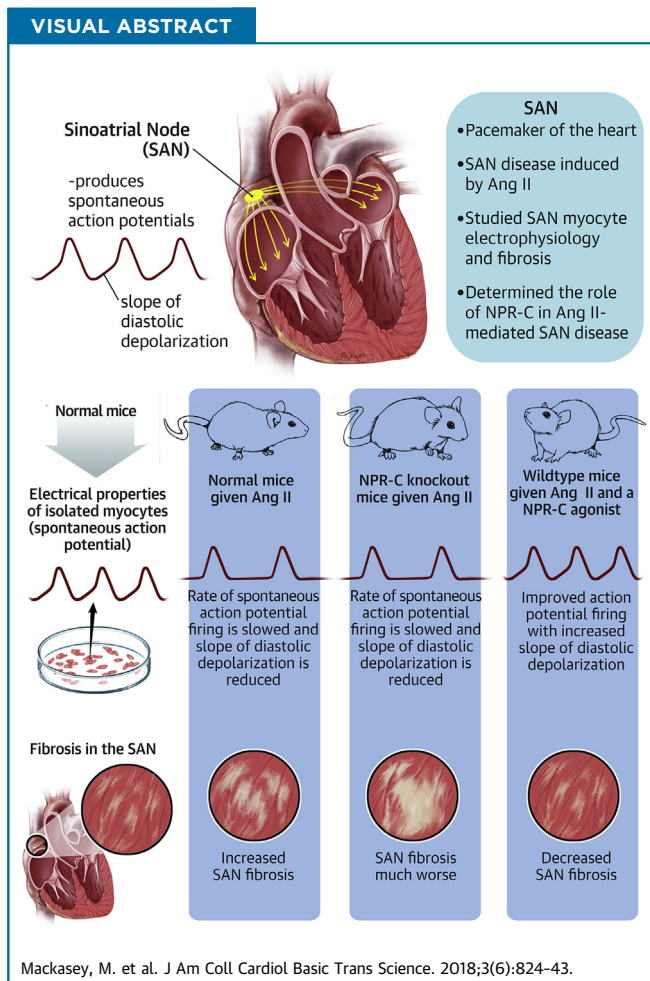


PRECLINICAL RESEARCH

# Natriuretic Peptide Receptor-C Protects Against Angiotensin II-Mediated Sinoatrial Node Disease in Mice



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**HIGHLIGHTS**

- SAN disease is prevalent in hypertension and heart failure and can be induced by chronic Ang II treatment in mice.
- Ang II caused SAN disease in mice in association with impaired electrical conduction, reduction in the hyperpolarization-activated current ( $I_f$ ) in SAN myocytes, and increased SAN fibrosis.
- Ang II-induced SAN disease was worsened in mice lacking NPR-C in association with enhanced SAN fibrosis.
- Mice co-treated with Ang II and an NPR-C agonist (cANF) were protected from SAN disease.
- NPR-C may represent a new target to protect against Ang II-induced SAN disease.

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## SUMMARY

Sinoatrial node (SAN) disease mechanisms are poorly understood, and therapeutic options are limited. Natriuretic peptide(s) (NP) are cardioprotective hormones whose effects can be mediated partly by the NP receptor C (NPR-C). We investigated the role of NPR-C in angiotensin II (Ang II)-mediated SAN disease in mice. Ang II caused SAN disease due to impaired electrical activity in SAN myocytes and increased SAN fibrosis. Strikingly, Ang II treatment in NPR-C<sup>-/-</sup> mice worsened SAN disease, whereas co-treatment of wild-type mice with Ang II and a selective NPR-C agonist (cANF) prevented SAN dysfunction. NPR-C may represent a new target to protect against the development of Ang II-induced SAN disease. (J Am Coll Cardiol Basic Trans Science 2018;3:824-43) © 2018 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Sinoatrial node (SAN) disease is a highly prevalent but poorly understood form of cardiovascular disease that occurs in a number of conditions including hypertension, hypertrophy, and heart failure (1-4). Arrhythmias associated with SAN dysfunction account for approximately 50% of sudden deaths in the hospital in heart failure patients (2,5). A common theme in these prevalent forms of cardiovascular disease is a pathological elevation in angiotensin II (Ang II) and enhanced Ang II signaling (6,7). SAN disease in hypertension and heart failure is characterized by sinus bradycardia and chronotropic incompetence due, at least in part, to the SAN's compromised ability to generate spontaneous action potentials (APs) and excite the surrounding atrial myocardium (5,8). Mechanisms responsible for SAN disease remain poorly understood, which severely limits therapeutic options. Currently, artificial pacemaker implantation is the major treatment approach for SAN disease; however, this comes with substantial costs and a number of patient risks, including infections, hematoma, pneumothorax, endocarditis, limited response to autonomic regulation, lead dislodgment, and lead failure. The burden of ongoing, lifelong complications from these devices, which can help sustain function but do affect underlying disease mechanisms, indicates a clear need for improved treatment options for patients with SAN disease.

Under normal conditions, the heart beat is initiated in the SAN due to the ability of this specialized region

of the heart to generate spontaneous APs characterized by the presence of a diastolic depolarization (DD) (9,10). The DD is produced by a number of underlying ionic mechanisms including the hyperpolarization-activated current ( $I_f$ ), T-type and L-type  $Ca^{2+}$  currents ( $I_{Ca,T}$  and  $I_{Ca,L}$ , respectively), a  $Na^+$ - $Ca^{2+}$  exchange current ( $I_{NCX}$ ) driven by sarcoplasmic reticulum  $Ca^{2+}$  release, and repolarizing  $K^+$  currents (9,11). Alterations in the activity and coordination of these currents can compromise SAN pacemaker activity. Pacemaker activity in the heart is also importantly affected by the amount of fibrosis in the SAN, and enhanced fibrosis can impair normal SAN function (6,12).

Natriuretic peptide(s) (NP) are a family of cardioprotective hormones with a number of effects in the cardiovascular system that are mediated by specific NP receptors called NPR-A, -B, and -C (13,14). Among this family, NPR-C remains the least understood. We have demonstrated that NPs have potent effects on cardiac electrophysiology, including in the SAN, and that some of these effects can be mediated by NPR-C (14-18). Furthermore, we have recently shown that NPR-C knockout (NPR-C<sup>-/-</sup>) mice develop SAN dysfunction in association with enhanced fibrosis (19). These findings suggest that NPR-C plays an integral role in maintaining normal SAN structure and function and that it could be a novel therapeutic target; however, the role of NPR-C

## ABBREVIATIONS AND ACRONYMS

- Ang II** = angiotensin II
- AP** = action potential
- cSNRT** = corrected sinoatrial node recovery time
- CV** = conduction velocity
- DD** = diastolic depolarization
- G<sub>max</sub>** = maximum conductance
- HR** = heart rate
- I<sub>Ca,L</sub>** = L-type calcium current
- I<sub>Ca,T</sub>** = T-type calcium current
- I<sub>f</sub>** = hyperpolarization-activated current
- I<sub>NCX</sub>** = sodium-calcium exchanger current
- IV** = current voltage relationship
- NP** = natriuretic peptide
- NPR** = natriuretic peptide receptor
- NPR-C** = natriuretic peptide receptor C
- SAN** = sinoatrial node
- SBP** = systolic blood pressure
- V<sub>1/2(acts)</sub>** = voltage for 50% channel activation

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All authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the JACC: Basic to Translational Science [author instructions page](#).

in the SAN in cardiovascular disease remains unexplored.

This study used a mouse model of hypertension induced by chronic Ang II treatment to investigate the mechanisms for SAN disease in the setting of elevated Ang II and the role of NPR-C in the progression of SAN disease. Our studies demonstrate that chronic Ang II treatment causes SAN disease in association with electrical and structural remodeling. We also demonstrate that the progression of SAN disease is enhanced in the absence of NPR-C and that chronic NPR-C activation can prevent development of SAN disease.

## METHODS

An expanded methods and materials section can be found in the [Supplemental Material](#).

**ANIMALS AND SURGICAL INTERVENTIONS.** This study used male wild-type and NPR-C<sup>-/-</sup> mice between 10 and 15 weeks of age. NPR-C<sup>-/-</sup> mice were initially obtained from the Jackson Laboratory (strain B6;C-Npr3lgj/J, Bar Harbor, Maine) and backcrossed into the C57Bl/6 line as we previously reported (15,19,20). Mice of each genotype were treated with Ang II (3 mg/kg/day) or saline for 3 weeks, using osmotic minipumps (Alzet, Cupertino, California). In some cases, the NPR-C agonist [des(Gln<sup>18</sup>, Ser<sup>19</sup>, Gly<sup>20</sup>, Leu<sup>21</sup>, Gly<sup>22</sup>)ANP<sub>4-23</sub>-NH<sub>2</sub>]; cANF<sub>4-23</sub>; catalog H-3134; Bachem, Torrance, California) was also delivered by osmotic minipump along with Ang II at doses of 0.07 or 0.14 mg/kg/day. cANF is a well-characterized NPR-C agonist that has no effects on the guanylyl cyclase-linked NPR-A and NPR-B receptors (13,16,21-23). Numbers of mice treated with cANF are noted in the relevant figure legends. To insert osmotic pumps, mice were anesthetized by isoflurane inhalation, and the pumps were inserted subcutaneously through a small midscapular incision. All experimental procedures were approved by the University of Calgary Animal Care and Use Committee and the Dalhousie University Committee for Laboratory Animals and were conducted in accordance with Canadian Council on Animal Care guidelines.

**ECHOCARDIOGRAPHY AND SYSTOLIC BLOOD PRESSURE ASSESSMENT.** Cardiac structure and function were assessed in anesthetized mice (isoflurane inhalation) by using transthoracic echocardiography at baseline and after 3 weeks of drug treatment. Echocardiography was performed using a high-resolution transducer and a Vivid 7 ultrasound machine (GE Healthcare, Laurel, Maryland) to measure 2-dimensional (2D) M-mode images from the parasternal short axis at the level of the midpapillary

muscle, as we have described previously (19,24). Systolic blood pressure (SBP) was measured in conscious, restrained mice at baseline and after 3 weeks of drug treatment, using a tail cuff apparatus (IITC Life Sci, Woodland Hills, California).

**IN VIVO ELECTROPHYSIOLOGY.** Surface ECGs were measured in anesthetized mice (2% isoflurane inhalation) using 30-ga subdermal needle electrodes (Grass Technologies, West Warwick, Rhode Island). In conjunction, a 1.2-F octapolar electrophysiology catheter (Transonic, Ithaca, New York) was inserted into the right heart through an incision in the jugular vein and used to measure corrected sinoatrial node recovery time (cSNRT), as we have described previously (19,24,25). Data were acquired using a Gould ACQ-7700 amplifier and Ponemah physiology platform software (Data Sciences International, St. Paul, Minnesota). Additional details are available in the [Supplemental Material](#).

**HIGH-RESOLUTION OPTICAL MAPPING.** To study activation patterns and electrical conduction in the right atrial posterior wall, high-resolution optical mapping was performed in isolated atrial preparations, using methods we have described in detail previously (17,19,24,25). Atrial preparations were immobilized using blebbistatin (1 μM) (26). Changes in fluorescence were captured using the voltage-sensitive dye Di-4-ANEPPS (10 μM) and a high-speed electron-multiplying charge-coupled device camera (Evolve 128 model; Photometrics, Tucson, Arizona) at ~900 fps. Spatial resolution was 45 × 45 μm/pixel. All experiments were performed at 35°C. Data were analyzed using customized software written in Matlab version 9.1 software (Mathworks, Natick, Massachusetts). Further details are available in the [Supplemental Material](#).

**PATCH CLAMPING IN ISOLATED SAN MYOCYTES.** Single pacemaker myocytes from the SAN were isolated using procedures we have described previously (15,19). Spontaneous APs were recorded using the perforated patch clamp technique in current clamp mode, while I<sub>f</sub> and I<sub>Ca,L</sub> were recorded in the whole-cell configuration in voltage clamp mode. Solutions and protocols for these measurements are described in detail in the [Supplemental Material](#).

**QUANTITATIVE POLYMERASE CHAIN REACTION.** Quantitative gene expression in the SAN (or right and left atria) was performed using established techniques we have described previously (15,25). Genes of interest that were measured include *Npr1* (encodes NPR-A), *Npr2* (encodes NPR-B), *Npr3* (encodes NPR-C), *HCN1*, *HCN2*, *HCN4*, *SCN5a* (encodes NaV1.5), *GJC1* (encodes Cx45), and *GJA1* (encodes Cx43). *GAPDH* was

used as a reference gene based on its stable expression levels across treatment groups. Additional information is available in the [Supplemental Material](#).

**WESTERN BLOTTING.** For Western blotting, SAN samples were extracted from 2 mice and pooled for each experimental replicate in order to ensure sufficient protein (27). In some cases, Western blotting was also performed on right and left atrial samples ( $n = 3$  for each region). Western blotting was used to measure the protein expression of NPR-A, NPR-B, NPR-C, and HCN4, as well as GAPDH as the control. Procedures for these experiments are provided in the [Supplemental Material](#).

**COLLAGEN STAINING AND IMMUNOHISTOCHEMISTRY.** Fibrosis in the SAN was assessed using picosirius red staining on paraffin-embedded sections (8  $\mu\text{m}$ ) through the SAN. Sections were cut perpendicularly to the crista terminalis, and the SAN region was confirmed by HCN4 immunostaining (28,29) in adjacent sections. The level of fibrosis was quantified using ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland). Additional details are provided in the [Supplemental Material](#).

**STATISTICS.** All data are means  $\pm$  SEM. Data were analyzed using Student *t*-test or 1- or 2-way ANOVA with Tukey post hoc test as indicated in each figure legend. A *p* value  $<0.05$  was considered significant. Statistical analysis was conducted using SigmaPlot version 11.0 software (Systat, San Jose, California). Sample sizes for each experimental approach ranged as follows: 11 to 17 mice/group for SBP analysis; 9 to 15 mice/group for echocardiography; 19 to 29 mice/group for intracardiac electrophysiology; 8 to 9 mice/group for autonomic blockade studies; 8 to 24 mice/group for optical mapping; 10 to 17 cells/group (minimum 5 mice/group) for patch clamping; 5 hearts/group for histology; 3 to 5 replicates/group for Western blotting; and 7 to 11 samples/group for quantitative polymerase chain reaction. Specific numbers for all experiments conducted are noted in the figure legends.

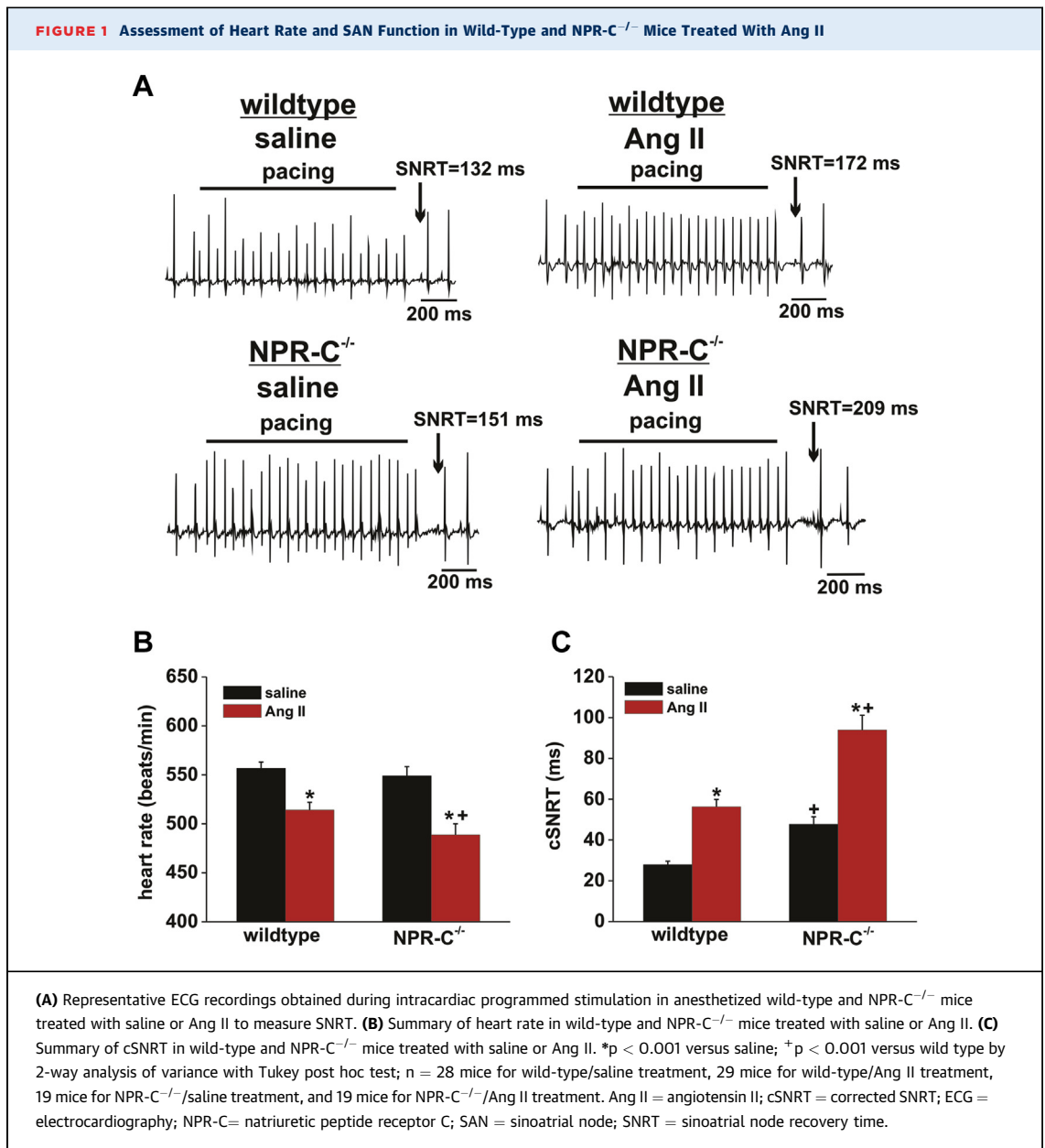
## RESULTS

**EFFECTS OF ANG II ON SBP AND CARDIAC STRUCTURE IN WILDTYPE AND NPR-C<sup>-/-</sup> MICE.** Initially we measured the effects of Ang II (or saline as a control) on SBP in wild-type and NPR-C knockout mice ([Supplemental Figure S1](#)). These data demonstrated that Ang II treatment increased ( $p < 0.001$ ) SBP to very similar extents in wild-type and NPR-C<sup>-/-</sup> mice, such that there were no differences ( $p = 0.115$ ) in the extent of hypertension between genotypes. There were also no differences ( $p = 1.000$ ) in systolic pressure between

wild-type and NPR-C<sup>-/-</sup> mice at baseline, as we have shown previously (19). As expected, saline treatment had no effect ( $p = 1.000$ ) on systolic pressure.

Next, we used echocardiography to assess overall changes in cardiac structure and function in wild-type and NPR-C<sup>-/-</sup> mice treated with Ang II compared with saline controls. M-mode imaging of the left ventricle ([Supplemental Figure S2](#), [Supplemental Table S1](#)) demonstrated that, in wild-type mice, Ang II treatment caused a hypertrophic response characterized by increases in thickness of the interventricular septum ( $p < 0.001$ ) and in the left ventricular posterior wall ( $p = 0.006$ ) and a reduction ( $p < 0.001$ ) in left ventricular internal diameter. These structural changes occurred in association with modest increases in ejection fraction ( $p = 0.007$ ) and fractional shortening ( $p < 0.001$ ). These data indicate that Ang II treatment in wild-type mice caused concentric hypertrophy without compromising systolic performance. Strikingly, Ang II treatment in NPR-C<sup>-/-</sup> mice caused a much more severe response, despite the observation that there were no differences in ventricular structure and function between wild-type and NPR-C<sup>-/-</sup> mice at baseline. Specifically, Ang II treatment in NPR-C<sup>-/-</sup> mice caused ventricular dilation as indicated by an increase ( $p = 0.004$ ) in left ventricular internal diameter and a tendency for the interventricular septum and the left ventricular posterior wall to be thinner. Furthermore, ejection fraction ( $p < 0.001$ ) and fractional shortening ( $p < 0.001$ ) were substantially reduced in NPR-C<sup>-/-</sup> mice treated with Ang II, indicating that, in contrast to wild-type mice, Ang II treatment in NPR-C<sup>-/-</sup> mice resulted in overt heart failure with ventricular dilation and impaired systolic function.

**EFFECTS OF ANG II ON SAN FUNCTION IN WILD-TYPE AND NPR-C<sup>-/-</sup> MICE.** Next, we used electrocardiography (ECG) recordings with intracardiac programmed stimulation to assess SAN function by measuring heart rate (HR) and corrected SAN recovery time (cSNRT) in anesthetized mice ([Figure 1A](#)). Ang II treatment reduced ( $p < 0.001$ ) HR ([Figure 1B](#)) and prolonged ( $p < 0.001$ ) cSNRT ([Figure 1C](#)) in wild-type and NPR-C<sup>-/-</sup> mice. Notably, following Ang II treatment, HR was lower ( $p < 0.001$ ) and cSNRT was more severely prolonged ( $p < 0.001$ ) in NPR-C<sup>-/-</sup> mice than in wild-types. SAN function was further assessed by measuring intrinsic HR in anesthetized mice after autonomic nervous system blockade with atropine (10 mg/kg) and propranolol (10 mg/kg) ([Supplemental Figure S3](#)). These data demonstrated that intrinsic HR (i.e., SAN function) was reduced ( $p < 0.001$ ) following Ang II treatment in wild-type and NPR-C<sup>-/-</sup> mice but



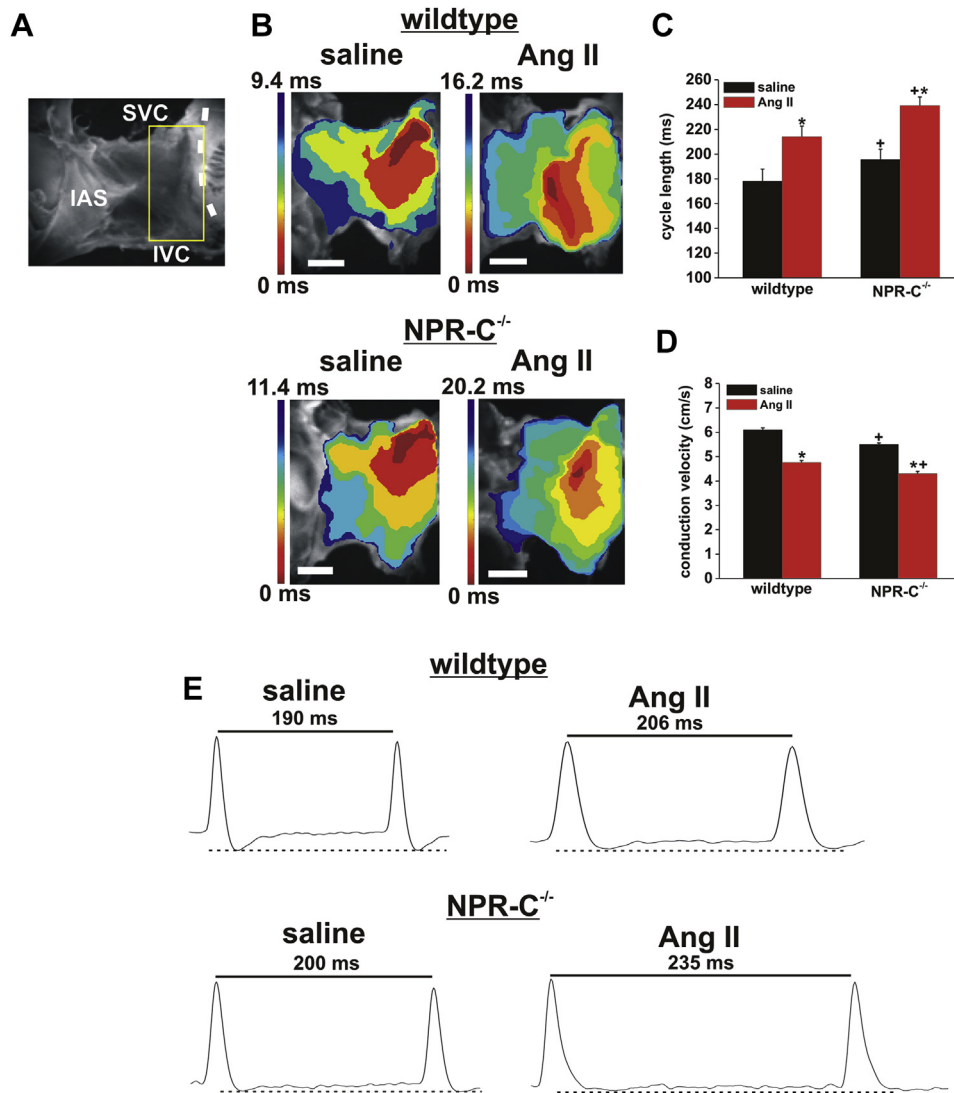
that this reduction is larger, resulting in lower ( $p = 0.004$ ) intrinsic HR in NPR-C<sup>-/-</sup> mice (Supplemental Figure S3B). Consistent with our prior studies (19), these data also demonstrated that cSNRT was longer ( $p < 0.001$ ) (Figure 1C) and intrinsic HR was lower ( $p = 0.037$ ) (Supplemental Figure S3) in saline-treated NPR-C<sup>-/-</sup> mice than saline-treated wild types. These observations demonstrate that Ang II treatment induces SAN disease and that this is exacerbated in NPR-C<sup>-/-</sup> mice.

Conduction in the right atrial posterior wall following Ang II treatment was investigated using high-resolution optical mapping techniques, which

we used previously (19,24,25). Activation maps, which were measured in the right atrial posterior wall adjacent to the crista terminalis (Figure 2A) in wild-type and NPR-C<sup>-/-</sup> mice treated with saline or Ang II, revealed distinct patterns in each group (Figure 2B). Specifically, these activation maps revealed that conduction time through the right atrial posterior wall adjacent to the crista terminalis was slowed in wild-type and in NPR-C<sup>-/-</sup> mice treated with Ang II compared with that in saline controls and that this slowing was greatest in NPR-C<sup>-/-</sup> mice. Furthermore, the hearts from mice treated with Ang II (wild type and NPR-C<sup>-/-</sup>) displayed activation sites



**FIGURE 2** Assessment of Electrical Conduction in the SAN in Wild-Type and NPR-C<sup>-/-</sup> Mice Treated With Ang II



(A) Atrial preparation used in optical mapping. Dashed line indicates the location of the crista terminalis. The SAN is located in the intercaval region of the right atrial posterior wall adjacent to the crista terminalis. The yellow box illustrates the area that was typically mapped. (B) Representative activation maps in atrial preparations from wild-type and NPR-C<sup>-/-</sup> mice treated with saline or Ang II. Bars = 1 mm. (C) Summary data for cycle length in wild-type and NPR-C<sup>-/-</sup> mice treated with saline or Ang II. \*p < 0.001 versus saline; +p = 0.048 versus wild type by 2-way analysis of variance (ANOVA) with Tukey post hoc test. (D) Summary data for SAN CV in wild-type and NPR-C<sup>-/-</sup> mice treated with saline or Ang II. \*p < 0.001 versus saline; +p < 0.001 versus wild type by 2-way ANOVA with Tukey post hoc test; n = 16 hearts for wild type/saline, 24 hearts for wild type/Ang II, 13 hearts for NPR-C<sup>-/-</sup>/saline, and 12 hearts for NPR-C<sup>-/-</sup>/Ang II. (E) Representative spontaneous SAN optical APs in wild-type and NPR-C<sup>-/-</sup> mice treated with saline or Ang II. Dashed lines run through the maximum diastolic potential. AP = action potential; CV = conduction velocity; IAS = interatrial septum; IVC = opening of inferior vena cava; SVC = opening of superior vena cava; other abbreviations as in Figure 1.

that were shifted inferiorly compared with those in saline-treated hearts.

We quantified cycle length and local conduction velocity (CV) within the right atrial posterior wall, using our previously established approaches (19,25).

These data demonstrated that cycle length (Figure 2C) was prolonged (p < 0.001) by Ang II treatment in wild-type mice, further confirming impairment in SAN function. Cycle length was also prolonged (p = 0.048) in saline-treated NPR-C<sup>-/-</sup> mice compared with that in

wild-type controls, whereas Ang II treatment in NPR-C<sup>-/-</sup> mice resulted in the longest ( $p = 0.048$ ) cycle lengths. As expected, CVs in this region of the heart were relatively low (i.e.,  $<10$  cm/s) (Figure 2D) (19,24). CV was reduced ( $p < 0.001$ ) in wild-type mice treated with Ang II and was also lower ( $p < 0.001$ ) in NPR-C<sup>-/-</sup> mice treated with saline than in wild-type controls. Ang II treatment in NPR-C<sup>-/-</sup> mice resulted in substantial slowing of CV in the right atrial posterior wall, which was lower ( $p < 0.001$ ) than that in Ang II-treated wild-type mice.

Optical APs from the pacemaker region of the right atrial posterior wall demonstrated the changes in cycle length, as well as alterations in AP morphology, in these treatment groups. Specifically, representative optical APs (Figure 2E) showed that wild-type mice treated with saline have the shortest cycle lengths (i.e., faster frequency of spontaneous AP firing) and a robust DD. Ang II treatment prolonged the cycle length between successive APs and reduced the slope of the DD in wild-type mice. AP cycle length was also increased in saline-treated NPR-C<sup>-/-</sup> mice, which is consistent with our prior work (19). Finally, Ang II-treated NPR-C<sup>-/-</sup> mice exhibited the longest intrinsic AP cycle length and a clear reduction in DD slope. The properties of SAN APs were further investigated in isolated cells as described subsequently.

**ANG II CAUSES ELECTRICAL REMODELING IN SAN MYOCYTES.** To further investigate the effects of Ang II on SAN AP firing, we measured spontaneous APs in isolated SAN myocytes from wild-type and NPR-C<sup>-/-</sup> mice treated with saline or Ang II (Figure 3A; Supplemental Table S2). Summary data demonstrated that Ang II treatment increased ( $p < 0.001$ ) AP cycle length (i.e., slowed AP frequency) (Figure 3B) in association with a reduction ( $p < 0.001$ ) in DD slope (Figure 3C) in wild-type and NPR-C<sup>-/-</sup> mice. Interestingly, in contrast to our findings in intact atrial preparations, there were no differences in AP cycle length ( $p = 0.557$ ) or DD slope ( $p = 0.517$ ) between genotypes following saline or Ang II treatment in isolated SAN myocytes.

The ionic basis for these alterations in SAN AP morphology was investigated next. First, we measured the hyperpolarization-activated current ( $I_f$ ) in SAN myocytes isolated from saline- and Ang II-treated wild-type and NPR-C<sup>-/-</sup> mice (Figure 3D).  $I_f$  IV curves for wild-type mice (Figure 3E) and NPR-C<sup>-/-</sup> mice (Figure 3F) demonstrate that Ang II reduced  $I_f$  density ( $p < 0.001$ ) similarly in both genotypes. Analysis of  $I_f$  activation kinetics (Figures 3G and 3H, Supplemental Figure S4) revealed that the voltage for 50% channel activation ( $V_{1/2(\text{act})}$ ) was not affected by Ang II ( $p = 0.416$ ) and not different between genotypes

( $p = 0.361$ ). Similarly, the slope factor ( $k$ ) for the  $I_f$  activation curves was not affected by Ang II ( $p = 0.057$ ) or genotype ( $p = 0.061$ ).

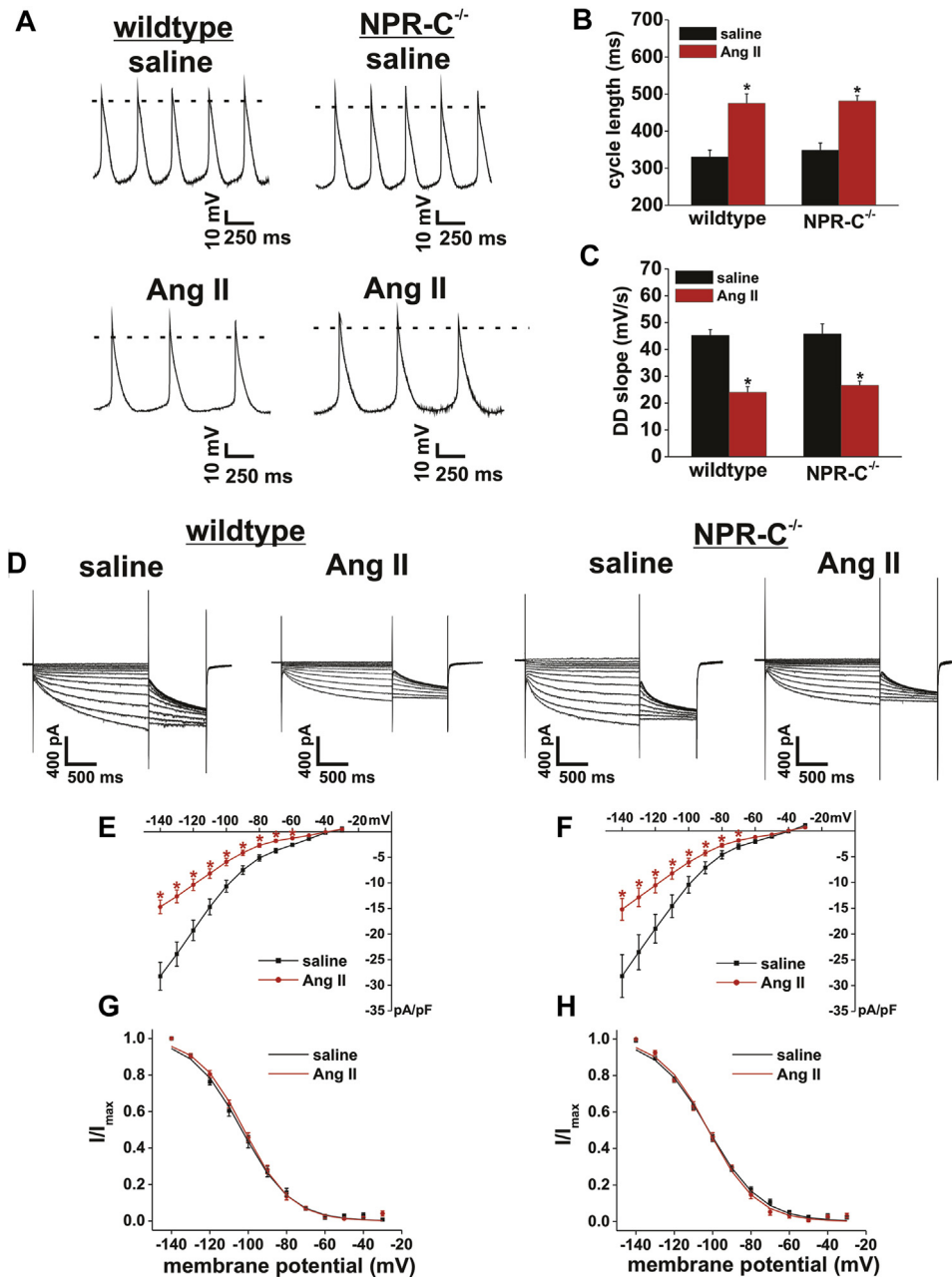
A reduction in  $I_f$  density without changes in activation kinetics suggests the current is reduced due to changes in ion channel expression. Accordingly, we next measured the mRNA expression level of the major *HCN4* channel isoforms in the SAN of wild-type mice treated with saline or Ang II (Supplemental Figure S5). *HCN4*, which is a major *HCN4* isoform in the mouse, was measured in the SAN as well as the right and left atria to ensure specificity of our SAN samples (Supplemental Figure S5A). From these data, it was apparent that our SAN samples expressed *HCN4* highly and that *HCN4* levels were very low or negligible in the right and left atria. Furthermore, *HCN4* mRNA expression was reduced ( $p < 0.001$ ) specifically in the SAN but not in the right ( $p = 0.676$ ) or left ( $p = 0.984$ ) atrium after Ang II treatment. *HCN1* and *HCN2* levels were measured in these same SAN samples. These data demonstrated that *HCN1* ( $p = 0.517$ ) (Supplemental Figure S5B) and *HCN2* ( $p = 0.067$ ) (Supplemental Figure S5C) mRNA expression levels were not altered in the SAN of Ang II-treated mice.

*HCN4* expression level in the SAN was also measured by Western blotting (Supplemental Figure S5D). Once again, we first confirmed that SAN samples used for Western blotting expressed *HCN4* protein highly compared with that in the right and left atria where *HCN4* protein expression was negligible (Supplemental Figure S5D). We then measured the expression level of *HCN4* protein in the SAN of saline- and Ang II-treated mice. These measurements confirmed that Ang II treatment caused a reduction ( $p = 0.044$ ) in *HCN4* protein in the SAN (Supplemental Figure S5E).

We also measured  $I_{\text{Ca,L}}$ , as carried by channels  $\text{Ca}_v1.2$  and  $\text{Ca}_v1.3$  (15,30,31), in SAN myocytes from wild-type mice treated with saline or Ang II (Supplemental Figure S6A). The  $I_{\text{Ca,L}}$  IV curves (Supplemental Figure S6B) demonstrate no differences ( $p = 0.503$ ) in  $I_{\text{Ca,L}}$  density between treatment groups. Similarly, analysis of  $I_{\text{Ca,L}}$  activation kinetics (Supplemental Figure 6C) shows that  $I_{\text{Ca,L}}$  maximum conductance ( $G_{\text{max}}$ ,  $p = 0.877$ ) (Supplemental Figure S6D),  $V_{1/2(\text{act})}$  ( $p = 0.342$ , Supplemental Figure 6E), and slope factor ( $k$ ,  $p = 0.270$ ) (Supplemental Figure S6F) were not affected by Ang II treatment.

Finally, we performed additional gene expression studies to assess whether there were additional contributors to impaired electrical function in the SAN following Ang II treatment. Specifically, we measured

**FIGURE 3** Effects of Ang II on AP Morphology and Hyperpolarization-Activated Current ( $I_f$ ) in Isolated SAN Myocytes From Wild-Type and NPR-C<sup>-/-</sup> Mice



(A) Representative spontaneous AP recordings in wild-type and NPR-C<sup>-/-</sup> SAN myocytes treated with saline or Ang II. (B, C) Summary data illustrating cycle length (B) and DD slope (C) in wild-type and NPR-C<sup>-/-</sup> mice treated with saline or Ang II. \* $p < 0.001$  versus saline;  $p = 0.557$  for AP cycle length and  $p = 0.517$  for DD slope between genotypes within treatment group;  $n = 16$  cells for wild type/saline, 11 cells for wild type/Ang II, 10 cells for NPR-C<sup>-/-</sup>/saline, and 13 cells for NPR-C<sup>-/-</sup>/Ang II for AP data. See Supplemental Table S2 for additional AP parameters. (D) Representative  $I_f$  recordings in SAN myocytes isolated from wild-type and NPR-C<sup>-/-</sup> mice treated with saline or Ang II. (E, F) Summary  $I_f$  IV curves for wild-type (E) and NPR-C<sup>-/-</sup> (F) mice treated with saline or Ang II. \* $p < 0.001$  versus saline at each membrane potential by 2-way repeated-measures analysis of variance with Tukey post-hoc test. (G, H)  $I_f$  activation curves for wild-type (G) mice and NPR-C<sup>-/-</sup> (H) mice treated with Ang II. See Supplemental Figure S4 for additional  $I_f$  kinetic analysis.  $n = 17$  cells for wild type/saline, 13 cells for wild type/Ang II; 13 cells for NPR-C<sup>-/-</sup>/saline, and 16 cells for NPR-C<sup>-/-</sup>/Ang II. IV = current voltage relationship; pA = picoamperes; pF = picofarads; other abbreviations as in Figures 1 and 2.



the mRNA expression levels of *SCN5a* (encodes  $\text{Na}_v1.5$  and has been shown to contribute to SAN conduction) (32); *GJC1* (Cx45), a major connexin in the SAN (33); and *GJA1* (Cx43), which can be detected at the mRNA in the SAN (34,35). These data demonstrated that there were no differences in the expression levels of *SCN5a* ( $p = 0.335$ ) (Supplemental Figure S7A) or *GJC1* ( $p = 0.680$ ) (Supplemental Figure S7B) in the SAN of Ang II-treated mice. *GJA1* showed a modest reduction ( $p = 0.002$ ) (Supplemental Figure S7C) in the SAN, although Cx43 was not thought to be a major contributor to SAN function (see the Discussion section).

**ANG II CAUSES STRUCTURAL REMODELING IN THE SAN.** The findings summarized in the preceding text demonstrate that SAN dysfunction following Ang II treatment occurred in association with impairments in spontaneous AP firing and reduced  $I_f$ . Notably, although the changes in AP cycle length and SAN conduction in NPR-C<sup>-/-</sup> mice treated with Ang II were greater than those in wild-type mice when measured in the intact atria, the impairments in AP firing (and  $I_f$ ) elicited by Ang II were similar between wild-type and NPR-C<sup>-/-</sup> mice in isolated SAN myocytes. Thus, the ionic alterations in SAN myocytes do not explain the more severe progression of SAN dysfunction in NPR-C<sup>-/-</sup> mice.

Accordingly, the basis for SAN dysfunction following Ang II treatment was further investigated by measuring the level of fibrosis in the SAN. This was done using picrosirius red staining in histological sections taken from wild-type and NPR-C<sup>-/-</sup> mice treated with saline or Ang II. Sections were cut perpendicularly to the crista terminals, and adjacent sections were immunostained for HCN4. Representative images for each treatment group demonstrate HCN4-positive regions in the SAN in the first image and picrosirius red staining in the second image (Figure 4A). SAN fibrosis was assessed specifically within regions that aligned with the HCN4-positive zone. These representative images and the summary data (Figure 4B) demonstrated that Ang II treatment increased ( $p < 0.001$ ) SAN fibrosis in wild-type and NPR-C<sup>-/-</sup> mice. Strikingly, the amount of SAN fibrosis was higher ( $p = 0.014$ ) in NPR-C<sup>-/-</sup> mice than in wild types. Ang II-treated NPR-C<sup>-/-</sup> mice had substantially higher ( $p = 0.014$ ) SAN fibrosis than Ang II-treated wild-type mice.

**EFFECTS OF NPR-C ACTIVATION ON SBP AND CARDIAC FUNCTION.** Results of experiments presented to this point demonstrated that Ang II causes SAN dysfunction in association with impairments in SAN electrical conduction and

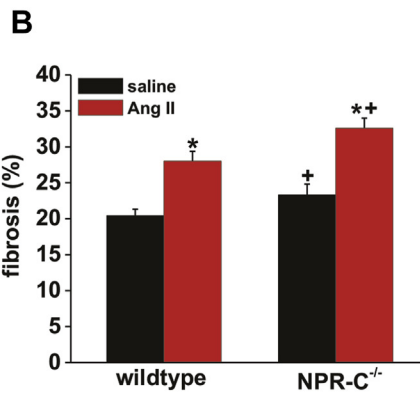
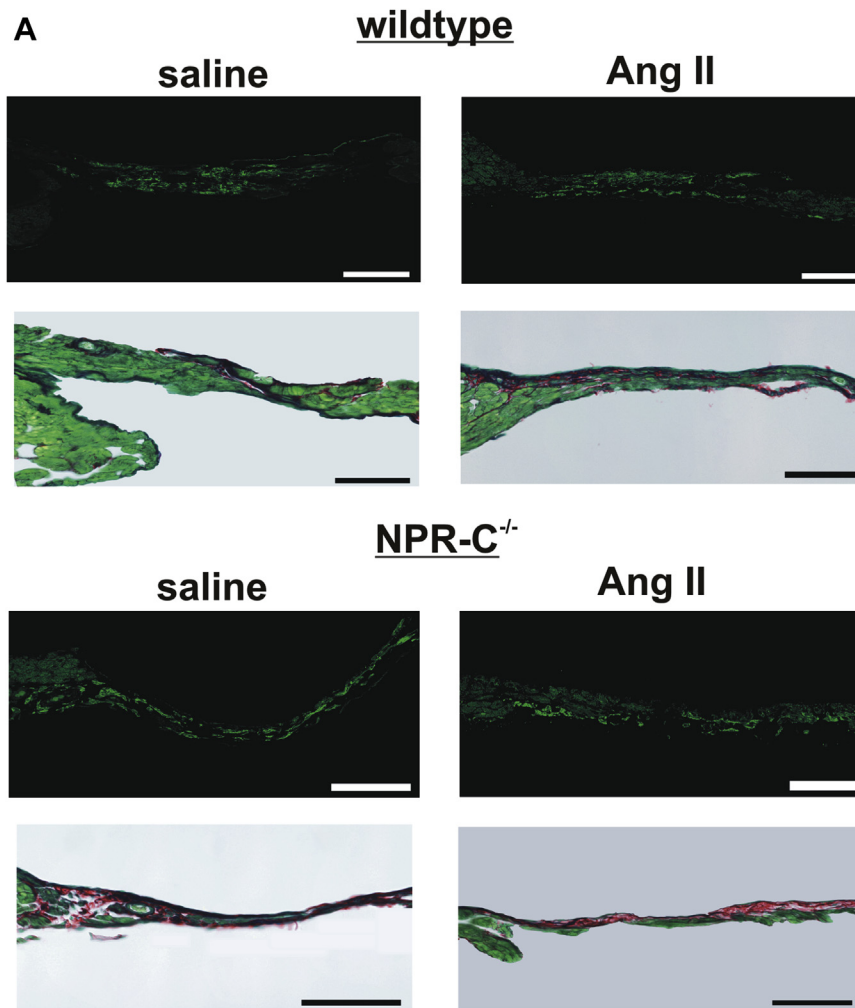
spontaneous AP firing as well as SAN fibrosis. Our data also demonstrated that Ang II treatment in the absence of NPR-C receptors exacerbates the progression to systolic heart failure and worsens SAN dysfunction, suggesting that NPR-C plays a central role in the progression of Ang II-mediated heart disease, including in the SAN.

To further test this hypothesis, we conducted a series of experiments in which wild-type mice were co-treated with Ang II and the selective NPR-C agonist cANF (21,22,36) at 2 different dosages (0.07 and 0.14 mg/kg/day). These dosages were selected to be comparable to those in other studies that administered NPs clinically and in animal studies (37-39). Initially we measured mRNA (Supplemental Figure S8A) and protein (Supplemental Figures S8B and S8C) expression for all 3 NPRs in the SAN of wild-type mice treated with saline or Ang II to determine whether NP receptor expression in the SAN was altered during chronic Ang II treatment. Ang II had no effects on the expression of NPR-A, -B, or -C in the SAN. We also measured SBP in mice treated with saline, Ang II, or Ang II plus cANF (both doses). These data (Supplemental Figure S9) demonstrated that SBP was elevated ( $p < 0.001$ ) in mice treated with Ang II and in Ang II plus cANF. There were no differences in SBP between Ang II alone and Ang II plus cANF at dosages of 0.07 ( $p = 0.859$ ) or 0.14 mg/kg/day ( $p = 0.740$ ), indicating that cANF co-treatment did not prevent Ang II hypertension.

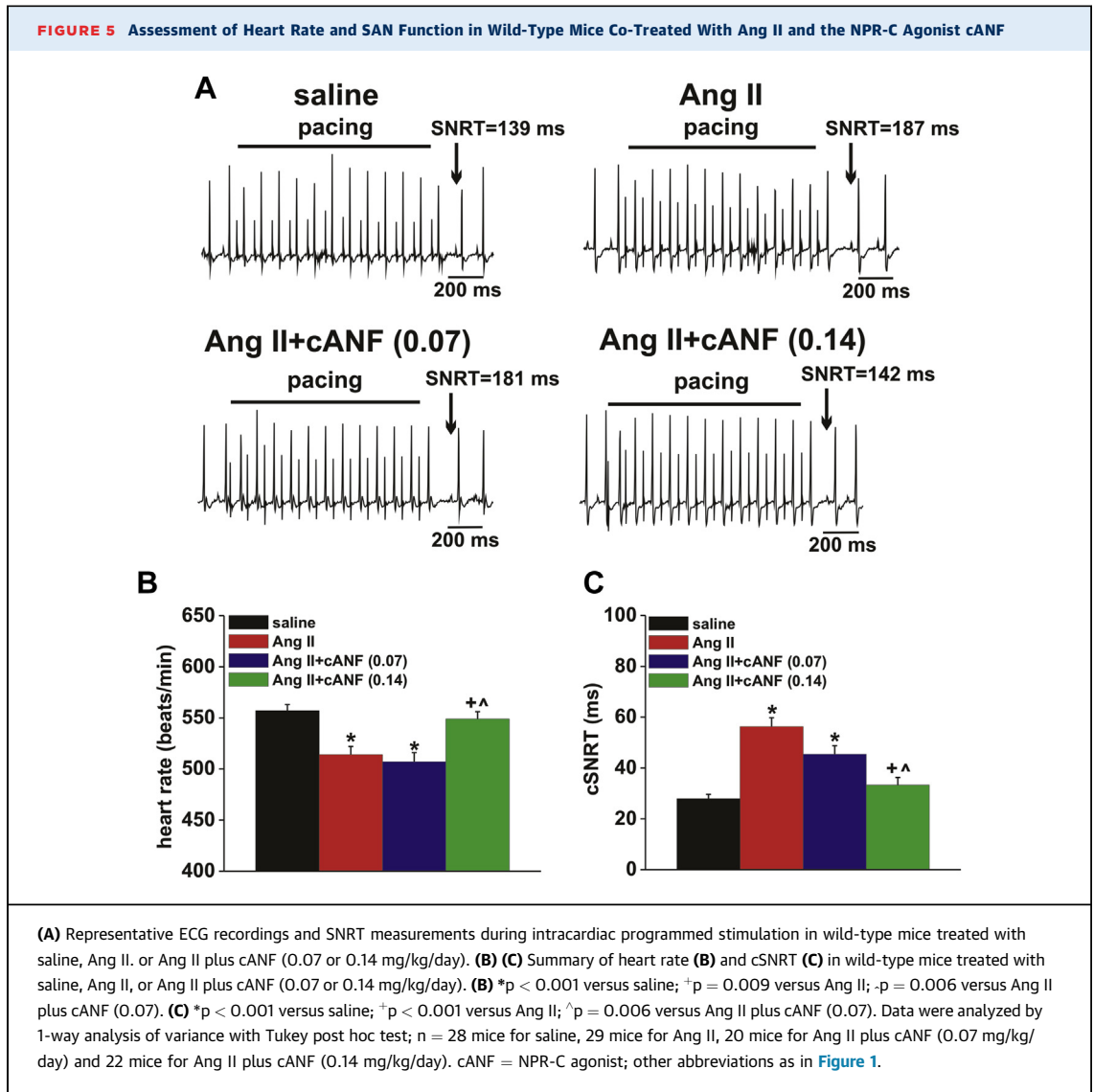
Next, we used echocardiography to determine the effects of cANF co-treatment on cardiac structure and function. M-mode images (Supplemental Figure S10) and summary data (Supplemental Table S3) demonstrated both dosages of cANF prevented the development of ventricular hypertrophy elicited by Ang II alone.

**cANF PREVENTS DEVELOPMENT OF ANG II-MEDIATED SAN DYSFUNCTION.** Using intracardiac electrophysiology in anesthetized mice, we measured the effects of co-treatment with Ang II and cANF on HR and SAN function (Figure 5A). Summary data illustrated that the lower dosage of cANF (0.07 mg/kg/day) did not prevent Ang II-mediated changes in HR ( $p = 0.915$ ) (Figure 5B); however, there was a clear trend toward a reduction in cSNRT ( $p = 0.067$ ) (Figure 5C) compared with Ang II alone. Furthermore, co-treatment with a higher dosage of cANF (0.14 mg/kg/day) potently prevented the reduction in HR ( $p = 0.009$ ) and prolongation in cSNRT ( $p < 0.001$ ) elicited by Ang II alone, such that HR ( $p = 0.915$ ) and cSNRT ( $p = 0.580$ ) were not different from those in saline controls (Figures 5B and 5C).

**FIGURE 4** Effects of Ang II on Interstitial Fibrosis in the SAN of Wild-Type and NPR-C<sup>-/-</sup> Mice



**(A)** Sections through the SAN in wild-type and NPR-C<sup>-/-</sup> mice treated with saline or Ang II. For each treatment group, the **top image** illustrates the HCN4-positive zone (**green**) used to identify the SAN region, and the **bottom image** illustrates the **picrosirius red** staining from an adjacent section used to identify fibrosis (**red**). Bars = 50  $\mu$ M. **(B)** Summary of SAN fibrosis measured in the HCN4-positive zone for wild-type and NPR-C<sup>-/-</sup> mice treated with saline or Ang II. \* $p < 0.001$  versus saline; † $p = 0.014$  versus wild type by 2-way analysis of variance with Tukey post hoc test;  $n = 5$  hearts for each group.

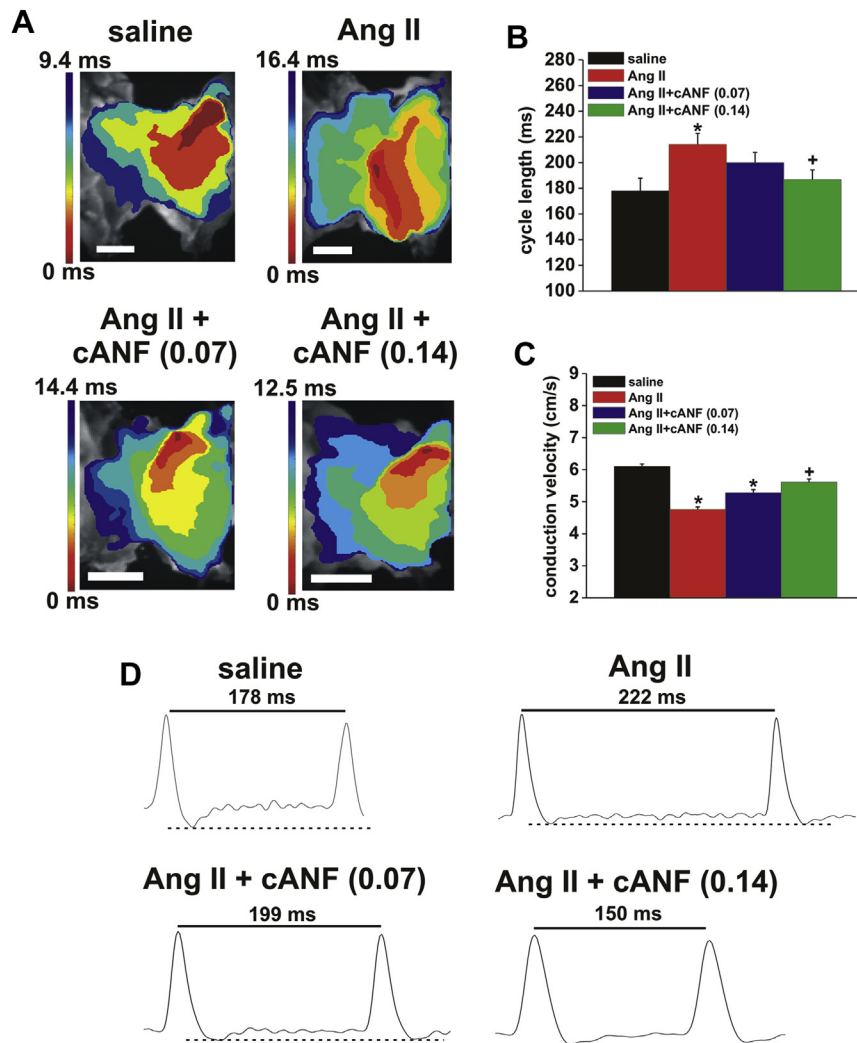


Similar effects of cANF co-treatment were observed using high-resolution optical mapping of the right atrial posterior wall ([Figure 6](#)). Specifically, representative activation maps ([Figure 6A](#)) demonstrate that conduction in the right atrial posterior wall was faster in atrial preparations from mice co-treated with cANF than with Ang II alone and that cANF co-treatment resulted in leading activation sites that were shifted back toward the superior portion of the right atrial posterior wall. Cycle length in atrial preparations from mice co-treated with the lower dose of cANF tended to be shorter than Ang II alone, whereas the higher dosage of cANF reduced ( $p < 0.001$ ) cycle length to values that were not different ( $p = 0.641$ ) from saline controls ([Figure 6B](#)). Furthermore, CV in the right atrial posterior wall following treatment with the low dosage of cANF tended to be

higher than that of Ang II alone, but this did not reach statistical significance. CV was increased ( $p < 0.001$ ) following co-treatment with the higher dose of cANF compared with that in Ang II alone to values that were not different from saline controls ([Figure 6C](#)). Optical APs further demonstrated that cANF dosage dependently shortened AP cycle length and increased the DD slope compared to those with Ang II alone ([Figure 6D](#)). These findings demonstrated that activation of NPR-C with cANF can dose-dependently prevent the development of SAN dysfunction elicited by Ang II.

**EFFECTS OF cANF ON SAN MYOCYTE ELECTROPHYSIOLOGY AND FIBROSIS.** To determine the mechanism(s) for the protective effects of NPR-C activation in the SAN during Ang II treatment, we

**FIGURE 6** Assessment of Electrical Conduction in the SAN in Wild-Type Mice Co-Treated With Ang II and the NPR-C Agonist cANF

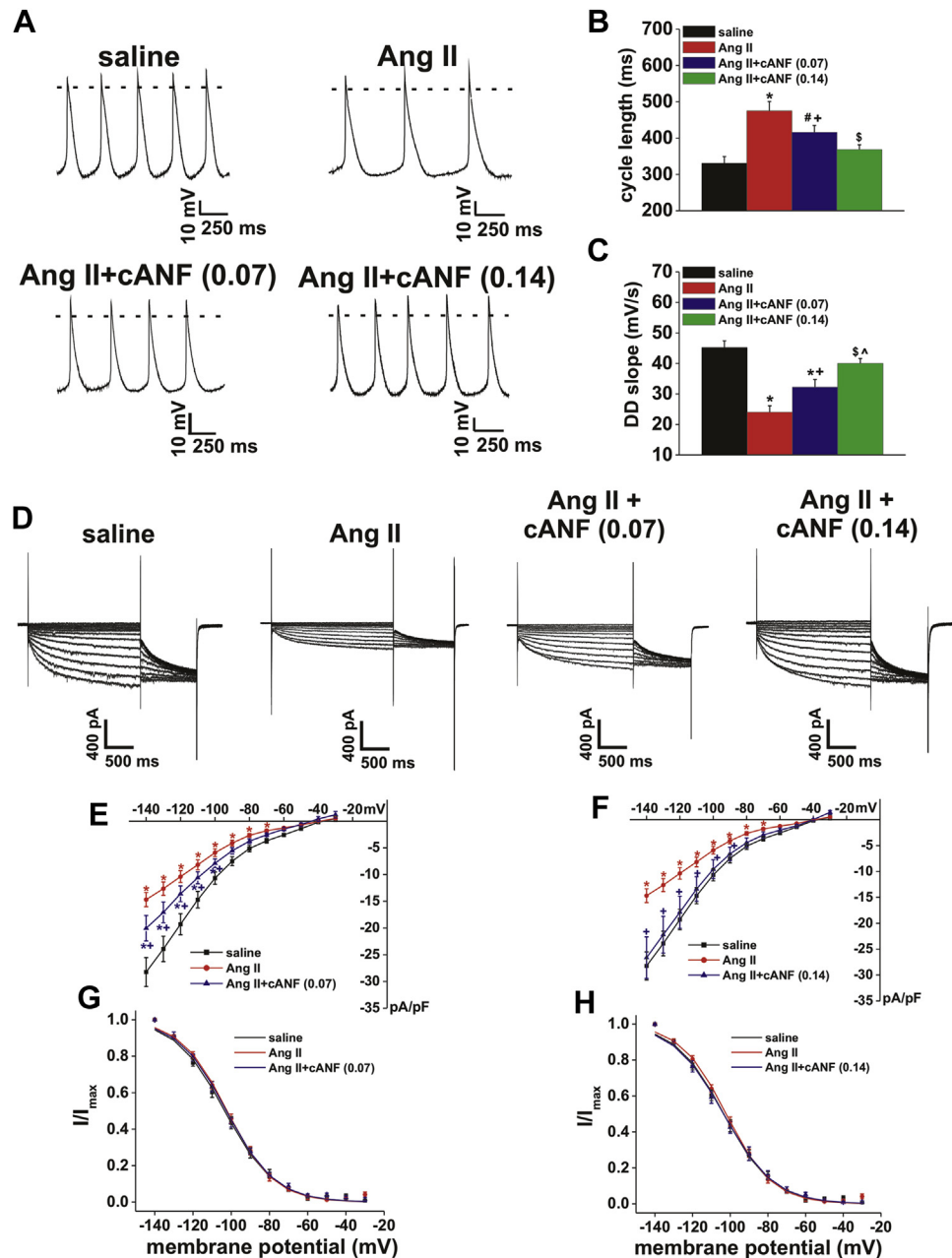


(A) Maps show wild-type mice treated with saline, Ang II, or Ang II plus cANF (0.07 or 0.14 mg/kg/day). (B) (C) Summary data for cycle length (B) and SAN CV (C) in wild-type mice treated with saline, Ang II, or Ang II plus cANF (0.07 or 0.14 mg/kg/day). (B) \* $p = 0.013$  versus saline; <sup>+</sup> $p < 0.001$  versus Ang II. (C) \* $p < 0.001$  versus saline; <sup>+</sup> $p < 0.001$  versus Ang II. Data were analyzed by 1-way analysis of variance with Tukey post hoc test;  $n = 16$  hearts for saline, 24 hearts for Ang II, 8 hearts for Ang II + cANF (0.07 mg/kg/day), and 8 hearts for Ang II plus cANF (0.14 mg/kg/day). (D) Representative spontaneous SAN optical APs in wild-type mice treated with saline, Ang II, or Ang II plus cANF (0.07 or 0.14 mg/kg/day). Dashed lines run through the maximum diastolic potential. Abbreviations as in Figures 1, 2, and 5.

measured spontaneous APs in SAN myocytes isolated from mice co-treated with cANF (Figure 7A, Supplemental Table S4). Summary data demonstrate that cANF dose-dependently prevented the increase in AP cycle length (i.e., prevented the slowing of AP firing) (Figure 7B) and the reduction in DD slope (Figure 7C). Specifically, the lower dosage of cANF had an intermediate effect such that AP cycle length was lower ( $p < 0.046$ ) than that of Ang II alone but still

longer ( $p = 0.006$ ) than in saline-treated mice. In contrast, the higher dose of cANF reduced ( $p = 0.008$ ) AP cycle length compared with Ang II alone, such that cycle length was not different ( $p = 0.573$ ) from that in saline-treated mice. Very similar effects were seen for DD slope.

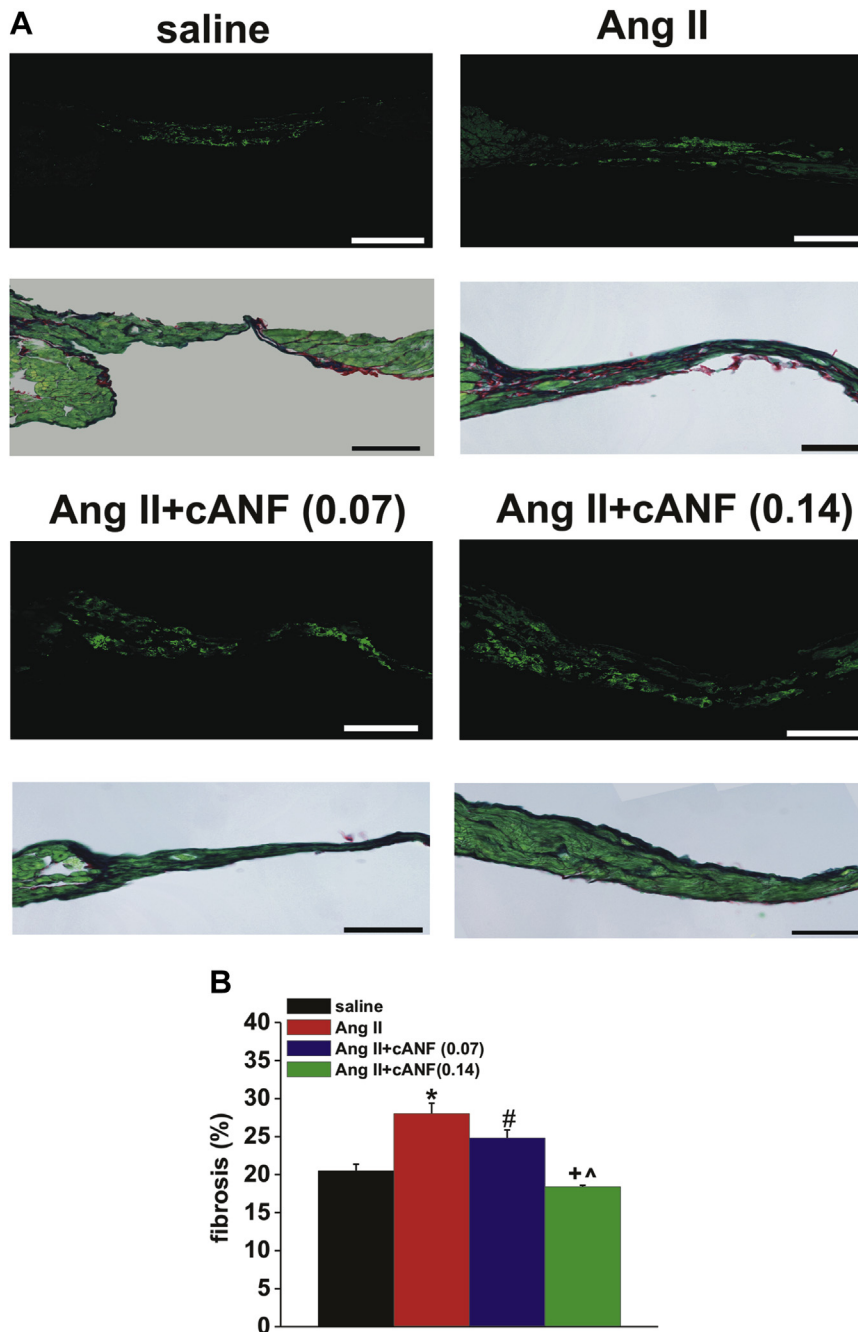
Consistent with these effects on AP morphology, cANF also dose-dependently prevented the reduction in  $I_f$  elicited by Ang II in SAN myocytes (Figure 7D).

**FIGURE 7** Effects of the NPR-C Activator cANF on Ang II-Mediated Alterations in AP Morphology and Hyperpolarization-Activated Current ( $I_f$ ) in Isolated SAN Myocytes

(A) Representative spontaneous AP recordings obtained in wild-type mice treated with saline, Ang II, or Ang II plus cANF (0.07 or 0.14 mg/kg/day). (B) (C) Summary of cycle length (B) and DD slope (C) illustrate the effects of cANF co-treatment on Ang II-mediated changes in SAN AP morphology. (B) \* $p < 0.001$  versus saline; # $p = 0.006$  versus saline; + $p = 0.046$  versus Ang II; \$ $p = 0.008$  versus Ang II. (C) \* $p < 0.001$  versus saline; + $p = 0.014$  versus Ang II; \$ $p < 0.001$  versus Ang II; ^ $p = 0.024$  versus Ang II plus cANF (0.07). Data were analyzed by 1-way analysis of variance with Tukey post hoc test;  $n = 16$  saline, 11 Ang II, 13 Ang II plus cANF (0.07), and 10 Ang II plus cANF (0.14) for AP data. See Supplemental Table S4 for additional AP parameters. (D) Representative  $I_f$  recordings from wild-type mice treated with saline, Ang II, or Ang II plus cANF (0.07 or 0.14 mg/kg/day). (E) (F) Summary IV curves for the effects of Ang II and cANF (0.07 and 0.14 mg/kg/day) on  $I_f$ . (G) (H)  $I_f$  activation curves illustrating the effects of Ang II and cANF (0.07 and 0.14 mg/kg/day) in SAN myocytes. (E) \* $p < 0.001$  versus saline; + $p = 0.002$  versus Ang II. (F) \* $p < 0.001$  versus saline; + $p < 0.001$  versus Ang II. Data were analyzed by 2-way repeated-measures analysis of variance with Tukey post hoc test;  $n = 17$  cells for saline, 13 cells for Ang II, 14 cells for Ang II+cANF (0.07), and 13 cells for Ang II plus cANF (0.14). See Supplemental Figure S10 for additional  $I_f$  kinetic analysis. Abbreviations as in Figures 1, 2, 3, and 5.



**FIGURE 8** Effects of the NPR-C Agonist cANF on Ang II-Mediated Alterations in SAN Fibrosis



**(A)** SAN immunohistochemistry for HCN4 (**top**) and picrosirius red staining of fibrosis (**bottom**) for wild-type mice treated with saline, Ang II, or Ang II plus cANF (0.07 or 0.14 mg/kg/day). Bars = 50  $\mu$ m. **(B)** Summary of the effects of cANF on Ang II-mediated fibrosis in the SAN. \* $p < 0.001$  versus saline; # $p = 0.037$  versus saline; + $p < 0.001$  versus Ang II; ^ $p = 0.004$  versus Ang II plus cANF (0.07) by 1-way analysis of variance with Tukey post hoc test;  $n = 5$  hearts for each group.

Once again, the lower dose of cANF had an intermediate effect, such that  $I_f$  density was larger ( $p = 0.002$ ) than that in SAN myocytes treated with Ang II alone but still reduced ( $p < 0.001$ ) compared with that in

saline-treated SAN myocytes (Figure 7E). Cotreatment with the higher dosage of cANF fully prevented the reduction in  $I_f$  whereby  $I_f$  density was larger ( $p < 0.001$ ) than Ang II alone and not different

( $p = 0.441$ ) from that in saline-treated SAN myocytes (Figure 7F). In agreement with the results presented earlier (Figure 3), neither Ang II or cANF (with either dose) had any effects on  $I_f$  activation kinetics (Figures 7G and 7H, Supplemental Figure S11).

Finally, we assessed the effects of cANF co-treatment on fibrosis in the SAN by using picrosirius red staining and HCN4 immunostaining (Figure 8). These data demonstrate that the lower dose of cANF resulted in an intermediate level of SAN fibrosis that was still higher ( $p = 0.037$ ) than that in saline-treated mice. In contrast, co-treatment with the higher dose of cANF potentially prevented the increase in SAN fibrosis. Specifically, fibrosis in the SAN following the higher dose of cANF was reduced compared with that of Ang II alone ( $p < 0.001$ ) and to that with the lower dose of cANF ( $p = 0.004$ ) and not different ( $p = 0.534$ ) from that of saline. Collectively, these findings demonstrate that selective NPR-C activation dose-dependently prevents Ang II-mediated impairments in SAN AP firing, the reduction in  $I_f$  and fibrosis of SAN.

## DISCUSSION

The present study used a mouse model of hypertensive heart disease induced by sustained Ang II delivery to investigate the impacts of Ang II and hypertension on SAN function. Chronic Ang II treatment in mice is a well-established model of hypertension and cardiac hypertrophy (40). We showed that, in addition to producing these expected responses, chronic Ang II also results in overt SAN disease, as indicated by a reduction in HR (baseline and following autonomic blockade), a prolongation of cSNRT, and impaired conduction in the right atrial posterior wall in isolated atrial preparations. This is consistent with clinical data showing associations among hypertension, hypertrophy, and SAN disease (6). In addition, we studied the role of NPR-C in the progression of SAN disease. Strikingly, we found that Ang II treatment in NPR-C<sup>-/-</sup> mice greatly exacerbated disease progression. Specifically, NPR-C<sup>-/-</sup> mice treated with Ang II developed ventricular dilation with overt systolic heart failure (as opposed to compensated concentric hypertrophy in wild-type mice) and substantially more severe decline in SAN function. On the other hand, co-treatment of wild-type mice with Ang II and a selective NPR-C agonist (cANF) was able to largely prevent development of SAN disease. Several prior studies have demonstrated that cANF activates NPR-C without activating guanylyl cyclase-linked NPR-A and NPR-B (21,22,36). Furthermore, we previously

showed that the acute effects of cANF in the heart are completely absent in NPR-C<sup>-/-</sup> mice (16,17), confirming the fact that cANF is a selective NPR-C agonist. Interestingly, our experiments suggest that Ang II treatment impaired SAN function in association with an inferior shift in the location of the leading pacemaker site (as assessed by optical mapping). This is consistent with studies in human and animal models of SAN disease that commonly show alterations in the location of the leading pacemaker site (6,25,41-43). Strikingly, co-treatment with cANF prevented this shift in leading activation site and resulted in SAN activations similar to that in saline-treated mice. Currently, there is a severe lack of therapeutic options for SAN disease. Our novel findings in mouse models suggest that NPR-C may be a viable new target for preventing the progression of SAN disease.

The responses observed in NPR-C<sup>-/-</sup> mice and in mice co-treated with cANF occurred without any differences in the hypertensive response to Ang II. NPR-C<sup>-/-</sup> mice treated with Ang II displayed the same degree of hypertension as wild-type mice, and cANF co-treatment did not prevent the increase in SBP elicited by Ang II. Some of the effects of Ang II on the hypertrophic response and cardiac remodeling are known to occur independently of blood pressure (44). Our findings suggest that this is also true in the context of SAN disease and indicate that NPR-C operates directly within the SAN, independently of SBP, to modulate the effects of Ang II. Interestingly, a previous study demonstrated that cANF could reduce blood pressure in spontaneously hypertensive rats, although the pressures observed in that model were substantially higher than those observed in our study of chronic Ang II treatment (23). If cANF can reduce blood pressure under some conditions, this may further contribute to the beneficial effects of NPR-C activation in hypertension. Collectively, our experiments indicate that cANF can modulate SAN function through direct effects in the SAN, independently of changes in SBP, supporting the idea that NPR-C is a viable target in the SAN in Ang II-mediated heart disease.

In previous studies (15,19) and the present one, we have shown that NPR-C is the most highly expressed NPR in the heart, including in the SAN. Our present study also demonstrates that chronic Ang II treatment does not alter the expression of NPR-C in the SAN, which is likely important in enabling NPR-C to be targeted with cANF. Nevertheless, there could be Ang II-induced changes in the expression of signaling molecules downstream of NPR-C, which could be affected differently in NPR-C<sup>-/-</sup> mice than in mice

co-treated with cANF. These will be useful areas for future investigation that will provide further insight into the mechanisms through which NPR-C and cANF affect Ang II-induced SAN dysfunction.

Our investigations of the mechanisms by which Ang II causes SAN disease revealed important roles for electrical remodeling in SAN myocytes (i.e., impaired spontaneous AP firing) as well as fibrosis of the SAN. In terms of electrical remodeling, our data illustrate that AP firing was impaired in association with reductions in the DD slope and  $I_f$ . Importantly, these Ang II-induced changes at the level of the SAN myocyte were indistinguishable between those in wild-type and NPR-C<sup>-/-</sup> mice. Rather, SAN disease was worse in NPR-C<sup>-/-</sup> mice due to a greater increase in SAN fibrosis in the absence of NPR-C. Interstitial fibrosis is a key component of SAN structure and a key determinant of SAN function, as studies have shown that the SAN contains higher levels of fibrosis than the working myocardium (10,12). Our data demonstrate levels of fibrosis in the SAN that are consistent with this. NPs are known to have potent antifibrotic effects in the heart, although most prior studies have focused on the roles of NPR-A and NPR-B in mediating these effects (45-49). Nevertheless, there is evidence that NPR-C can be involved in fibrotic signaling (50), and our previous work with NPR-C<sup>-/-</sup> mice shows that NPR-C regulates fibrosis by affecting collagen gene expression and collagen deposition in the heart, including in the SAN (19). The present study now demonstrates for the first time that loss of NPR-C greatly exacerbates the fibrotic response elicited by Ang II. Of particular importance is the novel finding that chronic, selective NPR-C activation potently prevents pathological collagen deposition in the SAN in Ang II-mediated heart disease. Collectively, these findings demonstrate a major role for NPR-C as a regulator of structural remodeling and fibrosis in the SAN and that this is a major determinant of the severity of SAN disease.

Studies of electrical remodeling at the level of the SAN myocyte (i.e., spontaneous AP morphology, ionic currents) in animal models of heart disease have been relatively scarce, and results have not always been consistent. For example, a study in a rabbit model of heart failure demonstrated SAN disease in association with a slowing of spontaneous AP firing, a reduction in DD slope and reduced  $I_f$  (1). Similarly, SAN disease has been described in canine studies of heart failure induced by atrial tachypacing (51,52). Although these studies did not directly measure SAN AP morphology, they did show that

$I_f$  density was reduced in association with reductions in expression of HCN2 and HCN4. Calcium currents were not altered in the SAN in any of those studies. On the other hand, a separate study using a chronic Ang II model reported that SAN disease occurred due to apoptosis of SAN myocytes (4). That study reported no differences in spontaneous AP firing or morphology in isolated SAN myocytes, and no ionic currents were investigated. Our experiments using a chronic Ang II model clearly demonstrate substantial changes in SAN AP firing patterns, as well as reductions in  $I_f$ . Consistent with the studies in rabbit and dog models of heart failure (1,51,52), we observed a reduction in expression of HCN4 in the SAN following Ang II treatment, which is consistent with the changes in AP cycle length, DD slope, and  $I_f$  density we observed. We also found no differences in  $I_{Ca,L}$  in SAN myocytes following Ang II. Overall, our study supports the conclusion that spontaneous AP firing is compromised in hypertensive/Ang II-mediated heart disease and heart failure and that changes in expression of HCN channels and  $I_f$  density are a critical determinant of SAN disease. This is also consistent with data for other factors that contribute to SAN disease and dysfunction. For example, aging is a major contributor to SAN disease (5,25), and this has been shown to occur, at least in part, with a reduction in  $I_f$  (53). Similarly, endurance athletes exhibit bradycardia and a higher incidence of SAN disease in association with reductions in the expression of HCN4 and  $I_f$  density (54).

We also examined other potential mediators of electrical dysfunction by measuring the mRNA expression levels of *SCN5a*, *GJC1*, and *GJA1*. *SCN5a* has been shown to be present in the periphery of the SAN and to play a role in electrical conduction from the SAN to the atrial myocardium (32,55). We observed no changes in *SCN5a* expression in the SAN following Ang II treatment. Cx45 is a critical connexin in the SAN where it facilitates cell-to-cell communication (33); however, we observed no differences in Cx45 expression after Ang II treatment. Cx43 is primarily located in the atria and is mostly absent in the SAN, although strands of Cx43-positive tissue have been found to extend into the SAN (28,33,56). Furthermore, numerous studies have shown that Cx43 mRNA can be detected in the SAN (34,35,57). Accordingly, we measured Cx43 expression and found it was modestly reduced after Ang II treatment. Because the SAN is considered to be primarily a Cx43-negative region and the reduction we measured was very small, it is unlikely that a down-regulation of Cx43 contributed substantially to the

effects of Ang II on electrical conduction in the SAN. Nevertheless, we measured mRNA levels only for SCN5a, Cx45, and Cx43, and these targets could be altered in ways that do not involve changes in gene expression, which could be investigated in future studies.

In addition to preventing SAN fibrosis, we found that co-treatment with the NPR-C agonist cANF also dose-dependently prevented the reduction in  $I_f$  and changes in AP morphology induced by Ang II in SAN myocytes. As mentioned earlier, the reduction in  $I_f$  observed following Ang II treatment occurred in association with reduced HCN4 expression and no changes in  $I_f$  activation kinetics, indicating that reduced expression is the major determinant of the reduction in  $I_f$ . Similarly, the improved  $I_f$  densities observed following cANF co-treatment with Ang II occurred without changes in  $I_f$  activation kinetics. This suggests that cANF prevented the reduction in levels of HCN4 expression in the SAN observed in Ang II alone. Additional studies will be required to assess the effects of chronic cANF on ion channel expression in the SAN in Ang II-mediated heart disease, as well as the molecular mechanisms for any such effects. We have previously shown that acute application of NPs can modulate  $I_f$  in SAN myocytes isolated from normal wild-type mice and that these effects do involve changes in the voltage dependence of activation (15). Because we observed no such changes in our cANF co-treatment experiments, our data suggest that the chronic effects of cANF in heart disease are distinct from the acute effects of NPs in the normal SAN.

As mentioned, the impairments in electrical conduction in the SAN occurred in association with shifts in the location of the leading pacemaker site. The SAN is a heterogeneous structure (56), and it is conceivable that differences in expression levels of ion channels such as HCN4 in the superior and inferior pacemaker locations could contribute the alterations in function we observed. Furthermore, studies in the human and canine heart have demonstrated the existence of distinct SAN conduction pathways that can be accessed depending on location of leading pacemaker site (10,58,59), and this may further contribute to changes in SAN conduction. Although a study in human heart showed that HCN4 expression levels were similar in the head, the center, and the tail of the SAN (60), it is possible that there may be alterations in the diseased heart. Thus, it will be useful to explore the effects of Ang II on regional expression of HCN4 and possibly other channels (e.g., HCNs, calcium channels) in different parts of the SAN in future studies.

It should be noted that our optical mapping studies did not resolve optical APs with double component upstrokes (i.e., an SAN component and an atrial component), which have been reported in other studies in large mammals and in mice (61-63). Identifying such APs would enable more specific assessments of SAN conduction velocities and thus could be pursued in future studies. We also note that the baseline beating rates in our isolated atrial preparations were lower than in *ex vivo* mouse hearts. This may be related to the effects of Di-4-ANNEPS, which can have some effects on sinus rate (64). Nevertheless, our isolated SAN myocyte and molecular studies, as well as histological assessments in the HCN-positive regions of the SAN, clearly support our conclusions from the effects of Ang II and NPR-C in the SAN.

**STUDY LIMITATIONS.** The present study focused on SAN dysfunction following Ang II treatment in mice. In addition, echocardiographic data demonstrated that Ang II treatment caused concentric ventricular hypertrophy in wild-type mice and ventricular dilation and heart failure in NPR-C<sup>-/-</sup> mice. These findings suggest that NPR-C might have affected electrical or structural remodeling in the ventricles as well. Similarly, Ang II treatment has been shown to cause atrial fibrillation (65,66), and it is possible that NPR-C affects atrial remodeling in addition to its effects in the SAN. Although our study demonstrates important effects of NPR-C in the SAN, it is also important to recognize that global ablation of NPR-C and systemic application of cANF can also have noncardiac effects. Although these were not the foci of our study, it is conceivable that treatment with NPs could have effects on the vasculature, kidneys, and other systems that may further affect disease progression. These areas will be important for additional studies. Our study was conducted in mouse models, which was essential for identifying new roles for NPR-C using knockout mice. It will be important to validate these findings in large animal models and ultimately in human patients.

Efforts to develop new therapeutic approaches for SAN disease are focused on the development of new pharmacological agents as well as the possibility of using biological pacemakers (67,68). Interestingly, several studies in the area of biological pacemakers have focused on strategies that up-regulate HCN channel expression, either directly or through specific transcription factors such as Tbx18 (69-71). Our results illustrate that targeting NPR-C offers another approach to modulating HCN expression and  $I_f$  density in SAN disease. There is

great interest in the design and use of synthetic NPs for the treatment of heart disease (72–75). Our findings indicate that compounds targeting NPR-C should be considered in the design of new agents and that these could be useful in the context of SAN disease.

## CONCLUSIONS

Data demonstrate that SAN disease in the setting of hypertension and elevated Ang II involves both electrical remodeling (i.e., changes in ion channel function in SAN myocytes) and structural remodeling (i.e., fibrosis) in mice. Our studies identified the novel approach of targeting NPR-C to prevent both electrical and structural remodeling in the SAN during chronic Ang II treatment, which had highly beneficial effects on SAN function *in vivo*. The observation that both of these processes are preventable by activation of NPR-C helps explain the potent beneficial effects of targeting this NP receptor and demonstrates that synthetic NPs that activate NPR-C could offer a 2-pronged approach to prevent major cellular and molecular determinants of the progression of SAN disease in the setting of Ang II-mediated hypertension, hypertrophy, and heart failure.

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## PERSPECTIVES

**COMPETENCY IN MEDICAL KNOWLEDGE:** SAN disease is prevalent in hypertension and heart failure, conditions characterized by elevated Ang II signaling. The mechanisms responsible for SAN disease remain poorly understood, which limits therapeutic options.

**TRANSLATIONAL OUTLOOK:** Our studies provide new insight into the mechanistic basis for Ang II-induced SAN disease in mice. We show that Ang II impairs spontaneous SAN myocyte action potential firing by decreasing the slope of the diastolic depolarization and reducing the hyperpolarization-activated current ( $I_p$ ). Ang II also causes SAN fibrosis. Our study shows that NPR-C may represent a new target to prevent these impairments in SAN function. Additional studies are required to determine how to translate these findings to human patients.

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**KEY WORDS** fibrosis, hypertension, ion currents, natriuretic peptide, sinoatrial node

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**APPENDIX** For an expanded Methods section, please see the online version of this paper.