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Neurogranin regulates calcium-dependent cardiac hypertrophy

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Abstract

Intracellular Ca^{2+}-calmodulin (CaM) signaling plays an important role in Ca^{2+}-CaM-dependent kinase (CaMKII) and calcineurin (CaN)-mediated cardiac biology. While neurogranin (Ng) is known as a major Ca^{2+}-CaM modulator in the brain, its pathophysiological role in cardiac hypertrophy has never been studied before. In the present study, we report that Ng is expressed in the heart and depletion of Ng dysregulates Ca^{2+} homeostasis and promotes cardiac failure in mice. 10-month-old Ng null mice demonstrate significantly increased heart-to-body weight ratios compared to wild-type. Using histological approaches, we identified that depletion of Ng increases cardiac hypertrophy, fibrosis, and collagen deposition near perivascular areas in the heart tissue of Ng null mice. Ca^{2+} spark experiments revealed that cardiac myocytes isolated from Ng null mice have decreased spark frequency and width, while the duration of sparks is significantly increased. We also identified that a lack of Ng increases $CaMKII_{\delta}$ signaling and periostin protein expression in these mouse hearts. Overall, we are the first study to explore how Ng expression in the heart plays an important role in Ca^{2+} homeostasis in cardiac myocytes as well as the pathophysiology of cardiac hypertrophy and fibrosis.

Keywords

Neurogranin; Calcium-calmodulin; Calcium spark; Cardiac hypertrophy; Fibrosis

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CRediT authorship contribution statement

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Disclosures

All authors declare no conflicts of interest.

1. Introduction

Intracellular calcium (Ca^{2+}) regulation is vital to the mechanism for contraction and relaxation of the heart chambers, termed excitation-contraction coupling (Bers, 2006). This contraction and relaxation, in cardiac myocytes, is triggered by a temporary rise and decline in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). During the travelling action potential, depolarization of the cell membrane triggers the opening of L-type Ca^{2+} channels (LTCCs) (Louch et al., 2012). The resulting Ca^{2+} influx triggers additional Ca^{2+} entry by binding to Ca^{2+}-release channels called ryanodine receptors (RyRs) in the sarcoplasmic reticulum (SR). This is the amplification process known as the Ca^{2+}-induced Ca^{2+} release (Fabiato, 1985), who's regulation is vital to cardiac myocyte stability.

There are significant changes in the handling of myocyte Ca^{2+} during heart failure, which are critical in dictating the typical characteristics of the disease's progression (Bers, 2006), such as cardiac hypertrophy and fibrosis. This is due to the critical role $[Ca^{2+}]_i$ plays in cardiac myocyte signal transduction in addition to its role in contraction/relaxation (Roe et al., 2015). Overactivity of the Ca^{2+}-calmodulin (CaM)-dependent phosphatase, calcineurin (CaN) pathway, for example, is crucially involved in the development of pathologic hypertrophy (Molkentin et al., 1998) and inflammation (Goonasekera and Molkentin, 2012). The Ca^{2+}-CaM-dependent kinase ($CaMKII_\delta$) has also been established as a mediator of heart failure development, and an increasing number of studies also implicate the role of elevated $CaMKII_\delta$ signaling during pathological cardiac remodeling, including increased fibrosis (Weinreuter et al., 2014; Zhong et al., 2017) and collagen deposition (Martin et al., 2014).

Neurogranin (Ng) is a small 7.5 kDa protein known to regulate intracellular Ca^{2+}- CaM formation in the brain (Mahoney et al., 1996; Miao et al., 2000), and its genetic variant is significantly implicated in several neurological diseases (Pohlack et al., 2011; Smith et al., 2011) that demonstrate increased cardiovascular mortality (Laursen et al., 2012; Obisesan et al., 2012; Roher et al., 2013). At high $[Ca^{2+}]_i$, Ng demonstrates a strong affinity for CaM, resulting in inhibition of Ca^{2+}- CaM complex formation. Protein kinase C (PKC)-mediated Ng phosphorylation at S37 during low $[Ca^{2+}]_i$ blocks Ng-CaM binding sites, which promotes Ca^{2+}-CaM complex formation (Miyakawa et al., 2001). Furthermore, CaMKII activation in the brain requires Ca^{2+}-CaM binding to its regulatory domain for autophosphorylation of the autoinhibitory domain (T286); which is known to be affected by Ng expression (Pak et al., 2000).

Although Ng is believed to be a brain-specific protein, increasing studies suggest that Ng can be produced outside of the brain, including in T-cells (Nielsen et al., 2009) and skeletal muscle (Fajardo et al., 2019). A recent study also identified Ng expression in human and mouse endothelial cells, which may be critical for cardiovascular function. Echocardiograms of Ng null ($Ng^{-/-}$) mice demonstrate heart failure through significantly decreased ejection fraction and fraction shortening, as well as increased systolic diameter and volume at 10 months of age (Cheriyan et al., 2020). Therefore, Ng could be a possible molecular target involved in the development of Ca^{2+}-dependent cardiac hypertrophy, which has never been studied before.

In the present study, we are the first to report that Ng plays an essential role in cardiac Ca^{2+} homeostasis and hypertrophy using $Ng^{-/-}$ mice. The goal of this study was to determine how Ng expression in the heart regulates Ca^{2+}-dependent signaling and to elucidate the mechanism of hypertrophy and cardiac failure. Ca^{2+} spark and western blotting experiments enabled us to examine the molecular pathology of hypertrophy in $Ng^{-/-}$ mice. Then, we validated our *in vivo* findings using Ng siRNA in the HL-1 cardiac muscle cell line, which is an immortalized mouse cardiomyocyte cell line able to continuously divide while maintaining a cardiac phenotype (Claycomb et al., 1998; White et al., 2004). Overall, the results of these approaches suggest that the Ng protein expression is an essential player in the pathophysiology of cardiac hypertrophy by regulating Ca^{2+}-dependent $CaMKII_{\delta}$ signaling.

2. Materials and methods

2.1. Animals

Male $Ng^{+/+}$ and $Ng^{-/-}$ mice (C57BL/6 J background, Jackson Laboratories, Bar Harbor, ME) aged 10-month-old were used. Mice were group housed in standard Plexiglas cages under a 12 h light/ dark cycle (lights on at 6:00 AM) at a constant temperature (24 ± 0.5 °C) and humidity (60 ± 2%) with food and water available *ad libitum*. The animal care and handling procedures were in accordance with LSUHSC institutional and National Institutes of Health (NIH) guidelines.

2.2. Ng siRNA in HL-1 cardiac muscle cell line

The HL-1 Cardiac Muscle Cell Line (Millipore Sigma, Burlington, MA) was purchased and maintained in HL-1 expansion medium (Claycomb Basal Medium) supplemented with 10% FBS, 0.1 mM Norepinephrine, 2 mM glutamine, and 1× penicillin/streptomycine (Millipore Sigma). HL-1 cells at 70% confluency were transfected with Ng siRNA (50 nmol/L) using Lipofectamine 3000 (Life Technologies, Carlsbad, CA) for 24 h. Experiments were performed after an additional hour of growth (Claycomb et al., 1998; White et al., 2004). To measure the efficiency of Ng siRNA, Ng expression and phospho-Ng expressions were examined using western blotting. Ng knockdown was validated by the comparison of Ng expression to mock HL-1 cells and lipofectamine treated HL-1 cells.

For immunofluorescence, HL-1 cells were cultured in 24-well plates on coverslips until 70% confluency. Then, they were fixed with 2% formaldehyde, permeabilized with 0.2% Triton X-100, and blocked in 1% BSA with azide. Primary antibodies for Neurogranin (#07–425-1, Millipore, Billerica, MA), Calmodulin (#MA3–917, Invitrogen, Carlsbad CA, USA), and Troponin T (#MABT368, Millipore) were incubated overnight. Images were obtained using an Olympus W1 Spinning Disk Confocal System.

2.3. Measurement of myocardial heart weight/body weight ratio

10-month-old mice were weighed, anesthetized with isoflurane (Patterson Veterinary, Greely, CO), and then euthanized *via* cervical dislocation. The chest cavity was opened, and the heart was quickly removed and rinsed in two washes of ice-cold PBS (Fisher Scientific).

Major blood vessels and connective tissues were also removed. The heart was blotted dry, weighed, and the heart weight/body weight ratio was calculated.

2.4. Immunofluorescence for Ng and wheat germ agglutinin (WGA) staining

For immunofluorescence analysis, mouse heart tissues were fixed with formalin and embedded in paraffin. After xylene dewaxing and citrate buffer (10 mM, Vectors Biolabs, Malvern, PA) antigen retrieval, 5 μm sections were incubated with primary Ng or WGA antibodies (1:200 dilution, MilliporeSigma, Burlington, MA) overnight at 4 °C, followed by staining with Alexa Fluor 488-conjugated secondary antibody (4 μg/mL) (Life Technologies, Carlsbad, CA). Nuclei were counterstained with DAPI. Immunofluorescence images were acquired using LSM 710 Confocal microscope (Carl Zeiss, Oberkochen, Germany).

2.5. Histological analysis for Masson's trichrome and Sirius red staining

Hearts from deeply anesthetized 10-month-old mice were harvested in diastole, fixed in 10% buffered formalin, and embedded in paraffin. Serial 5-μm heart sections from each group were stained with Masson trichrome and Sirius red. Fibrosis and collagen deposition were quantified using ImageJ software (National Institutes of Health, Bethesda, MD) as described previously (Bhuiyan et al., 2016). We used 5 mice per each genotype. To identify fibrosis and collagen deposition in the mouse heart, we examined at least 30 slices per mice and selected around 15 tissues slices having average vessels size. Fibrosis was quantified by blue-stained areas, and non-stained myocyte areas from each section using color-based thresholding. To quantify collagen deposition, red-stained areas and non-stained myocyte areas were determined using color-based thresholding. The percentage of total fibrosis area was calculated as the blue-stained areas divided by total surface area from each section. The percentage of collagen deposition area was calculated as the red-stained areas divided by the total surface area from each section (Abdullah et al., 2018).

2.6. Calcium sparks on isolated cardiac myocytes

Atrial cardiac myocytes were isolated as previously described (Voigt et al., 2013). In brief, 3-month-old mice were euthanized using isoflurane followed by cervical dislocation after anesthesia was confirmed. The heart was quickly removed and placed in Complete Tyrode's solution. The left and right atrial tissue were dissected away and placed in 2.5 mL of 35 ± 1 °C Low Ca^{2+}/Mg^{2+} Tyrode's solution. The atrial tissue endured three gentle washes before being digested in Low Tyrode's with elastase and a collagenase/protease blend (containing 140 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 1.2 mm KH_2PO_4, 50 mM taurine, 5.5 mM D-glucose, 5 mM Hepes-NaOH, pH 6.9. Collagenase type II, elastase, protease, and BSA). Following the enzyme digestion of the tissue strips for 20 min at 37 °C by gentle mechanical agitation, the tissue was gently washed three times in 2.5 mL of Modified Kraft-Brühe (KB) solution. Single cells were then dissociated by constant trituration at approximately 0.5–1 Hz for 10–15 min. After dissociation, calcium up to 1.3 mM was reintroduced using multiple concentrations of a NaCl/ $CaCl_2$ adaptation solution. Following calcium re-adaptation, the cardiac myocytes were collected by centrifugation at a rate of 2000 x*g* for 3 min. The cardiac myocytes were then plated onto a laminin coated glass bottom 4 well slide. Cells were allowed to adhere for 1–2 h. Fluo-4 AM (1:1000, Invitrogen) at room temperature in the dark was loaded to the cardiac myocytes

and allowed to incubate for 30 min. After deesterification, the cells were perfused with Complete Tyrode's solution. Confocal imaging was performed using a Nikon Eclipse Ti-E inverted confocal microscope equipped with 40 × 0.75 NA objective. The cells were excited using a 488-nm laser line. Calcium sparks were measured during resting conditions by performing line scan recording at a sampling rate of 2FPS along the longitudinal axis of the cardiomyocyte. Calcium sparks were quantified in the following areas: sparks (sparks/100 μm/s), width (μm), and full duration (ms).

2.7. NOx measurements

Nitric oxide (NO) metabolites (NOx) were measured using Sievers Nitric Oxide Analyzer 280i in Redox Molecular Signaling Core in LSUHSC-Shreveport. An aliquot of plasma was placed in NO preservation solution (800 mmol/L potassium ferricyanide, 17.6 mmol/L N-ethylmaleimide, 6% Nonidet P-40) for tri-iodide NO chemiluminescent analysis. Nitrite was reduced using the tri-iodide method. NO was measured using an ozone-based chemiluminescent assay (Sievers Nitric Oxide Analyzer 280i, Weddington, NC) (Kolluru et al., 2015). Aliquots of samples were tested for sulfanilamide resistance following the addition of an acidic sulfanilamide solution to a final concentration of 0.5% v/v and sitting in the dark for 15 min prior to injection into the analyzer. Brain tissue (positive control) was homogenized in 500 μL NO preservation buffer (1.25 mol/L potassium ferricyanide, 56.9 mmol/L N-ethylmaleimide, 6% Nonidet P-40 substitute in PBS).

2.8. Measurement of mouse blood pressure

Systolic and diastolic blood pressure as well as blood pulse were measured on unanesthetized mice using a Hatteras Instruments SC1000 Dual-Channel Blood Pressure Analysis System (Cary, NC). The blood pressure measurement experiments were conducted in a designated quiet area (22 ± 2 °C), where mice acclimatized for a 1-h period. To measure blood pressure, the occlusion cuff is inflated to 250 mmHg and deflated over 20 s. The sensor cuff detected changes in blood pressure. Mice were habituated for at least 3 consecutive days before baseline blood pressure measurements. To measure baseline blood pressure, total 6 cycle were measured but first 3 cycle were not used in the analysis.

2.9. Cytosolic fractionation and Western blot analysis

5-month-old $Ng^{+/+}$ mice and $Ng^{-/-}$ mice were subjected to isoflurane gas to induce unconsciousness, followed by PBS perfusion and isolation of heart ($n = 4$ per genotype). The extracted tissue was snapfrozen on dry ice and stored at −80 °C until it was processed for Western blotting. Each sample was homogenized in an extraction buffer containing 250mM Sucrose, 50mM Tris–HCl (pH 7.4), 5mM $MgCl_2$ and subcellular fractionation was performed on mouse heart tissues using the protocol from (Dimauro et al., 2012). Tissue lysates were separated into cytoplasmic and nuclear fractions and prepared for western blot. The purity of fractions were confirmed with western blot analysis probing markers for each fraction. Protein concentration was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher, Waltham, MA). Cytoplasmic proteins were separated using 4–12% SDS-PAGE (BioRad, Hercules, CA) at 100 V for 1.5 h, transferred onto PVDF membranes at 25 V for 7 min using a Trans-Blot Turbo Transfer System (BioRad, Hercules, CA), and incubated with antibodies against Neurogranin #07–425-I, Calcineurin #07–1490,

and Ryanodine Receptor 2 #AB9080 (Millipore, Billerica, MA), Periostin #PA5–34641 (Invitrogen, Carlsbad, CA, USA), Calmodulin #4830S, Phospho-CaMKII (T286) #12716S, and CaMKII (pan) #4436S (Cell Signaling, Danvers, MA), and GAPDH #SC-32233 (Santa Cruz, Dallas, TX). Chemiluminescent bands were detected on an Image Station and quantified using NIH Image J software.

2.10. Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Statistics were performed using either a two-tailed Student's *t*-test (Prism, GraphPad Software, La Jolla, CA) or two-way repeated measures ANOVA followed by a Tukey *post hoc* test (SigmaStat, SYSTAT software, Point Richmond, CA). The criterion for statistical significance was $p < 0.05$.

3. Results

3.1. Depletion of Ng induces heart failure in mice

$Ng^{-/-}$ mice have a reduced lifespan compared with their wild-type littermate controls measured by Kaplan–Meier survival curve (Fig. 1A). The Gehan-Breslow-Wilcoxon test indicated a significant survival difference between genotypes with a 6.94 Chi-squared value ($p = 0.0084$). $Ng^{-/-}$ mice display a median survival age of 177 days while $Ng^{+/+}$ mice show a median survival age of 415 days. This curve was originally designed to measure mouse survival for up to 1 year. Later, older mouse data was added in order to see the trend up to 2 years. This caused the large fluctuation in the wild-type mouse curve. However, the significantly decreased survival rate in the $Ng^{-/-}$ mice is still evident.

Because $Ng^{-/-}$ mice demonstrate heart failure at 10-months old with significantly lower ejection fraction and fraction shortening (Cheriyan et al., 2020), we examined the Ng protein expression in the heart. Although Ng is known as brain-specific protein, we identified that a significant amount of Ng is expressed in both the atria and ventricles of wildtype mouse hearts with western blotting (Fig. 1B). Using Immunofluorescence (IFF) we were able to confirm Ng expression (red) in the heart tissue of wild-type mouse. We observed Ng protein expression not only in the vasculature of the heart, but also cardiac muscle. To further validate Ng expression in the cardiac myocytes, we employed *in vitro* experimental model using the HL-1 immortalized mouse cardiomyocyte cell line (Claycomb et al., 1998; White et al., 2004) (Fig. 1C). In the HL-1 cell line, Ng protein is expressed and co-localized with cardiac Troponin T, which regulates muscle contraction in response to alterations in cellular Ca^{2+} concentration. Ng protein is also merged with cardiac calmodulin (CaM) expression, which implicates the possible role of Ng in CaM-dependent excitation-contraction coupling in cardiac myocytes (Yang et al., 2003).

We then measured heart size of 10-month-old $Ng^{+/+}$ and $Ng^{-/-}$ mice. $Ng^{-/-}$ mouse hearts appear obviously larger than wild-type hearts compared to $Ng^{+/+}$ mice (Fig. 1D). $Ng^{-/-}$ mice also showed significantly increased heart weight ($t(12) = 2.64$, $p = 0.021$) as well as an increased heart to body weight ratio ($t(12) = 2.58$, $p = 0.027$), while there were no body weight differences between genotypes (Fig. 1E). These results demonstrated that Ng protein

is expressed in the cardiomyocyte and depletion of Ng expression may contribute to heart failure in mice.

3.2. Ng null mice demonstrate hypertrophy and fibrosis in the heart

Since $Ng^{-/-}$ mice demonstrated significant heart failure and enlarged hearts at 10 months of age, we wanted to determine pathological hypertrophy through histological evaluation. First, wheat germ agglutinin (WGA) staining was used to determine individual cardiac myocyte size from $Ng^{+/+}$ and $Ng^{-/-}$ mice ($n = 5$ mice per group, 200 cardiomyocytes analyzed per mouse). Since WGA binds to glycoproteins of the cell membrane, cross-sectional area of cardiac myocytes was measured to determine cellular hypertrophy. Our results indicate that $Ng^{-/-}$ mice cardiac myocytes have a significantly increased cross-sectional area compared to that of $Ng^{+/+}$ mice ($t(16) = 6.07$, $p < 0.0001$) (Fig. 2A).

To evaluate the pathology of the surrounding cardiac myocyte tissue, we conducted Masson's trichrome staining (n = 5 mice per group, 6–10 sections analyzed per mouse). $Ng^{-/-}$ mice showed significantly increased fibrosis deposition near blood vessels (blue) and cardiac myocyte swelling (red) compared to $Ng^{+/+}$ mice ($t(98) = 3.92$, $p < 0.0001$) (Fig. 2B). Finally, to determine collagen deposition, we conducted Sirius Red staining (n = 5 mice per group, 14–18 sections analyzed per mouse). Compared to wild-type mice, $Ng^{-/-}$ mice displayed significantly increased collagen deposition near the perivascular areas (red), which appeared thicker around the vasculature of the heart ($t(99) = 5.92$, $p < 0.0001$) (Fig. 2C). Overall, these pathological results indicate that dysregulation of Ng in mice induces cardiac hypertrophy as well as fibrosis, which contributes to the heart failure phenotype that has never been reported.

3.3. Ng null mice demonstrate Ca^{2+} dysregulation in cardiac myocytes

It is known that Ng expression regulates Ca^{2+} dynamics in the neuronal cells (Petersen and Gerges, 2015). Having shown that lack of Ng in mouse hearts has a detrimental effect on cardiac function and hypertrophy, we postulated if Ng expression alters Ca^{2+} responses in the heart. To test this, we isolated cardiac myocytes from 3-month-old wild-type and $Ng^{-/-}$ mice and evaluated intracellular calcium release using Ca^{2+} spark imaging (Fig. 3A).

Spontaneous Ca^{2+} sparks occurred with an average frequency of 23.41 (100um/s) in wild-type cardiac myocytes, while an average frequency in $Ng^{-/-}$ cardiac myocytes significantly decreased to 17.47 (100um/s; $t(114) = 3.13$, $p = 0.002$) (Fig. 3B). In addition, the width of Ca^{2+} sparks also significantly decreased in the cardiac myocytes from $Ng^{-/-}$ mice ($t(114) = 3.97$, $p < 0.0001$) (Fig. 3C). Interestingly, the duration of Ca^{2+} sparks significantly increased in the cardiac myocytes lacking Ng ($t(114) = 2.27$, $p = 0.025$), which indicates significant dysfunction in Ca^{2+} handling in cardiac myocytes without Ng expression (Fig. 3D).

It is known that Ng expression in endothelial cells regulates Ca^{2+}-mediated endothelial nitric oxide synthase (eNOS) regulation (Cheriyan et al., 2020). To validate the possible role of Ng-mediated NO regulation in the heart, we assessed NO metabolites in the atrium and ventricle using a nitric oxide analyzer. We found that there are no significant changes in levels of NO metabolites in the heart of $Ng^{-/-}$ mice compared to those in wild type animals (Fig. 3E). In addition, since cardiac failure can be possibly induced by NO-mediated

hypertension, and Ng expression alters NO production, we examined blood pressure of 10-month-old $Ng^{+/+}$ and $Ng^{-/-}$ mice. Using tail-cuff blood pressure measurement, no significant changes in blood pulse (Fig. 3F) or blood pressure (Fig. 3G) were seen between the mouse groups. Based on these findings, we hypothesize that depletion of Ng expression in the heart induces dysregulation of Ca^{2+} dynamics in the cardiac myocyte leading to heart failure, while there is no NO-mediated regulation.

3.4. Lack of Ng increases CaMKII signaling and periostin expression in the heart

Since Ng is known to regulate the Ca^{2+}-CaM pathway, we examined Ca^{2+}-CaM-dependent pathway changes in the mouse heart. Hearts were isolated from both 5-month-old $Ng^{+/+}$ mice and $Ng^{-/-}$ mice and changes in protein expression were measured using western blotting (Fig. 4A). Cytosolic fractions were isolated to quantify CaM, Ca^{2+}/CaM-dependent protein kinase II (CaMKII), and Ca^{2+}/CaM-dependent phosphatase (calcineurin; CaN) expression change in $Ng^{-/-}$ mice. Overall, we observed the difference of Ca^{2+}-CaM signaling in the heart of $Ng^{-/-}$ mice. Although heart tissues showed increased trends of protein expression of CaM (Fig. 4B), CaMKIIδ (Fig. 4D), and CaN (Fig. 4E), only phospho-CaMKII expression (T286) was significantly increased in $Ng^{-/-}$ mice ($t(6) = 2.48$, $p < 0.05$) which indicates increased activation of CaMKII activity in the heart (Fig. 4C).

We then tested if Ng expression directly regulates ryanodine receptors (RyRs) expression, since RyRs in the sarcoplasmic reticulum regulates Ca^{2+} spark responses (Fig. 3A–D). However, no expression change in RyR between genotypes was observed (Fig. 4F). We also examined the expression change of periostin, which is considered a marker for fibrosis and possibly hypertrophy (Oka et al., 2007; Zhao et al., 2014). 5-month-old $Ng^{-/-}$ mice display significantly increased periostin expression in the heart ($t(6) = 3.02$, $p < 0.05$) compared to wild-type mice (Fig. 4G), consistent with increased cardiac fibrosis and hypertrophy in 10-month-old $Ng^{-/-}$ mice (Fig. 2).

To validate molecular findings from the heart lysate of $Ng^{-/-}$ mouse, we developed an *in vitro* Ng knockdown model using siRNA in the HL-1 mouse cardiac myocyte cell line (Fig. 4H). Ng siRNA knockdown in HL-1 showed significantly decreased protein expression of Ng and phospho-Ng. Consistently, phospho-CaMKII expression (T286) was significantly increased in Ng siRNA compared to that of lipofectamine control samples ($t(7) = 2.66$, $p < 0.05$) (Fig. 4I). Both *in vivo* and *in vitro* results indicate that Ng expression plays an important role in Ca^{2+}-CaM signaling in the cardiac myocyte and may play an essential role in the pathophysiology of cardiac hypertrophy and cardiac fibrosis.

4. Discussion

In humans, the Ng gene is associated with brain disorders, including Alzheimer's disease and schizophrenia. Ng expression and function are known to decrease during the aging process in Alzheimer's disease; and human genomics studies have reported that Ng genetic variants (*rs12807809*) or decreased Ng mRNA expression correlate with an increased risk of schizophrenia (Donohoe et al., 2011; Ohi et al., 2013). Furthermore, patients with schizophrenia display an increased risk of developing cardiovascular disease. This may suggest that Ng-induced cardiotoxicity, as a genetic risk factor, could possibly explain the

increased rate of cardiac failure observed in patients with neurological disease (Hennekens et al., 2005; Ifteni et al., 2014). Although there is no clinical evidence that supports Ng-mediated heart failure yet, our preclinical molecular and pathological findings focusing on Ng-mediated cardiac hypertrophy may provide a clue into the relationship between cardiovascular complications and neurological disease.

In this study, we first identified Ng expression as a new Ca^{2+}-CaM modulator in the mouse heart and showed how depletion of Ng promotes Ca^{2+}-dependent cardiac hypertrophy and fibrosis. Although it has been believed that Ng is a brain-specific protein, new results suggest that Ng can be produced outside of the brain (Fajardo et al., 2019; Nielsen et al., 2009). The most recent study has identified that Ng is expressed in both human and mouse endothelial cells, where it regulates nitric oxide homeostasis and endothelial activation (Cheriyan et al., 2020). This indicates that Ng-mediation of Ca^{2+} signaling in the vasculature may be related to the pathophysiology of cardiovascular disease. Furthermore, this previous study also revealed significant Ng mRNA expression using RT-PCR in wild-type mouse hearts. We have now been able to detect Ng protein expression in both the atria and ventricles of wild-type mouse hearts using western blot, whereas the preceding study could not. We have identified similar Ng expression profiles between atria and ventricles of wild-type mouse hearts. Therefore, we believe that the current western blots using whole heart lysates are sufficient, and signaling does not differ between cardiac regions. Moreover, we validated Ng protein expression in the cardiac myocyte using the HL-1 mouse cardiac myocyte cell line. Ng siRNA knockdown in HL-1 alters Ca^{2+}-mediated CaMKII signaling transduction, which is consistent with findings from $Ng^{-/-}$ mice.

However, the main limitation of our study is the use of conventional $Ng^{-/-}$ mice only. In this study, we report the possible role of Ng in cardiac myocyte and heart failure. Further research into the role of Ng in the cardiovascular system requires a cardiac myocyte-specific Ng knockout mouse model as well. Since recently generated endothelial-specific Ng knockout mice do not present cardiac failure, we believe that depletion of Ng in the cardiac myocyte may contribute to the hypertrophy and fibrosis seen in our knockout mice. $Ng^{-/-}$ mice were previously seen to display heart failure through significantly decreased ejection fraction, and increased systolic diameter and volume *via* electrocardiogram (EKG) measurement (Cheriyan et al., 2020). This technique was also used to measure left ventricular mass in the previous study. Our results of significantly increased heart weight (Fig. 1E) contradict this study's results. However, we believe this is due to our improved method of measuring heart weight directly, as opposed to using EKG. In addition, there was no change in blood pressure using the tail-cuff method between genotypes. Our method of blood pressure measurement has been limited to the use of tail-cuff, which has been found to induce stress in animals and affect readings (Wilde et al., 2017). Therefore, these results need to be validated by utilizing the *in vivo* telemetry technique in the future.

Lack of Ng was seen to significantly increase both fibrosis and collagen deposition in $Ng^{-/-}$ mouse hearts using Masson's Trichrome and Sirius Red staining, respectively. Sirius Red staining revealed increased collagen deposition not only in the perivascular areas, but also in between cardiac myocytes as well. Compared to mice with high levels of cardiac fibrosis, such as mice subjected to TAC (transverse aortic constriction) (Richards et al., 2019), our

$Ng^{-/-}$ mice display less cardiac myocytes fibrosis. However, our combined molecular and histological data does suggest strong pathological evidence for cardiac hypertrophy in $Ng^{-/-}$ mice. These mice display a significantly increased heart-to-body weight ratio compared to wild-type and their hearts are visibly larger. WGA staining of cell membranes enabled us to quantify cross-sectional area of cardiac myocytes from wild-type and $Ng^{-/-}$ mice. With this, we were able to observe significantly larger $Ng^{-/-}$ cardiac myocytes compared to control, indicating hypertrophy. In addition, western blotting for periostin, a fibrosis and hypertrophy marker, showed significantly increased expression in 5-month-old $Ng^{-/-}$ mice.

Having shown that lack of Ng in mouse hearts has a detrimental effect on cardiac function and hypertrophy, we then examined if Ng expression alters Ca^{2+} dynamics in the cardiac myocyte isolated from the atria of our $Ng^{+/+}$ and $Ng^{-/-}$ mice. Ca^{2+} spark imaging measures the cardiac Ca^{2+} dynamics that occur through RyRs in the SR membrane. Abnormal Ca^{2+} spark dynamics have been implicated in cardiac pathologies such as heart failure and cardiac arrhythmia (Hoang-Trong et al., 2015). Specifically, decreased Ca^{2+}-induced Ca^{2+} release (CICR) has been seen to be an important contributor to the impaired contractility of the failing heart through failure in maintaining Ca^{2+} homeostasis (Kubalova et al., 2005). Decreased Ca^{2+} spark frequency indicates decreased CICR functioning, which could lead to cardiac arrhythmias. In addition, decreased spark width paired with increased duration, implicates increased Ca^{2+} leak from RyRs that is common in heart failure (Bers et al., 2003). Therefore, future Ca^{2+}-spark results using cardiac myocytes specific Ng knockout mice will enable us to elucidate a physiological role for Ng expression in heart failure.

Although depletion of Ng in the heart decreases Ca^{2+} spark frequency, we observed that RyR expression did not change between mouse genotypes. These results suggest that Ng plays an important role in regulating Ca^{2+} handling cardiac myocytes, and dysfunctions in cardiac Ng may cause heart failure. Furthermore, these results support that Ng plays an important role in regulating Ca^{2+} dynamics and contributing intracellular Ca^{2+}-CaM pathway. It is possible that Ng is influencing CICR through CaMKII regulation in the heart, since Ng expression plays an essential role in regulating CaMKII activation in both the mouse brain (Wu et al., 2003) and aorta (Cheriyan et al., 2020). In this study, we observed that compared to $Ng^{+/+}$, $Ng^{-/-}$ mice displayed increased expression of free CaM as well as $CaMKII_{\delta}$ and p-CaMKII (T286) in their hearts. Since increased CaMKII activity in the heart has been known to increase Ca^{2+} leakage from RyRs (Dobrev and Wehrens, 2010), we expect that Ng expression is more critical for modulating CaMKII-mediated RyRs functioning.

CaMKII is a relatively new target in the study of cardiovascular disease. Its increased activity in the heart is associated with the development of pathological cardiac hypertrophy (Zhang et al., 2005). The delta (δ) isoform of CaMKII is of particular interest in this pathogenesis, as it is the major cardiac isoform (Zhang et al., 2003). In this study, we used a (pan) CaMKII antibody to quantify CaMKII expression in the heart. This antibody detects for all four isoforms of CaMKII. In the heart, the γ and δ isoforms are present, which explains the two bands seen in our western blots. $CaMKII_{\gamma}$ expression can be seen at around 60kD, whereas $CaMKII_{\delta}$ is expressed at around 50kD and is the predominant band. Increased expressions of $CaMKII_{\delta}$ and p-CaMKII (T286), the active form of CaMKII, were

seen in our $Ng^{-/-}$ mice, which is further evidence of pathological hypertrophy in these mice. These findings may provide a better understanding of cardiac CaMKII signaling.

We also checked CaN expression, because increases in this phosphatase are also implicated in cardiac hypertrophy (Molkentin et al., 1998) (Asakawa and Komuro, 2001) (Saito et al., 2003). Moreover, recent studies have demonstrated Ng's mechanical role in CaN signaling. For example, Ng expressed in mammalian skeletal muscle and the mouse aorta inhibits CaN by sequestering CaM (Fajardo et al., 2019). In this study, we observed an increasing trend of CaN expression by Ng knockout in the heart, which supports the aforementioned study (Cheriyan et al., 2020) (Fajardo et al., 2019). This finding further suggests that Ng may be a common regulator of CaN in skeletal and cardiac muscle tissue. Our data also indicates that Ng is a direct mediator of cardiac Ca^{2+} signaling. Lack of Ng in the heart, allows more Ca^{2+}-CaM formation, which then increases the activities of CaM-dependent CaN and CaMKII.

There is evidence suggesting that elevation of cardiomyocyte-produced nitric oxide increases Ca^{2+}-spark frequency and amplitude through the increased opening of RyR (Petroff et al., 2001). Yet, it is unclear if this is due solely to nitric oxide's effect on RyR or if other Ca^{2+}-handling molecules are involved. Our $Ng^{-/-}$ mice do not have altered cardiac nitric oxide levels compared to wildtype, but they do have decreased Ca^{2+}-spark responses with increased activity of Ca^{2+}-dependent signaling. This indicates that nitric oxide regulation may not be as important as overall Ca^{2+} homeostasis in the cardiac myocyte. Interestingly, Ng does not seem to regulate nitric oxide production in cardiac tissue, however it does in the brain and endothelium where nitric oxide activity is critical. Furthermore, we have seen that Ng directly mediates Ca^{2+}-dependent signaling in the heart, which is vital to RyR function during CICR.

In conclusion, $Ng^{-/-}$ mice display a decreased survival rate due to cardiac failure caused by hypertrophy. Depletion of Ng expression in the heart tissues increases CaMKII activity, as well as alters Ca^{2+} spark responses, indicating Ca^{2+} dysregulation. These findings strongly suggest that decreased Ng expression in cardiac tissue induces heart failure. Moreover, it is required to further study the mechanism of the Ng-mediated CaMKII pathway using the cardiac myocyte-specific Ng knockout mice and its clinical relevance in human cardiovascular disease.

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Abbreviations:

Ca^{2+}	calcium
Ng	neurogranin
CaM	calmodulin
RyR	ryanodine receptor
CaMKII	Ca^{2+}-CaM-dependent kinase II
CaN	Calcineurin
WGA	wheat germ agglutinin

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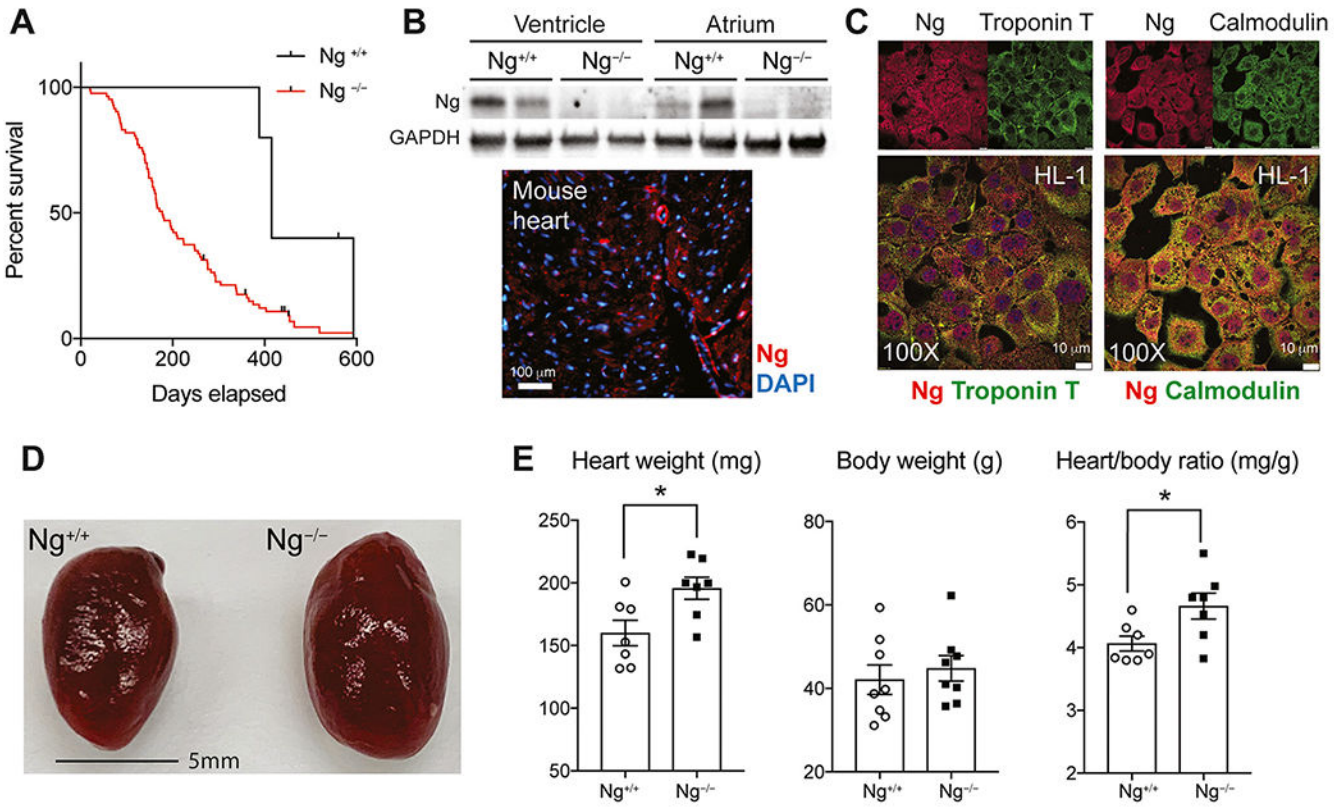

Fig. 1.

$Ng^{-/-}$ mice display reduced survival and enlarged hearts.

A. Kaplan-Meier curve showing 50% survival of $Ng^{-/-}$ mice (red line) at 177 days old ($Ng^{+/+}$ mice; $n = 144$ and $Ng^{-/-}$ mice; $n = 177$). B. Ng protein is expressed in both the atrium and ventricle of $Ng^{+/+}$ mice. Ng protein (red) is expressed in the heart tissue of $Ng^{+/+}$ mouse. Scale bar = 100 μm denoted by white line. C. Ng protein is expressed in the mouse cardiac myocyte cell line (HL–1). Immunofluorescence results indicate that Ng protein is co-localized with Troponin T and calmodulin. Scale bar = 10 μm denoted by white line. D. Hearts of 10-month-old $Ng^{-/-}$ mice are significantly larger than $Ng^{+/+}$ mice hearts E. $Ng^{-/-}$ mice exhibit a higher heart weight and heart/body weight ratio compared to wild-type mice. There are no changes in body weight between groups ($n = 7–8$). *$p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

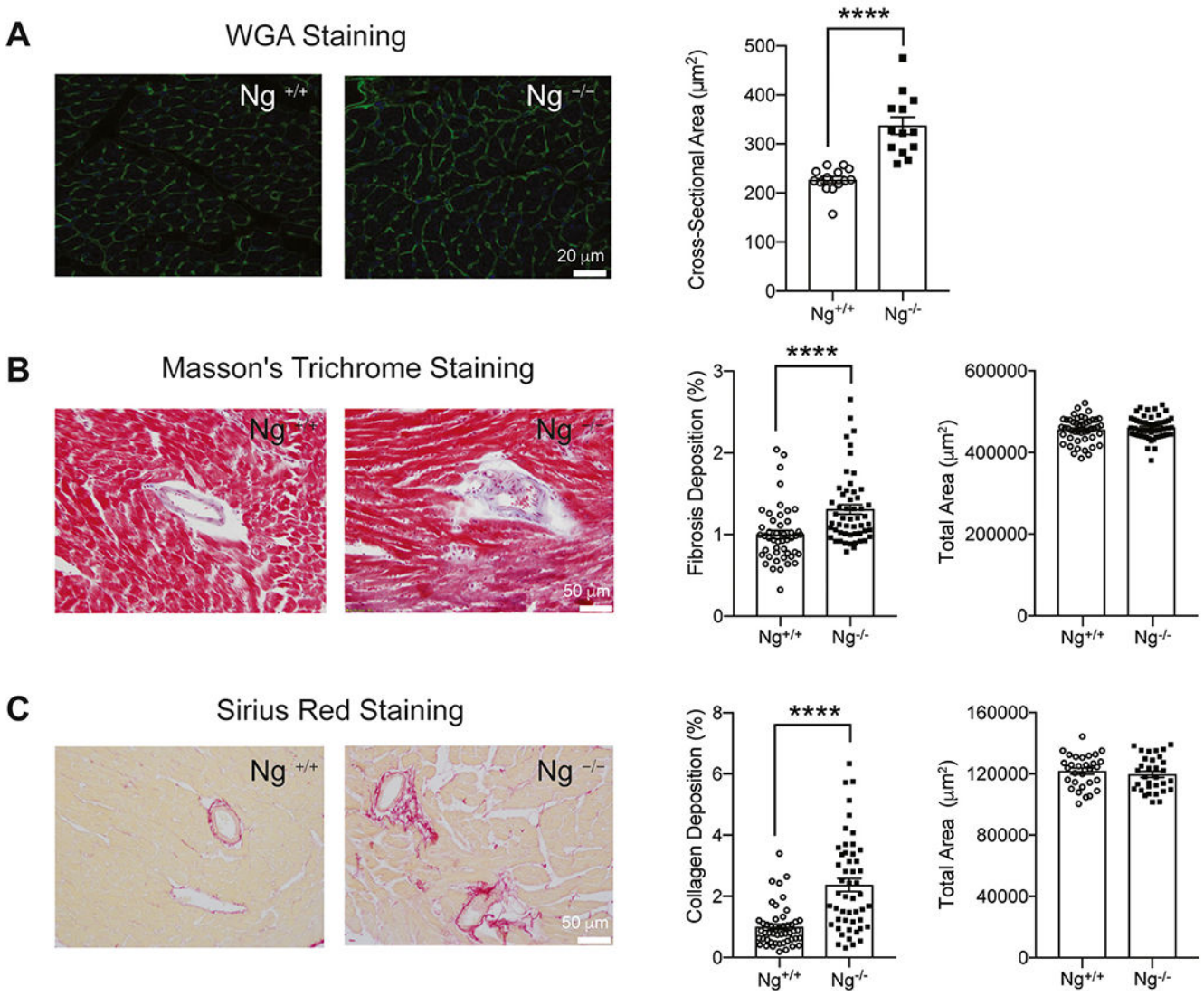

Fig. 2.

Significant hypertrophy, fibrosis, and collagen deposition is seen in the hearts of 10-month-old $Ng^{-/-}$ mice.

A. WGA staining shows that $Ng^{-/-}$ mice have significantly enlarged cardiac myocytes compared to wild-type (n = 5 mice per group, 3 sections analyzed per mouse). Scale bar = 20 μm. B. $Ng^{-/-}$ mice display significantly increased fibrosis deposition (purple) with Trichrome staining (n = 5 mice per group, 6–10 sections analyzed per mouse). Scale bar = 50 μm. *C. Sirius* Red staining in $Ng^{-/-}$ mice hearts show significantly increased collagen deposition (red) near the perivascular area compared to wild-type (n = 5 mice per group, 14–18 sections analyzed per mouse). Scale bar = 50 μm. ****$p < 0.0001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

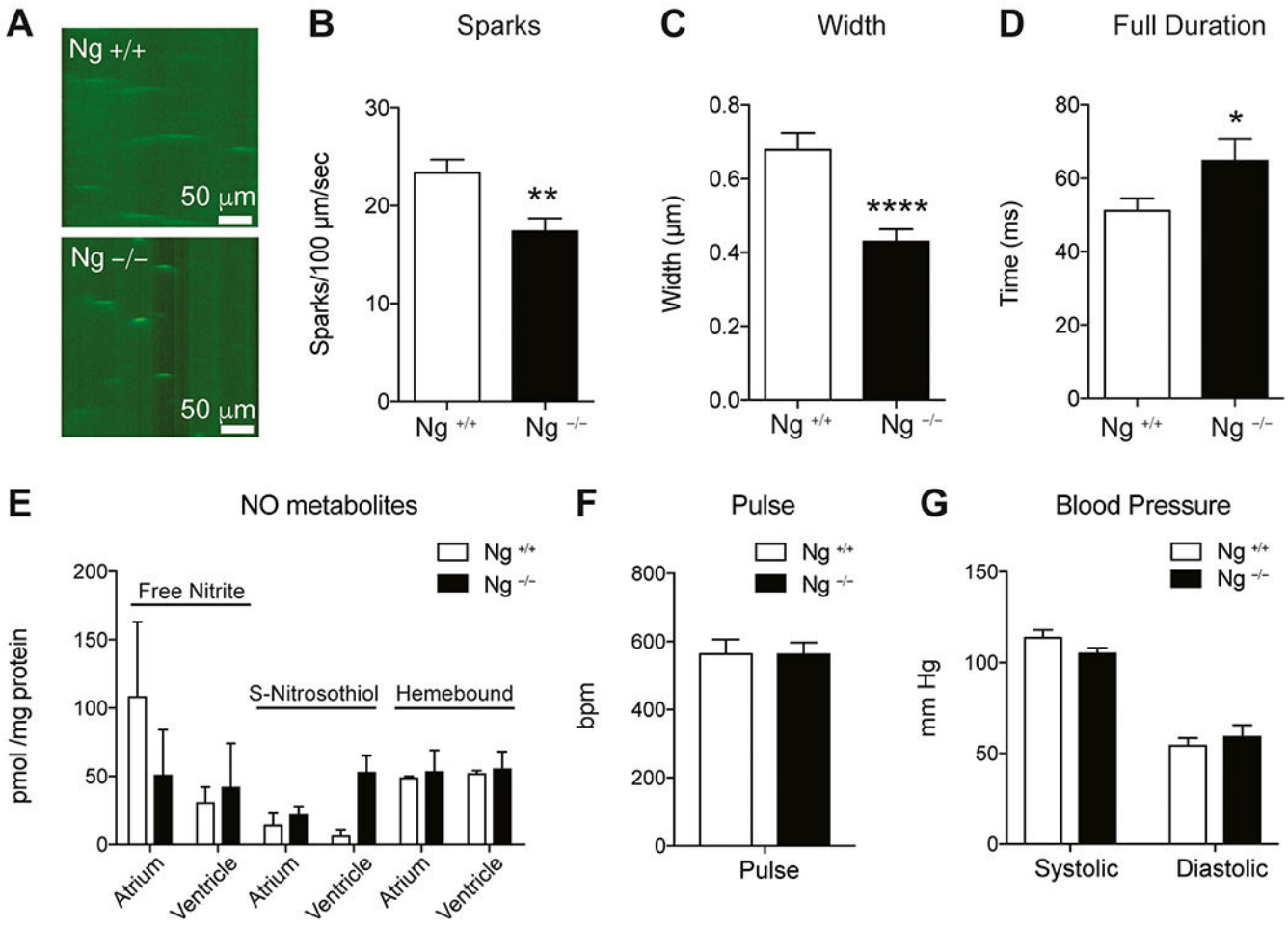

Fig. 3.

Lack of Ng disrupts calcium spark responses in cardiomyocytes but does not affect blood pressure.

A. Representative images of Ca^{2+} spark measurements. B and C. Cardiac myocytes from $Ng^{-/-}$ mice display significantly decreased Ca^{2+} spark frequency and width compared to those of wild-type mice. D. Full duration of spark is significantly increased in the cardiac myocytes from $Ng^{-/-}$ mice (72 data points for $Ng^{+/+}$ mice ($n = 4$) and 44 data points for $Ng^{-/-}$ mice ($n = 3$)). E. There is no change in cardiac nitric oxide levels between $Ng^{+/+}$ mice and $Ng^{-/-}$ mice. F and G. $Ng^{-/-}$ mice do not exhibit altered levels of blood pulse or blood pressure (n = 4 per genotype). *p < 0.05, **p < 0.01, ****p < 0.0001.

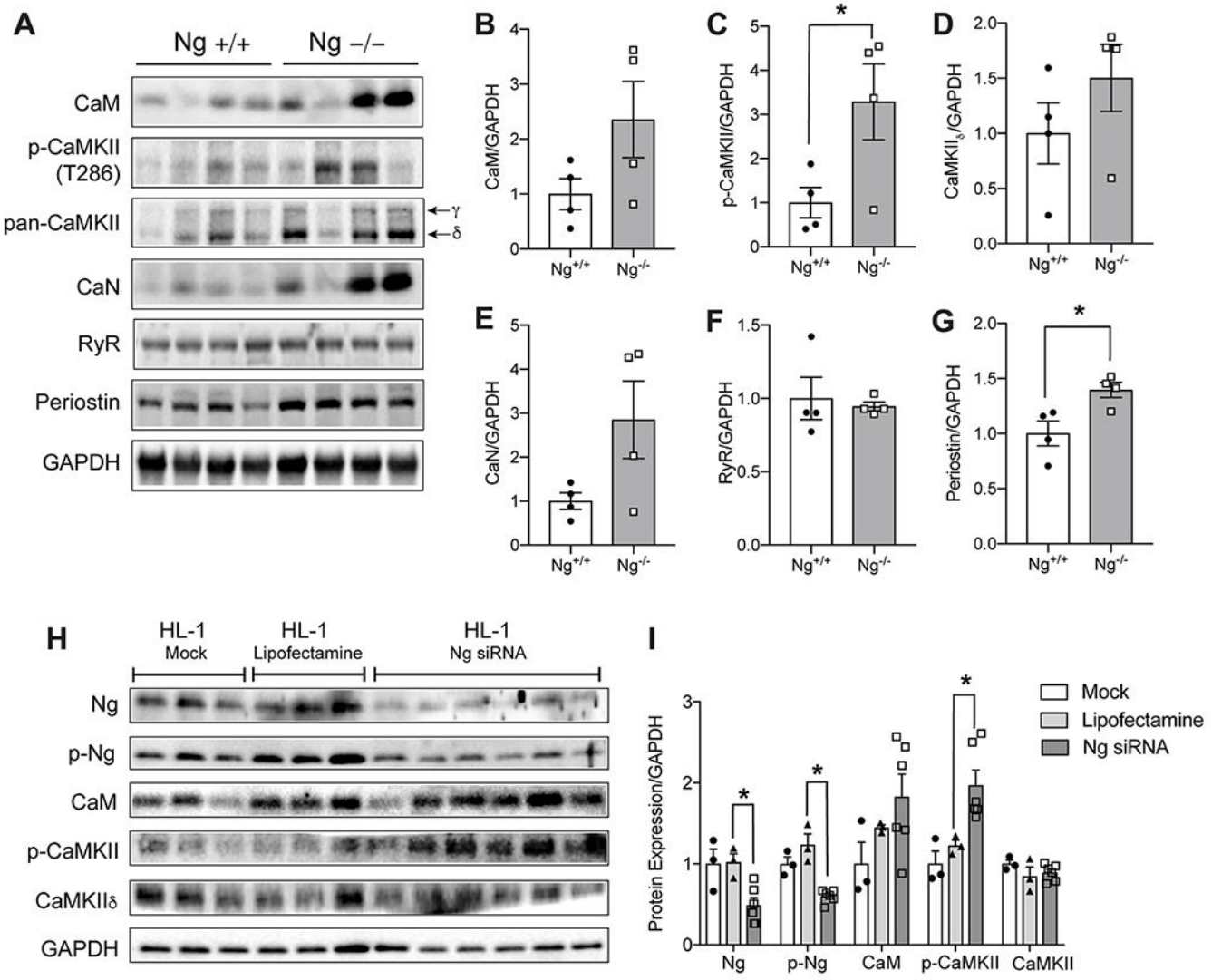

Fig. 4.

Lack of Ng increases CaMKII signaling and periostin expression in the heart.

A. Heart lysates from $Ng^{-/-}$ mice exhibit significant increases in Ca^{2+}-CaM signaling. B. $Ng^{-/-}$ mice exhibit increased trends for CaM expression in the heart lysate. C. Phospho-CaMKII expression (T286) is significantly increased in the heart of $Ng^{-/-}$ mice. D-E. There are increased trends of protein expression of pan-CaMKII and CaN. F. Lack of Ng does not affect RyR expression in the heart. G. Significantly increased periostin expression is observed in the heart of $Ng^{-/-}$ mice (n = 4 per genotype). H. Ng siRNA knockdown (n = 6) in HL-1 cell line exhibits altered CaMKII activity compared to those of mock (n = 3) and lipofectamine (n = 3) controls. I. Phospho-CaMKII expression (T286) was significantly increased by Ng siRNA in HL-1, while there was no change in CaMKIIδ expression. *$p < 0.05$.