed with PBS. Serum was pre-incubated overnight with biotinylated p305 peptide. Free biotin was then added to the serum solution to block all non-specific biotin binding sites, after which the solution was applied to the streptavidin column. The column was then washed 3 times with PBS to remove antibodies not specific for peptide, and antibodies of interest were then eluted with 0.1 M glycine, pH 2.5 and immediately transferred into 1M Tris, pH 8.0 in a 1:10 ratio.

Peptides and ELISA

ELISA epitope mapping was performed using α_{1G} peptide PEPscreen Custom peptide libraries (Sigma, St. Louis, MO, USA). We investigated fifty overlapping 15aa peptides corresponding to aa 130–385. Thirty-six 15aa peptides corresponding to aa774–963, and forty-four 15aa peptides covering aa1308–1536 were studied. Peptides were dissolved (80% DMSO/20% water) and 96-well ELISA plates were coated with antigen (20 μg/ml) in carbonate coating buffer pH 9.6 (0.03 M Na_2CO_3, 0.07 M $NaHCO_3$, 0.1% NaN_3), and incubated overnight at 4°C. Plates were washed and blocked with 5% BSA for 2 h and incubated for 2 h with sera at room temperature. Plates were washed 3× with washing buffer and incubated for 1 h with goat anti-human IgG AP-conjugate (Sigma, St. Louis, MO, USA). Plates were then developed with AP substrate buffer (R&D systems) and read at OD 405 nm minus that of the reagent blank. All samples were run in duplicate. Controls were from 10 anti-Ro/La positive mothers who were at-risk for CHB and followed in the rheumatology clinic, but with healthy pregnancy outcomes, as well as control sera (anti-Ro/La negative) for assay development. Cut-offs for epitope mapping were determined by calculation of HC average+3× standard deviation.

The time at which ELISA experiments were read by spectrophotometer OD (405 nm) was optimized for each peptide using two criteria: (i) $OD_{405}<0.3$ in healthy control samples, and (ii) OD_{405} as high as possible among test samples within spectrophotometric limits ($OD_{405}<2.0$). OD values shown in Figures 7 and 8 were accordingly obtained at various incubation times. Peptide ELISA reading times were as follows: 50 min for p305; 2 h 30 min for p300 and p315; 1 hr 30 min for p310 and p305/p310.

Comparison of α_{1G} and α_{1H} peptides was accomplished with peptide amines modified at their N- and C-termini with lysine residues to enhance water solubility [79] (α_{1G}, sequence H_2N-KKKYNSSSNTTCVNWNQYKKK-NH_2; α_{1H}, H_2N-KKKVG-AARNACINWNQYKKK-NH_2). These peptides were dissolved in water and coated at 1 μg/ml and blocked with 1% fish serum for 2 h before incubating with primary sera for 2 h. Otherwise the procedure was identical to the above.

Electrophysiological recordings of T-type calcium current

Mice were cared for and housed in the Animal Care Facility at University of Toronto and maintained in accordance with university regulations and Canadian Council on Animal Care (CCAC) guidelines. The use of mice for electrophysiology studies was approved by The Animal Care Committee of University of Toronto under Protocol 20009885.

After heparinized mouse (C57BL/6, 8 weeks old, male, Charles River Inc. Canada) was anesthetized, heart was quickly removed and retrogradely perfused through aorta with 37°C Ca^{2+}-free Tyrode's solution, containing (in mM) 137 NaCl, 5.4 KCl, 1.0 $MgCl_2$, 0.33 NaH_2PO_4, 22 D-glucose, 10 HEPES, pH 7.3, for 8∼10 min. Then, collagenase (1.0 mg/mL, Worthington, type II) and elastase (0.15 mg/mL, Worthington) were added. Heart was digested for 35∼50 min. SAN region (as described in [80]) was cut and cells were dissociated and kept in solution which contained (in mM) 120 potassium glutamate, 20 KCl, 20 HEPES, 1.0 $MgCl_2$, 10 D-glucose, 0.5 K-EGTA, and 0.1% bovine serum albumin, pH 7.35 with KOH.

Ca^{2+} current was introduced by 400 ms test pulse from −70 mv to +30 mV with 10 mV step from holding potential at −80 mV or −40 mV. Cells were bathed in solution containing (in mM) 140 CsCl, 1 $MgCl_2$, 1.8 $CaCl_2$ 10 HEPES, 10 D-glucose, pH 7.35 with CsOH. SAN cells were picked as described previously [68]. Whole-cell patch clamp recording (Axopatch 200B and Clampex 8 software, Axon Instrument, CA, USA) was performed with 3.0∼4 MΩ pipette when filled with solution containing (in mM): 135 CsCl, 5 TEA, 1 $MgCl_2$, 4 MgATP, 10 HEPES, 10 EGTA, 0.3 Na_2GTP, pH 7.2 with CsOH. Serial resistance was 80%∼85% compensated. Data were analyzed with Clampfit 10 (Axon Instrument, CA, USA).

In order to evaluate the effect of serum on T-type calcium current, positive (CHB^+) or control serum (normal serum) was added into external solution (1:50 dilution) and kept for 5 min for full reaction, then serum was fully washed out to remove Na^+ from external solution.

Statistical analysis

For ELISA assay, Mann-Whitney U-test or Kruskall-Wallis ANOVA were used for statistical analysis. Mann-Whitney analysis was used for gene expression comparison. For analysis of

electrophysiology data a paired T-test was used. The level of significance was set at $p<0.05$.

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Author Contributions

Conceived and designed the experiments: LSS RMH XC PHB JL ES. Performed the experiments: LSS XC XL VS BBS JYY AA WY MC FQ KL JL CA LB. Analyzed the data: LSS XC AR JL. Contributed reagents/materials/analysis tools: RMH EDS CMD VS PHB. Wrote the paper: LSS XC RMH AR CMD PHB. Characterized maternal sera: EDS. Diagnosed CHB pregnancies: ETJ.

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