Structural remodeling and collagen deposition in the heart of NPR-C knockout mice

by

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Abstract

Human natriuretic peptides (NPs), including ANP, BNP and CNP, regulate many important processes in the body including blood pressure control, bone homeostasis, and protection against cardiac hypertrophy and cardiac fibrosis. Natriuretic peptides mediate their effects through three receptors, NPR-A, NPR-B and NPR-C. While the natriuretic peptide-mediated effects of NPR-A and NPR-B have been well characterized, NPR-C is poorly understood. It was initially thought to function as a 'clearance receptor' with no signaling function; however, numerous studies have demonstrated NPR-C actually activates inhibitory G proteins (G_I) Data from the Rose laboratory demonstrates that mice lacking functional NPR-C receptors (NPR-$C^{-/-}$) exhibit SAN dysfunction, slowed atrial conduction, and increased susceptibility to atrial fibrillation. A possible mechanism for these conditions could be fibrotic remodeling in the heart of the NPR-$C^{-/-}$ mouse.

Accordingly, the purpose of this project was to assess the patterns of collagen deposition in hearts of NPR-$C^{-/-}$ mice using histological techniques. It was hypothesized that there would be an increase in interstitial collagen in the atria of NPR-$C^{-/-}$ mice when compared to wild type littermates. The atrial appendages of 5 NPR-$C^{-/-}$ and 5 wild type littermates, and the whole heart of 1 NPR-$C^{-/-}$ and 1 wild type littermate were surgically removed, sectioned and stained with picrosirus red and fast green solution. This solution stains collagen red and cell cytoplasm green. The percent area of collagen was calculated using digital images of the tissue and *ImageJ* software.

NPR-$C^{-/-}$ mice showed increased levels of interstitial collagen compared to wild type littermates in the right ($P< 0.001$) and left ($P<0.008$) atrial appendages of the heart. Patterns of collagen deposition were also measured in the left ventricles of wildtype and NPR-C-/- mice. This preliminary data demonstrate that there was no difference in the amount of interstitial collagen in the ventricles of the NPR-$C^{-/-}$ mouse. Increased interstitial collagen in the atrial appendages of the NPR-$C^{-/-}$ mice suggests that fibrotic remodeling is a likely mechanism for the SAN dysfunction, slowed atrial conduction and increased susceptibility to atrial fibrillation. Normal levels of interstitial collagen in the ventricles of the NPR-$C^{-/-}$ mouse suggest that fibrotic remodeling is limited to the atrial appendages of the heart in NPR-$C^{-/-}$ mice.

Abbreviations

AMDM: acromesomelic dysplasia, type maroteaux

AF: atrial fibrillation

ANP: atrial natriuretic peptide

BNP: b-type natriuretic peptide

cGMP: cyclic guanine monophosphate

CNP: c-type natriuretic peptide

CT-1: cardiotropin-1

DAG: diacylglycerol

ECM: extracellular matrix

ET-1: endothelin-1

FGF2: fibroblast growth factor 2

GC: guanylyl cyclase

Gi: inhibitory guanine protein

IP3: inositol triphosphate

MMP: matrix metalloprotease

NEP: neutral endopeptidase

NPR: natriuretic peptide receptor

NPR-A: natriuretic peptide receptor type-A

NPR-B: natriuretic peptide receptor type-B

NPR-C: natriuretic peptide receptor type-C

NPR-C WT: natriuretic peptide receptor type-c wild type

NPR-C$^{-/-}$ or NPR-C KO: natriuretic peptide receptor type-c knockout

PDGF: platelet-derived growth factor

PIP2: phosphoatidyl inositol biphosphate

PKC: phosphokinase C

PLC: phospholipase C

RAAS: renin-angiotensin-aldosterone system

SAN: sinoatrial node

TGF-β: transforming growth factor-Beta

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1. INTRODUCTION

1.1 Natriuretic peptides and their receptors

1.1.1 Natriuretic peptides

The natriuretic peptide family is a functionally, structurally and phylogenetically related group of peptides found in vertebrate animals (Kimmenade et Januzzi, 2009) that were first isolated by researchers in the 1980s (deBold *et al,* 1981 and Flynn *et al* 1983). Natriuretic peptides (NPs) have numerous effects including regulation of blood pressure and volume, cardiac electrophysiological effects, muscular contractions of the gastrointestinal tract, and maintenance and formation of bone (Rose and Giles, 2008).There are three main types of NPs found in humans including atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) (Kimmenade and Januzzi, 2009). BNP can also be referred to as Brain Natriuretic Peptide in the literature since it was first isolated from porcine brain tissue, however it is found in highest concentration in cardiac tissue (Potter et al, 2009).

All three NPs are made up of a 17 amino acid ring linked by a disulfilde bond connecting two cysteine residues (Fig. 1). Receptor recognition and biological function of NPs is dependent upon their ring structure (Silberbach and Roberts, 2001). All three NPs are first synthesized as preprohormones, which are cleaved to produce a prohormone and further processed to generate the biologically active NP (Potter et al, 2009).

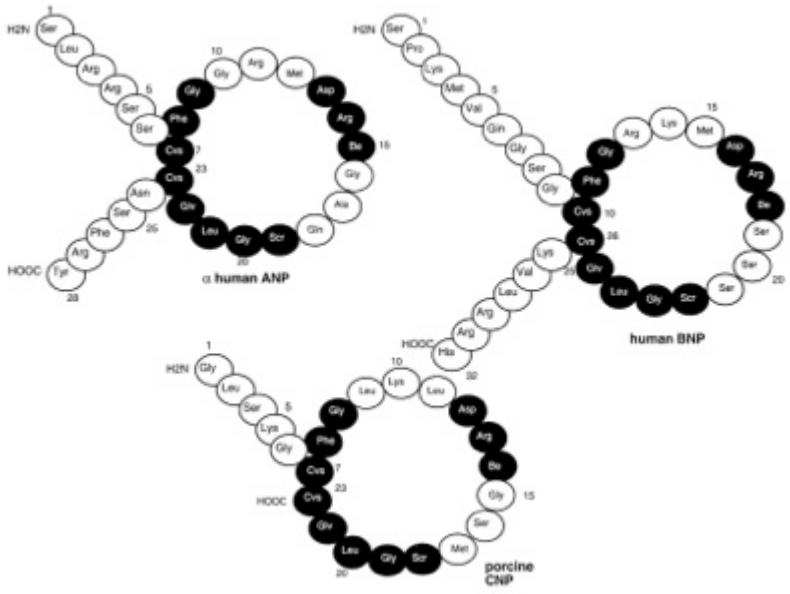

Figure 1*:* Schematic diagram of natriuretic peptides ring structure. Diagram taken from Silberbach and Roberts, 2000.

The genes coding for ANP and BNP, *NPPA* and *NPPB* respectively, are found on chromosome one in humans. In mice, *NPPB* is located on chromosome four. Both genes are about two Kb long with three exons and two introns (Potter et al, 2009). The mRNA transcript for ANP and BNP codes for a 151 and 134 amino acid preprohormone peptide respectively. PreproANP and preproBNP are cleaved into a 126 and 108 amino acid prohormone respectively (Potter et al, 2009). ProANP is stored in atrial granules in the atrial appendages of the heart. It is released from granules in response to atrial wall stretch and, once in the circulation, is cleaved into the 26-amino acid peptide ANP, Atrial wall stretch can be induced by an increase in intravascular volume or by pressor hormones (Potter et al, 2009). Some ProBNP is stored in atrial granules, but it is predominantly synthesized in the ventricles of the heart as needed, i.e. during cardiac stress, such as volume overload. In humans, proBNP is cleaved into 22-amino acid, BNP, but in mice and rats, BNP is composed of only 14 amino acids (Potter et al, 2009). In

healthy individuals ANP and BNP levels are normally low, but they both increase drastically during congestive heart failure (Potter et al, 2009).

CNP is the most highly conserved of the NPs amongst species, which may suggest that CNP was the ancestral prototype of the NP family (Silberbach and Roberts, 2001). CNP is found predominantly in the brain, but is also found in chondrocytes and endothelial cells. The gene coding for CNP, *NPPC*, is found on chromosome two instead of chromosome one and has two exons and one intron (Potter et al, 2009). PreproCNP is 126 amino acids long, and is cleaved into proCNP, which is 103 amino acids long. ProCNP is cleaved into a 53-amino acid or 22-amino acid CNP, 53-CNP or 22-CNP respectively. 53-CNP is found in highest concentration in the brain, and 22-CNP in highest concentration in circulation (Potter et al, 2009). CNP is not stored in atrial granules, but is synthesized as needed in vascular endothelial cells in due to shear stress such as injury in vascular tissue or in response to growth factors (Potter et al, 2009). In healthy individuals CNP levels are also low, but are elevated in congestive heart failure, however to a lesser degree than ANP and BNP. In humans, abnormally elevated levels of CNP are associated with Marfornoid-like skeletal overgrowths (Potter et al, 2009).

Circulating NP levels are thought to be regulated by two mechanisms, neutral endopeptidase (NEP) and Natriuretic Peptide Receptor type-C (NPR-C). NEP is a membrane-bound metalloprotease, which inactivates the NPs through hydrolysis (Kimmenade and Januzzi, 2009), breaking the ring structure (Daniels and Maisle, 2007). NEP has the highest specificity for CNP, followed by ANP, then BNP. NPR-C also binds to all three NPs, with highest specificity for ANP, followed by CNP, then BNP. As a

result of the specificity of NEP and NPR-C, ANP and CNP have short half-lives of about 2 to 3 minutes. BNP has a half-life of about 20 minutes (Potter et al, 2009).

NPR-C was originally described as a 'clearance receptor,' and its only function was thought to be reducing the circulating NP levels (i.e. it was thought that NPR-C did not activate signaling pathways) (Kimmenade and Januzzi, 2009). It is now known that NPR-C plays additional physiological roles including mediating anti-proliferative and anti-fibrotic effects of NPs in cardiac fibroblasts (Kimmenade and Januzzi, 2009). These additional roles of NPR-C will be discussed in more detail in the following section.

1.1.2 Natriuretic peptide receptors

Similarly to NPs there are three types of natriuretic peptide receptors (NPRs). They include: natriuretic peptide receptor type-A (NPR-A), Natriuretic Peptide Receptor type-B (NPR-B), and NPR-C (Fig. 2). All three receptors have a large extracellular ligand-binding domain and a single membrane-spanning domain in common; they differ in their intracellular domains. NPR-A and NPR-B have an equally large intracellular domain, while NPR-C has a much smaller intracellular domain (Kimmenade and Januzzi, 2009).

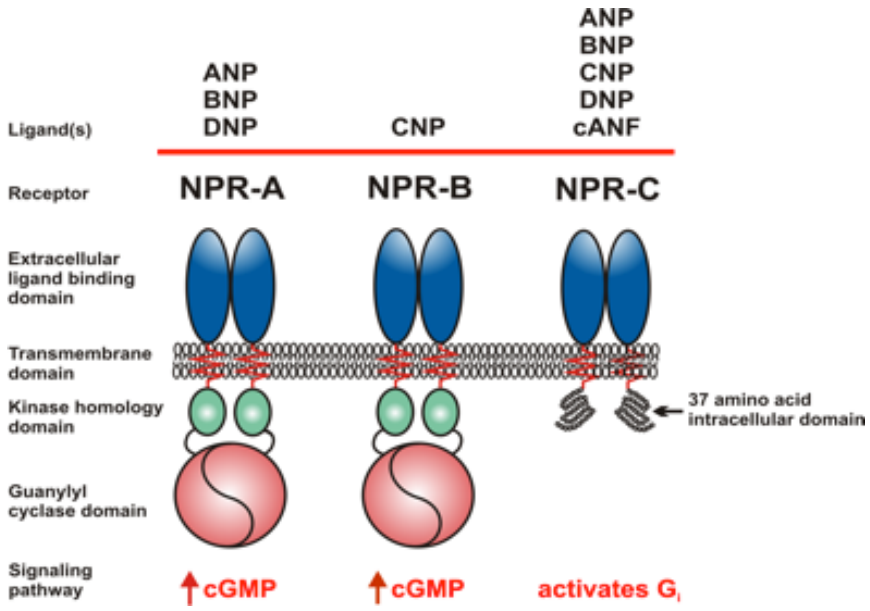

Figure 2*:* Schematic diagram of natriuretic peptide receptors, NPR-A, NPR-B and NPR-C.

NPR-A binds with equal affinity to ANP and BNP, and it is inactivated by dephosphorylation by protein kinase C. The kinase domain is present on the intracellular portion of NPR-A. Dephosphorylation inactivation is a mechanism of desensitization utilized during prolonged exposure to ANP. Mice that are lacking NPR-A exhibit chronic salt-resistant hypertension (Potter et al, 2009).

NPR-B is very similar to NPR-A, but binds with highest affinity to CNP. NPR-B is also inactivated by dephosphorylation by protein kinase C. Dephosphorylation occurs as a result of prolonged CNP exposure and elevated intracellular calcium levels (Potter et al, 2009). Mice that are lacking NPR-B exhibit dwarfism, female infertility, progressive blood-pressure-independent cardiac hypertrophy, and an elevated heart rate. In humans, NPR-B loss-of-function mutations cause a specific type of dwarfism called Acromesomelic Dysplasia, Type Maroteaux (AMDM dwarfism; Potter et al, 2009).

NPR-C is the most widely expressed NPR in the body (Potter et al, 2009). It is expressed in platelets, vascular smooth muscle, glomeruli, collecting ducts, pituitary ducts, adrenal glands, cerebral cortex, brain striatum, ciliary process of the eye, purkinje fibres, Leydig tumor cells, and the heart (Anand-Srivastava, 2005 and Springer et al, 2012). As previously mentioned NPR-C binds with highest affinity to ANP, followed by CNP, and then BNP. It was originally thought to be a clearance receptor, functioning through receptor-mediated internalization and then degradation (Potter et al, 2009). To date, studies have shown that mice lacking NPR-C (NPR-$C^{-/-}$) exhibit longer ANP half-lives, bone overgrowth, and hypotension. (Potter et al, 2009). In addition, unpublished data from the Rose laboratory has indicated that NPR-$C^{-/-}$ mice also exhibit sinoatrial node dysfunction, increased susceptibility to atrial fibrillation and slowed electrical conduction in the atria (Egom et al, 2014).

NPR-A and NPR-B mediated effects occur by stimulating an increase in intracellular levels of cGMP through guanylyl cylcase (GC) activation. NPR-C inhibits adenylyl cyclase (AC) via inhibitory G (G_i) proteins. The G_i protein has three subunits, α, β, and γ. The α subunit is involved with AC inhibition, while the β and γ subunits are involved with activating Phospholipase C (PLC; Rose and Giles, 2008). NPR-C is also involved in phosphitidyl inositol turnover, which is important in mobilizing intracellular calcium levels. Activation of PLC results in conversion of phosphatidyl inositol biphosphate (PIP2) to inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 is involved in calcium mobilization and DAG in PKC activation (Anand-Srivastava, 2005).

1.1.3 Biological roles of natriuretic peptides

Two main physiological roles of NPs in the cardiovascular system include blood pressure regulation, and protection from cardiac hypertrophy and fibrosis. The role of NPs in blood pressure regulation has been well studied; it occurs primarily through the ANP/NPR-A pathway and functions by altering the renin-angiotensin system (Potter et al, 2008). Studies have shown that mice lacking either ANP or NPR-A are hypertensive (John et al 1996, Lopez et al, 1995, and Oliver et al, 1997) and mice overexpressing ANP or BNP are hypotensive (Ogawa et al, 1994 and Steinhelper et al, 1990).

The function of NPs most significant to this project involves their protective role in cardiac hypertrophy and fibrosis. Studies have indicated that cardiac hypertrophy is regulated primarily via the ANP/NPR-A pathway, and that structural remodeling is regulated through the NPR-B and NPR-C pathways (Potter et al, 2009, Daniels and Maisel, 2007).

1.2 Cardioprotective role of natriuretic peptides

1.2.1 Cardiac remodeling and fibrosis

Generally speaking, cardiac remodeling involves an alteration to the myocardium. It is an essential adaptation process to altered mechanical, chemical or electrical signals, and is part of the normal process of cardiac growth. It is particularly essential during neonatal to adult development (Baudino et al, 2006). There are two types of cardiac remodeling, electrical remodeling and fibrotic remodeling. Electrical remodeling involves changes in the function and expression of ion channels, exchangers, calcium handling proteins and gap junctions. Fibrotic remodeling involves cardiomyocyte hypertrophy,

fibroblast proliferation and increased deposition of extracellular matrix components (Rohr, 2009 and Kapoun et al, 2004). These components include primarily fibronectin, collagen and matrix metalloproteinases (MMPs) (Souders et al, 2009 and Huntley et al, 2006). Fibrotic remodeling is a main component of the cardiac remodeling occurring during heart failure (Kapoun et al, 2004). It causes mechanical stiffness in the heart leading to abnormalities in diastolic function and disruption the electrical signaling between cardiomyocytes (Baudino et al, 2006). Interstitial cardiac fibroblast proliferation and increased deposition of components of the extracellular matrix are the relevant aspects of fibrotic remodeling in this project.

Fibrotic remodeling begins with increased secretion of cytokines and growth factors in heart. As a result, cardiac fibroblasts proliferate and migrate, leading to increased secretion of collagen and MMPs in the interstitium of the heart. This process is cyclical: cardiac fibroblasts then secrete more cytokines and growth factors leading to further proliferation and ECM component deposition. Initially, this process is essential to the adaptation process as a result of altered mechanical, chemical and electrical signals. However, over time these adaptations become detrimental, causing a decrease in cardiac function (Souders et al, 2009).

1.2.2 The Cardiac Fibroblast

Cardiac fibroblasts are the most abundant cell type in the myocardium, but cardiomyocytes occupy the most volume (Huntley et al, 2010). The primary role of cardiac fibroblasts is to regulate the ECM in order to maintain the three-dimensional structure of the myocardium. The ECM forms a scaffolding network that surrounds and

interconnects myocardial cells. Correct three-dimension structure allows proper cardiac form and function to be achieved (Souders et al, 2009).

Cardiac fibroblasts secrete three main components: several types of collagen, fibronectins and MMPs. MMPs function to degrade interstitial collagen (Souders et al, 2009). There are 20 types of MMP; they contain zinc and are functionally dependent on calcium. MMP-1 and MMP-13 have both been shown to be responsible for collagen degradation. MMP-2 is responsible for degrading gelatins, which is denatured collagen (Tsuruda et al, 2002). Cardiac fibroblasts also secrete all three NPs and express all three NPRs (Nishikimi et al, 2006),

1.2.3 The role of natriuretic peptides

The literature on the antifibrotic and antiproliferative role of NPs in the heart has mainly focused on NPR-A and NPR-B. The role of NPR-C in regulating fibroblast biology and fibrosis is not well understood although there is some data implicating this receptor in these processes (Huntley et al, 2006).

There are multiple growth factors that are involved in cardiac fibrosis development; some of these factors include: angiotensin II, endothelin-1 (ET-1), cardiotropin-1 (CT-1), norepinephrine, aldosterone, fibroblast growth factor 2 (FGF2), platelet-derived growth factor (PDGF), transforming growth factor- Beta (TGF-β), acidic fibroblast growth factor, and insulin-like growth factor I (Kapoun et al, 2004 and Nishikimi et al, 2006). However, the focus will be limited to angiotensin II, ET-1 and TGF-β for this thesis. TGF-β modulates fibroblast proliferation and secretion of collagen and fibronectin; it is typically upregulated during heart failure. TGF-β functions by

increasing expression of ECM genes, such as those coding for fibronectin synthesis (*fibronectin*) and collagen synthesis (*COL1A2, COL15A,* and *COL7A1*). Cardiac fibroblast secretion of TGF-β is further stimulated by Angiotensin II and ET-1 (Kapoun et al, 2004).

One of the mechanisms used by NPs in antiproliferation and antifibrosis of cardiac fibroblasts is through increasing the intracellular levels of cGMP, which is mediated by NPR-A and NPR-B. All three NPs are present in the heart and contribute to the increase in intracellular cGMP; CNP is the most potent, and causes the greatest increase in cGMP. cGMP inhibits DNA synthesis induced by growth factors, angiotensin II, ET-1, and TGF-β (Nishikimi et al, 2006, Tamura et al, 2000, and Kapoun et al, 2004). In addition to inhibiting the effects of angiotensin II, ET-1 and TGF-β, NPs also induce the expression of MMP-1, MMP-2 and MMP-3, and induce the secretion of MMP-2 by cardiac fibroblasts (Tsuruda et al, 2002).

The ECM may also play a role in effects of NPs on cardiac fibroblasts. Huntley et al (2006) suggested that in the presence of fibronectin, BNP produced a higher level of intracellular cGMP. Thus, fibronectin may play a modulatory role in the BNP/ cGMP, pathway.

There is some knowledge of the meditative role of NPR-C in the cardioprotective role of NPs. Huntley et al (2006) established all three NPRs are present in cultured human fibroblasts and compared the antifibrotic and antiproliferative effects of BNP on cultured human fibroblasts. When cANF, a NPR-C antagonist, was added with BNP to cultured human fibroblasts, the antiproliferative effects of BNP was significantly

reduced. They concluded that NPR-A and NPR-B mediated the antifibrotic effects, and NPR-C mediated the anti-proliferative effects of BNP (Huntley et al, 2006).

It is unclear whether NPs are acting as paracrine or autocrine factors. NPs may be secreted by cardiomyocytes and act on cardiac fibroblasts, reducing proliferation and secretion of ECM components. NPs may also act as autocrine factors and be secreted by cardiac fibroblasts and then act upon themselves to reduce proliferation and secretion of ECM components.

1.3 The NPR-C$^{-/-}$ mouse

The mutations causing a lack in functional NPR-C in mice is naturally occurring and involves an in-frame deletion of 36 base pairs, resulting in a protein short of 12 amino acids, on the extracellular domain of the NPR-C protein. (Jaubert et al, 1999). Increased body length, long digits, thinness, arachnodactyly and frequent tail and sacral kinks characterize mice lacking functional NPR-C (NPR-C$^{-/-}$), as seen in Fig. 3 (Jaubert et al, 1999). Heterozygotes of the condition do not show any of the phenotypic abnormalities, suggesting that the condition is completely recessive (Jaubert et al, 1999).

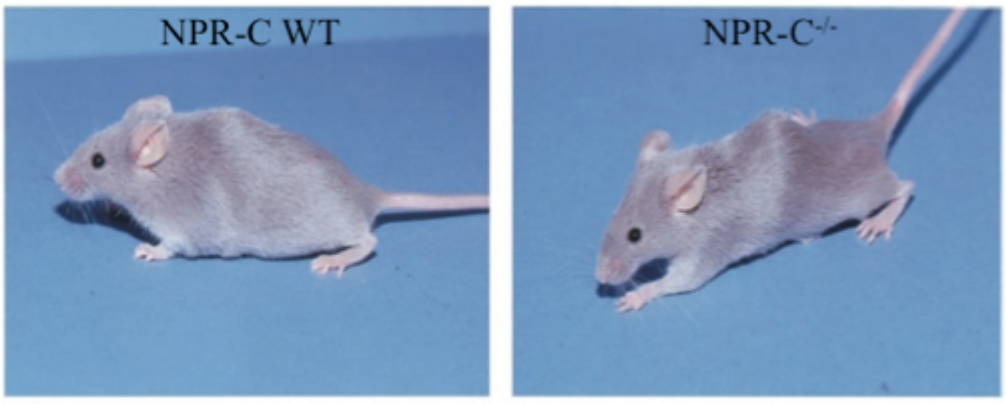

Figure 3. Phenotypic comparison of wildtype mouse (NPR-C WT) and NPR-C knockout mouse (NPR-C$^{-/-}$). Photo taken from Jaubert et al, 1999.

1.4 Purpose and Hypothesis

1.4.1 Purpose

Results from an unpublished study by the Rose Laboratory on the chronic effects of lacking NPR-C, have indicated that NPR-C may play a much larger physiological role than simply a 'clearance' receptor Their results have suggested that NPR-$C^{-/-}$ mice exhibit SAN dysfunction and increased susceptibility to atrial fibrillation (AF). In addition, high-resolution optical mapping data indicates that NPR-$C^{-/-}$ mice have slowed atrial conduction (Egom et al, 2014).. One possible mechanism for increased susceptibility to AF and slowed atrial conduction is fibrotic remodeling (Baudino et al, 2006 and Kapoun et al, 2004). The Rose laboratory has also confirmed that mRNA expression of collagen is increased in the atria of NPR-$C^{-/-}$ mice in comparison to wild type littermates (Egom et al, 2014). Interestingly, there was not an increased susceptibility to ventricular fibrillation in NPR-$C^{-/-}$ mice, suggesting that structural remodeling is only occurring in the atria of NPR-$C^{-/-}$ mice (Egom et al, 2014). The purpose of this project was to determine, using histological techniques, if there was an increase in intestinal collagen deposition, indicative of structural remodeling, in the hearts of NPR-$C^{-/-}$ mice.

This project will contribute to the Rose laboratory's study on the chronic effects of lacking NPR-C. It will also contribute to the growing bulk of literature outlining the physiological roles of NPR-C beyond that of a 'clearance receptor'.

1.4.2 Hypothesis

Based on the unpublished results of the Rose laboratory outlined above, it was hypothesized that the NPR-C$^{-/-}$ mice would show an increased deposition of collagen in the atrial appendages as compared to wild type littermates. Preliminary studies were also conducted in the ventricles of the NPR-C$^{-/-}$ mice; it was hypothesized that there would be no difference in collagen deposition in the ventricle of NPR-C$^{-/-}$mice in comparison to wild type littermates.

2. METHODS

2.1 Atrial appendages

Atrial preparations (Fig. 4) including both atrial appendages of 5 NPR-C$^{-/-}$ and 5 wild type, 10-14 week old, male littermates were surgically removed and fixed in formalin for a minimum of 48 hours. The tissues were then put through an automated tissue processor, where they were dehydrated, cleared and infiltrated with embedding media. The dehydration process involved 6 washes (1.5 hours each) in increasing concentrations of ethanol, as follows: 70%, 95%, 95%, 100%, 100%, 100%. The clearing process involved a 50:50 100% ethanol:xylene wash (1 hour) and two xylene washes (1 hour each). The infiltration process involved 2 washes (1 hour each) in paraffin wax (obtained from Fisher Scientific)

Following tissue processing, the atrial appendages were embedded in paraffin and allowed to solidify at 4°C (15 minutes). Using a Reichert-Jung rotary microtome the embedded atrial appendages were sectioned from bottom to top at 5 μm intervals. The

5µm sections were placed in a 45°C water bath, and set on silinated slides. Atrial appendage slides were dried for a minimum of 12 hours in a 37°C oven prior to staining.

The slides were stained in Fast-Green solution, which was prepared with Picric acid, Fast Green FCF, and Direct Red 80 (obtained from Sigma Aldrich, Lot#MKBH2276V, Lot#MKBH7165V, Lot#120M1516V respectively). Picric acid solid (2.4g) was added to water (200 mL) and shaken for 5-10 minutes. Direct Red 80 (0.2 g) and Fast Green FCF (0.2 g) powders were added to the picric acid solution and shaken for another 5-10 minutes.

Prior to staining, the slides were deparaffinized and rehydrated by washing twice in xylene (5 minutes each), twice in 100 % ethanol (2 minutes each), twice in 95% ethanol (2 minutes each), once in 70% (2 minutes), once in 50% ethanol (2 minutes), and twice in water (2 minutes each). The slides were then coated with the Fast-Green solution and incubated (1 hour). Fast-Green solution stains the cell cytoplast green and collagen red.

Following staining, the slides were resined once in water (10 dips), once in 70% ethanol (20 seconds), twice in 100% ethoanol (10 dips each) and twice in xylene (10 dips each). Coverslips were mounted in clean mounting medium, and slides were allowed to dry for a minimum of 12 hours.

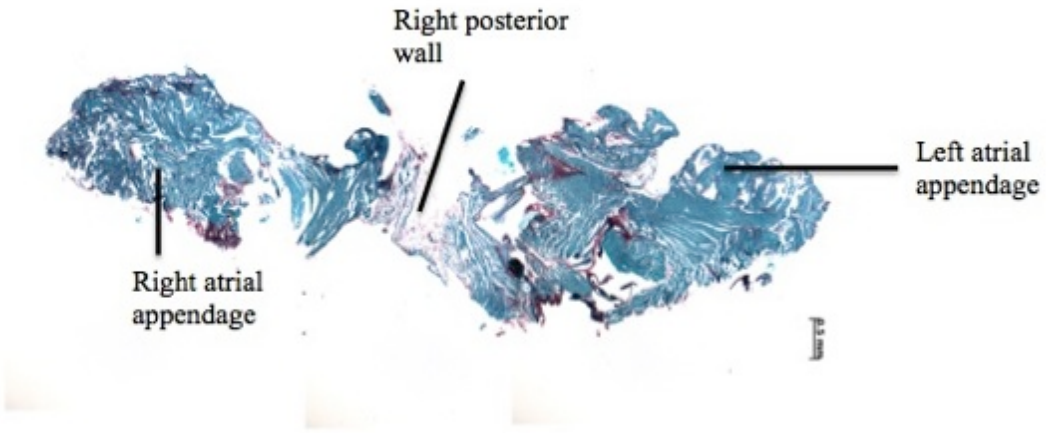

Figure 4. Digital image of atrial preparation at 5 times magnification stained with picrosirius red and fast green solution. Collagen was stained red and cell cytoplasm was stained green.

Digital images of the right and left atrial appendages were captured at 40x magnification using a Zeiss Axioplan 2 microscope. The digital images were analyzed semi-quantitatively using ImageJ software to capture the percent area of interstitial collagen. A T-test was used to statistically compare the difference in percent area of interstitial collagen between the wild type and NPR-$C^{-/-}$ right atrial appendages and again between the left atrial appendages.

2.2 Ventricles

The whole hearts of one male wild type and one male NPR-$C^{-/-}$ mouse were surgically remove and fixed in formalin. Both mice were between 10 to 12 weeks old. The same tissue processing and embedded procedures were performed for the whole heart tissues as for the atrial appendages. The tissue was sectioned from the front to back of the heart using the automated rotary microtome. The same histological procedure was

followed for the whole hearts as for the atrial appendages. Similarly, digital images were captured at 40x magnification of the left and right ventricles using a Zeiss Axioplan 2 microscope. The digital images were analyzed semi-quantitatively using ImageJ Software to capture the percent area of collagen.

Ventricular studies were preliminary involving only 1 wild type and 1 NPR-$C^{-/-}$ mouse. Thus, statistical analysis was not conducted on preliminary results of percent area coverage of collagen.

3. RESULTS

3.1 Right Atrial Appendage

Representative images of interstitial collagen for the NPR-$C^{-/-}$ and wild type right atrial appendage are shown in Fig. 5 (A). The average percent area of interstitial collagen in right atrial appendages of the NPR-$C^{-/-}$ mice was 5.54 ± 0.22. This is significantly higher than the average percent area of interstitial collagen of the wild type littermates, which was 1.14 ± 0.05 (p<0.001) (Fig. 5 B).

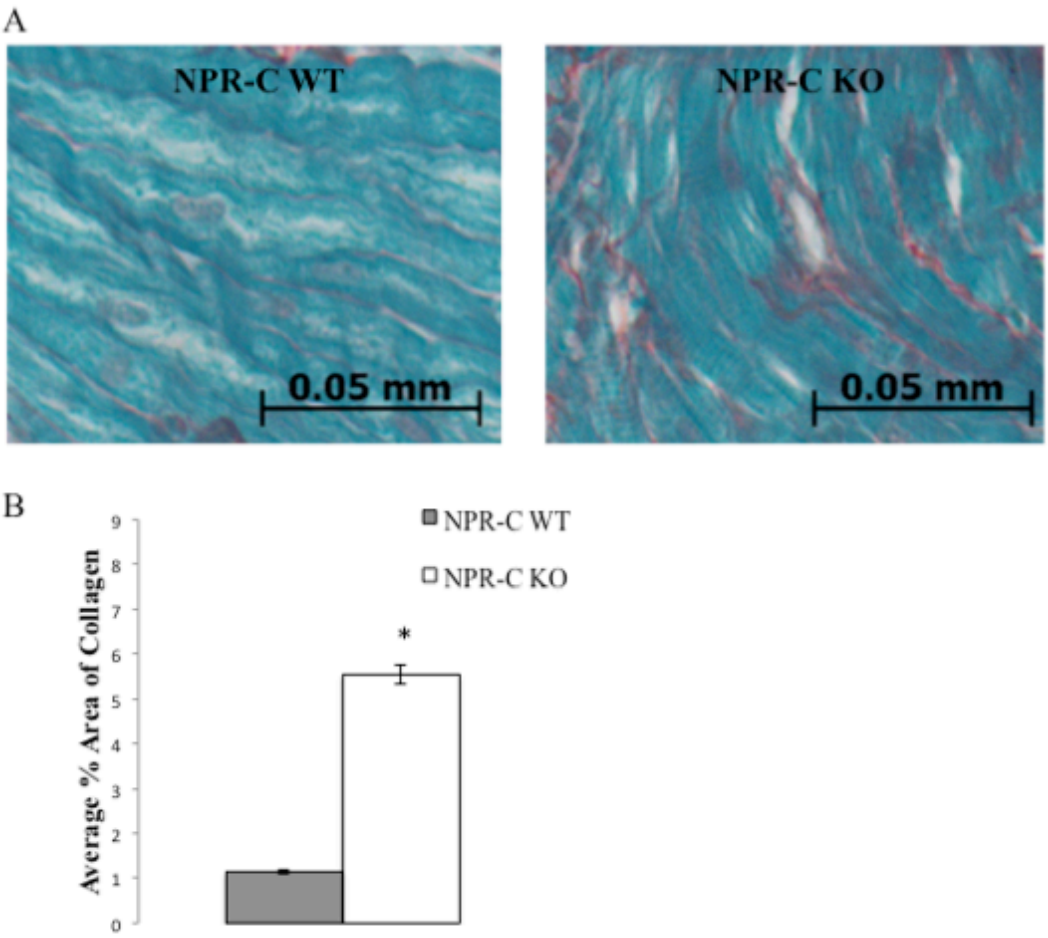

Figure 5 Collagen deposition in the right atrial appendage of wildtype and NPR-C$^{-/-}$ heart. A. Representative images of interstitial collagen in right atrial appendage of wild type and NPR-C$^{-/-}$ littermates. B..Average percent area of collagen deposition in right atrial appendages of wild type and NPR-C$^{-/-}$ littermates, * indicates significant difference ($p<0.001$).

3.2 Left Atrial Appendage

Representative images of interstitial collagen for the NPR-C$^{-/-}$ and wild type left atrial appendage are compared in Fig. 6. The average percent area of interstitial collagen in left atrial appendages of the NPR-C$^{-/-}$ mice was 6.39 ± 0.53. This is significantly higher than the average percent area of interstitial collagen of the wild type littermates, which was 0.95 ± 0.04 ($p<0.008$) (Fig. 6 B).

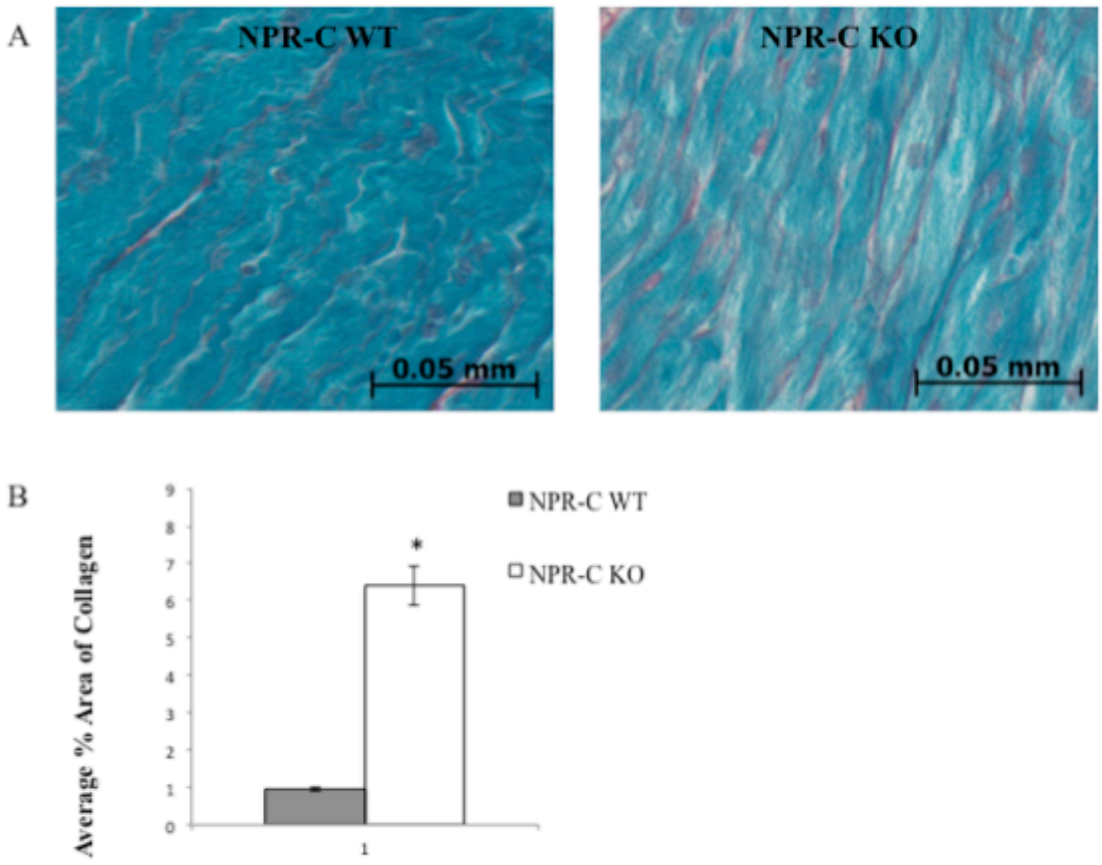

Figure 6. **Collagen deposition in the left atrial appendage of wildtype and NPR-C$^{-/-}$ heart.** A. Representative images of interstitial collagen in left atrial appendage of wild type and NPR-C$^{-/-}$ littermates. B. Average percent area of collagen deposition in left atrial appendages of wild type and NPR-C$^{-/-}$ littermates, * indicates significant difference ($p<0.008$).

3.3 Ventricles

Representative images of the right and left ventricles of the wild type and NPR-C$^{-/-}$ mouse are shown respectively in Fig. 7 (A and B respectively). The percent area of interstitial collagen in the right ventricle was 1.20 ± 0.23 for the wild type mouse and 1.33 ± 0.23 for the NPR-C$^{-/-}$ mouse. The percent area of interstitial collagen in the right ventricle was 1.49 ± 0.21 for the wild type mouse and 1.26 ± 0.17 for the NPR-C$^{-/-}$ mouse (Fig. 7 C).

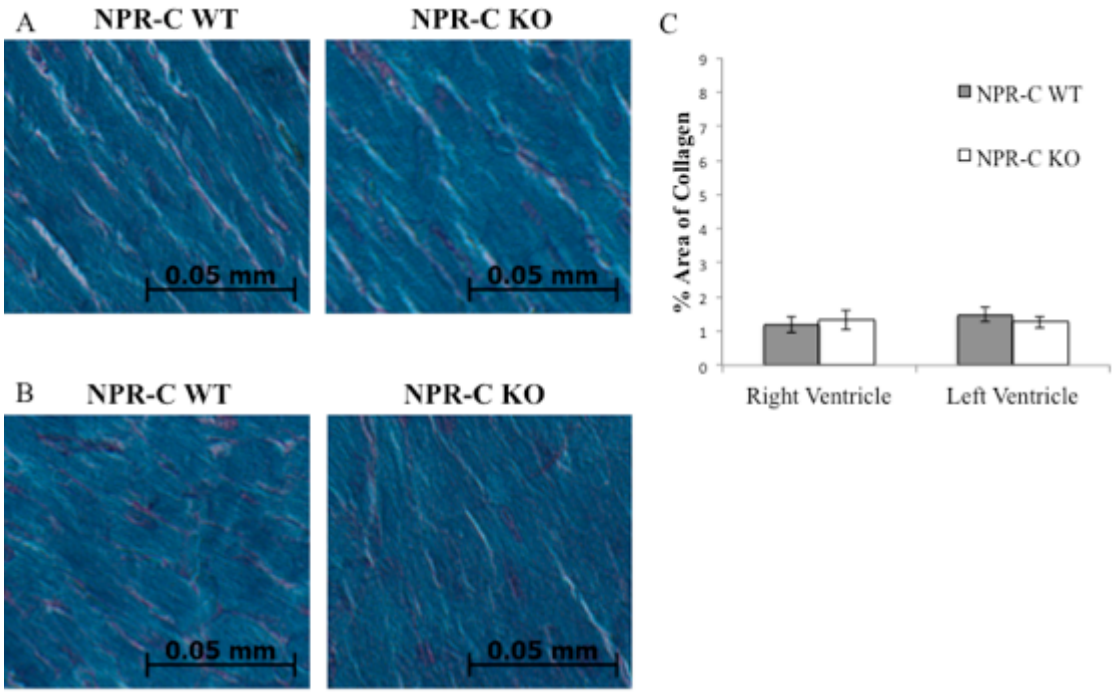

Figure 7. Collagen deposition in the ventricles of wildtype and NPR-C$^{-/-}$ heart. A. Representative images of interstitial collagen in right ventricle of wild type and NPR-C$^{-/-}$ littermates. B. Representative images of the interstitial collagen in the left ventricle of the wild type and NPR-C$^{-/-}$ littermates. C Percent area of interstitial collagen in left and right ventricles of 1 individual wild type (NPR-C WT) and NPR-C$^{-/-}$ (NPR-C KO) littermates.

4. DISCUSSION

It was hypothesized that the NPR-C$^{-/-}$ mice would have an increased amount of interstitial collagen in the atrial appendages, but not in the ventricles of the heart as compared to wild type littermates. This was based on the observations that NPR-C-/- mice are characterized by increased susceptibility to atrial, but not ventricular arrhythmias (unpublished data from our laboratory; Egom et al, 2014). NPR-C$^{-/-}$ mice showed a significantly higher amount of interstitial collagen in the atrial appendages as compared to wild type littermates. Preliminary studies in the ventricles indicated that

there is no difference in the amount of interstitial collagen in the ventricles of NPR-$C^{-/-}$ mice versus wild type littermates.

These results suggest that the chronic lack of NPR-C can lead to increased deposition of interstitial collagen indicative of structural remodeling that is characterized by cardiac fibrosis. The Rose laboratory is working on a larger study, to determine the overall chronic effects of missing NPR-C. They have established that NPR-$C^{-/-}$mice exhibit SAN node dysfunction, increased susceptibility to AF, slowed electrical conduction in the atrial, and increased mRNA expression of collagen in the atrial appendages (Egom et al, 2014). The increased atrial mRNA expression, in conjunction with the increased deposition of collagen shown in this project, suggest that fibrotic remodeling is a possible mechanisms causing the SAN dysfunction, increased susceptibility to AF and slowed atrial conduction.

Similar results were found by Wolf et al (2012) in a study on the human ankyrin-B syndrome in mice. Human ankyrin-B syndrome involves SAN dysfunction and an increased susceptibility to both inducible AF and spontaneous AF. Histological studies of the level of interstitial collagen in human ankyrin-B syndrome mice indicated they exhibited an increase in atrial fibrosis by three-fold (Wolf et al, 2012). Fibrotic remodeling can slow electrical conduction by altering the continuity between cardiomyocytes disrupting lateral gap junctions (Rohr, 2009 and Yue et al, 2010). In addition, activated fibroblasts are capable of coupling to cardiomyocytes via gap junctions, resulting in arrhythmogenic consequences. Activated fibroblasts can cause partial depolarization or repolarization of cardiomyocytes, which could alter proper

action potential induction and propagation (Rohr, 2009, Yue et al, 2010, and Zlochiver et al, 2008).

The results of this project have implications in the clinical setting of heart failure. Heart failure is defined as being a complex syndrome that results from a dysfunction in ventricular filling or ejection of blood (Soussie et al, 2014). Heart failure is highly prevalent in today's society and has poor outcomes, making it a major public health concern (Krum and Abraham, 2009). The leading precursor to heart failure is hypertension, however other precursors include myocardiac infarction, diabetes, valvural heart disease, left ventricular heart disease, and cardio myopathies (Levy et al, 1996). Cardiac fibrosis occurs in the final stages of heart failure just prior to cardiac arrhythmias and pump failure (Krum and Abraham, 2009). NPs are currently being used four ways in the clinical setting of heart failure– as a diagnostic tool, as a prognostic tool, as a heart failure management tool, and as a treatment option (Volpe et al, 2014). Concentration of BNP in the blood is being used to diagnose heart failure and to predict the clinical outcome for a patient. BNP levels are also used to personalize treatment for heart failure treatments (Volpe et al, 2014).

NPs are also being used as a treatment option. *Nesiritide* is a synthetic BNP, which can be administered orally or via intravenous infusion. *Nesiritide* was approved in North America in 2001, and has shown to have beneficial actions on myocardial structure and function. These benefits are thought to function through NPR-A and NPR-B and cause alterations in intracellular levels of cGMP. However, Nesiritide has not been shown to have a significant impact on heart failure and has also been linked to decreased renal function and increased short-term mortality (Volpe et al, 2014). NPs also bind to NPR-C

(Anand-Srivastava, 2005), and as the results of this project suggest, the NPR-C pathway plays a very important in role in protecting the heart from cardiac fibrosis and proliferation. Thus, during the design of synthetic NPs as a treatment option, the NPR-C pathway should also be taken into account.

The lack of fibrosis in the ventricles of NPR-C$^{-/-}$ mice suggests that perhaps the chronic effects of missing NPR-C are limited to the atrial appendages of the heart. The atrial appendages are more susceptible to fibrosis than the ventricles (Yue et al, 2010). A study in mice lacking functional TGF-β described similar results to this project, where fibrosis was limited only to atrial appendages and not present in the ventricles (Nikajima et al, 2000). One possible mechanism for the difference between atrial and ventricular susceptibility to fibrosis include a comparatively higher content of fibroblasts in the atria (Burstein et al, 2008). In addition, atrial fibroblasts exhibit greater proliferation than ventricular fibroblasts (Yue et al, 2010). Increased proliferation could be caused by increased expression of some growth factors in the atria as compared to the ventricles (Burstein et al, 2008).

Future directions of study could include using histological techniques to determine the amount of interstitial collagen deposition in the right atrial posterior wall. The SAN is located in the right atrial posterior wall, and since NPR-C$^{-/-}$ mice are exhibiting SAN dysfunction it is possible that they might show fibrosis in the right atrial posterior wall (Rose and Giles, 2008). In addition, a more extensive study of the collagen deposition in the ventricles of NPR-C$^{-/-}$ mice might be worthwhile. Preliminary results suggest that there is no difference in collagen deposition in the ventricles of the NPR-C$^{-/-}$ mouse when compared to a wild type littermate; however, these measurements were only

performed on 1 wildtype and 1 NPR-C-/- mouse. A more complete study of the NPR-$C^{-/-}$ ventricles using additional animals is needed to confirm this finding. It would also be interesting to determine how collagen deposition changes with age in the hearts of NPR-$C^{-/-}$ mice. Similarly, it would also be interesting to determine how collagen deposition changes during induced heart failure in NPR-$C^{-/-}$ mice. Finally, more research is needed to determine the mechanisms by which NPs, via NPR-C, regulate fibroblast proliferation and collagen deposition.

5. CONCLUSIONS

NPR-$C^{-/-}$ mice exhibit increased collagen deposition in the atrial appendages of the heart. These findings agree with unpublished data from the Rose laboratory indicating the NPR-$C^{-/-}$ mice have SAN dysfunction, increased susceptibility to AF and slowed atrial conduction. Increased collagen deposition, indicative of fibrosis, suggests that NPR-$C^{-/-}$ mice have undergone structural remodeling in the atria appendages.

There were no differences in the preliminary studies of collagen deposition in the ventricles of an NPR-$C^{-/-}$ mouse compared to a wild type littermate. Similarly this finding matched the unpublished results from the Rose laboratory that indicated that the NPR-$C^{-/-}$ mice do not show increased susceptibility to ventricular fibrillations. Taken together, the atrial and ventricular findings of this project suggest that the chronic effects of lacking NPR-C seem to be isolated to the atrial appendages of the heart.

These findings indicate that the role of NPR-C involves more than simply a ‘clearance receptor’. The anti-fibrotic effects and anti-proliferative effects of natriuretic

peptides mediated through NPR-C could prove to be important in the future development of treatments for cardiovascular disease.

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