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Original article

# Natriuretic peptides regulate heart rate and sinoatrial node function by activating multiple natriuretic peptide receptors

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ABSTRACT

Natriuretic peptides, including BNP and CNP, elicit their effects via two guanylyl cyclase-linked receptors denoted NPR-A and NPR-B as well as a third receptor, NPR-C. The relative contributions of these receptors to the overall effects of NPs on heart rate (HR) and sinoatrial node (SAN) function are very poorly understood. The effects of BNP and CNP (10–500 nM) on HR and SAN myocyte spontaneous action potential (AP) firing were studied using wildtype mice and mice lacking functional NPR-C receptors (NPR-C$^{-/-}$). In basal conditions and 10 nM doses of the β-adrenergic receptor (β-AR) agonist isoproterenol (ISO) BNP and CNP increased HR and AP firing in SAN myocytes. The NPR-C selective agonist cANF (10–500 nM) had no effects in basal conditions, but decreased HR and SAN AP frequency in the presence of ISO. These effects of cANF were completely absent in NPR-C$^{-/-}$ mice. Strikingly, in the presence of 1 μM doses of ISO, BNP and CNP switched to causing decreases in HR and SAN AP frequency. These decreases were not as large as those elicited by cANF and were absent in NPR-C$^{-/-}$ hearts, where BNP instead elicited a further increase in HR. Inhibition of NPR-A with A71915, in the presence of 1 μM ISO, enabled BNP to signal exclusively through NPR-C and to decrease HR as effectively as cANF. Together these data demonstrate that BNP and CNP affect HR and SAN function by activating multiple receptor subtypes. NPR-A/B mediate increases in HR and SAN function, but these effects are opposed by NPR-C, which plays an increasingly important signaling role in the presence of β-AR stimulation.



## 1. Introduction

Natriuretic peptides (NPs), including atrial (ANP), B-type (BNP) and C-type (CNP) NPs, are powerful regulators of the cardiovascular system. They are best known for their ability to regulate intravascular volume and blood pressure through their effects on the kidneys (natriuresis and diuresis), on vascular tone, and on the renin–angiotensin–aldosterone system [1–3]. Through these effects NPs can importantly regulate cardiac output by affecting loading conditions on the heart. We have shown that NPs, including BNP and CNP, can potently affect heart rate (HR) and sinoatrial node (SAN [4]) function [5,6] suggesting that NPs can also affect cardiac output directly by modulating chronotropic status in the heart.

NPs elicit their biological effects by binding to specific NP receptors (NPRs) called NPR-A, NPR-B and NPR-C [2,7,8]. NPR-A (which binds ANP and BNP) and NPR-B (which binds CNP) are membrane bound guanylyl cyclase (GC) receptors that increase intracellular cGMP when a peptide is bound. NPR-C, which binds all NPs, has been a controversial receptor. It was initially classified as a clearance receptor with no signaling function [9] and although it is still commonly referred to in this context [2,10] NPR-C is also known to be coupled to the activation of inhibitory G proteins ($G_i$) [11–14]. In agreement with a signaling role for NPR-C we have shown that activation of this receptor can decrease L-type $Ca^{2+}$ current ($I_{Ca,L}$) in cardiomyocytes [3,6,15].

NPs are able to simultaneously bind to their guanylyl cyclase-linked receptors (NPR-A and NPR-B) and NPR-C; however, the ways in which these three NPRs work together to control the effects of NPs are very poorly understood. It is essential that this relationship be clarified because synthetic NPs are currently in use [16] or in development [17] for the treatment of heart failure. Although most of the beneficial effects of these drugs have been attributed to cGMP-dependent (i.e. NPR-A and NPR-B) signaling [18], NPR-C may also contribute prominently in some conditions.

The purpose of this study was to determine how all three NPRs affect chronotropic status in the heart by measuring the effects of BNP (binds NPR-A and NPR-C) and CNP (binds NPR-B and NPR-C) on HR and SAN function using mice lacking functional NPR-C receptors (NPR-C$^{-/-}$). Our data provide definitive proof of a signaling role for NPR-C in the heart using these NPR-C$^{-/-}$ mice and demonstrate that NPR-C counteracts the NPR-A and NPR-B dependent effects on heart rate and SAN function in a condition specific fashion. Some of these data have been presented in abstract form [19].

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## 2. Methods

An expanded Methods section is available in the online supplement.

### 2.1. Animals

This study utilized male wildtype C57Bl/6 mice (Charles River) and mutant mice lacking functional NPR-C receptors (NPR-C$^{-/-}$; The Jackson Laboratory) [20] between the ages of 10–14 weeks. Experimental procedures were in accordance with the regulations of The Canadian Council on Animal Care and were approved by Dalhousie University.

### 2.2. Experimental approaches

HR and SAN function were studied using ECG recordings in Langendorff-perfused mouse hearts and patch-clamp recordings in isolated mouse SAN myocytes using protocols we have described previously [5,6,21,22]. Mice were anesthetized by isoflurane inhalation and cervically dislocated before hearts were removed. ECGs were recorded in isolated retrogradely perfused hearts at 37 °C and standard intervals, including R–R interval, P wave duration, P–R interval, and Q–T interval, were analyzed. SAN myocytes were enzymatically dissociated and spontaneous action potentials (APs) were recorded using the perforated patch-clamp technique in current clamp mode. APs were recorded at room temperature (22–23 °C), which must be noted when comparing these data to the isolated heart ECG experiments.

### 2.3. Statistical analysis

All summary data are presented as means ± SEM. Data were analyzed using a one way ANOVA with Tukey's post hoc analysis or Student's *t*-test as appropriate. $P<0.05$ was considered significant.

## 3. Results

### 3.1. Effects of natriuretic peptides in basal conditions

We recently demonstrated that BNP and CNP can potently increase HR in basal conditions by activating their GC-linked NPR-A and NPR-B receptors in the sinoatrial node [5]. These effects are confirmed in Supplemental Fig. S1, which demonstrates the ability of BNP and CNP (10 nM and 500 nM doses) to increase HR in Langendorff-perfused mouse hearts. On average, at 500 nM doses, BNP increased ($P<0.05$) HR from 350 ± 15 to 439 ± 18 beats/min and CNP increased ($P<0.05$) HR from 380 ± 22 to 470 ± 13 beats/min. At low 10 nM doses BNP increased ($P<0.05$) HR from 331 ± 3 to 347 ± 2 beats/min and CNP increased ($P<0.05$) HR from 334 ± 4 to 353 ± 4 beats/min.

The synthetic NP cANF has no capacity to activate NPR-A or NPR-B and thus has no capacity to directly stimulate GC enzymes [23]. Conversely, cANF has been shown to activate NPR-C and its downstream $G_i$-dependent signaling pathways [11,13,14]. Accordingly, cANF has been widely used as a selective agonist for NPR-C [3,12]. Supplemental Fig. S2 demonstrates that cANF has no effect on HR in isolated hearts ($P=0.93$) or spontaneous AP firing in isolated SAN myocytes ($P=0.88$) in basal conditions. Additional analysis of ECG parameters (Supplemental Table 1) and spontaneous AP parameters (Supplemental Table 2) further demonstrates the absence of effects of cANF in these conditions. These data demonstrate that BNP and CNP elicit their positive chronotropic effects entirely via NPR-A and NPR-B, and that NPR-C has no functional role in basal conditions.

### 3.2. NPR-C-dependent effects on heart rate and sinoatrial node function

Although NPR-C has no apparent signaling role in the SAN in basal conditions we have demonstrated that activation of NPR-C in the presence of a 1 μM dose of the β-adrenergic receptor (β-AR) agonist isoproterenol (ISO) can lead to a reduction in HR following activation of $G_i$ and a reduction in $I_{Ca,L}$ in SAN myocytes [6]. These experiments depended heavily on the use of cANF as a selective NPR-C agonist. Given the controversies around NPR-C (clearance receptor vs. signaling role) [2,10] it is essential to prove that NP effects attributed to this receptor are absent in NPR-C$^{-/-}$ mice, which has never been done in the heart. Accordingly, we have measured the effects of cANF on HR and SAN function in wildtype and NPR-C$^{-/-}$ mice in the presence of ISO (1 μM).

Figs. 1(A–C) illustrate representative ECG recordings and the time course of the effects of ISO and cANF (500 nM) on HR in Langendorff-perfused hearts from wildtype mice. Summary data (Fig. 1C) show that ISO increased ($P<0.05$) HR from 359 ± 10 to 442 ± 19 beats/min. Subsequent application of cANF reduced ($P<0.05$) HR to 385 ± 14. These changes in HR were associated with changes in electrical conduction whereby cANF also increased ($P<0.05$) the P wave duration and P–R interval on the ECG (Supplemental Table 3). We also examined the dose dependence of these cANF responses by measuring the effects of 10 nM and 100 nM cANF on HR in Langendorff-perfused hearts (Supplemental Fig. 3; Supplemental Table 4). On average, cANF decreased HR in the presence of 1 μM ISO by 25.4 ± 4.9 beats/min at the 10 nM dose, 38.2 ± 4.7 beats/min at the 100 nM dose and 57.3 ± 5.7 beats/min at the 500 nM dose.

Changes in HR in isolated hearts are indicative of effects within the SAN; therefore, we also measured the effects of ISO (1 μM) and cANF (100 nM) on spontaneous AP firing in isolated SAN myocytes, which has not been previously done (Fig. 1D). On average, ISO increased ($P<0.05$) AP frequency from 152 ± 8 to 189 ± 8 APs/min. Subsequent application of cANF decreased ($P<0.05$) AP frequency to 161 ± 8 APs/min (Fig. 1E). These changes in AP frequency were associated with changes ($P<0.05$) in the slope of the diastolic depolarization (DD slope; 32.4 ± 2.9 mV/s in control, 55.9 ± 3.2 in ISO, 38.9 ± 2.8 in ISO + cANF; Fig. 1F) but no change in maximum diastolic potential (MDP; $P=0.33$) or other AP parameters (Supplemental Table 5).

Next we measured the effects of ISO and cANF on HR in Langendorff-perfused NPR-C$^{-/-}$ hearts (Figs. 2A–C). As expected, ISO increased ($P<0.05$) HR from 323 ± 5 to 413 ± 18 beats/min; however, the effects of cANF in the presence of ISO were completely absent ($P=0.61$) in NPR-C$^{-/-}$ hearts (HR remained at 412 ± 18 beats/min; Fig. 2C). Supplemental Table 3 shows that cANF had no effects on any ECG parameters in NPR-C$^{-/-}$ hearts.

Fig. 2D and Supplemental Table 5 illustrate the effects of ISO and cANF on spontaneous AP firing in SAN myocytes from NPR-C$^{-/-}$ mice. In agreement with our isolated heart data, cANF had no effect on AP firing in NPR-C$^{-/-}$ SAN myocytes in the presence of ISO. Specifically, AP frequency (Fig. 2E) was increased ($P<0.05$) from 154 ± 10 to 205 ± 12 beats/min in ISO and remained unchanged (202 ± 10 beats/min; $P=0.98$) following application of cANF. DD slope (Fig. 2F) was increased ($P<0.05$) from 31.2 ± 5.6 to 58.9 ± 4.7 mV/s in ISO and was not changed (58.3 ± 3.7 mV/s; $P=0.99$) after application of cANF. These data represent the first definitive evidence of a signaling role for NPR-C in the heart using NPR-C$^{-/-}$ mice and confirm the utility of cANF as a selective NPR-C agonist.

### 3.3. Effects of BNP and CNP on heart rate and sinoatrial node function in the presence of 1 μM isoproterenol

Taken together, our data demonstrate that BNP and CNP increase HR in basal conditions via NPR-A and NPR-B, whereas in the presence of ISO (1 μM) cANF elicits a decrease in HR via NPR-C. Since the native peptides BNP and CNP are able to simultaneously bind NPR-A/B as well as NPR-C [7,12] we next sought to determine the effects of BNP and CNP on HR and SAN function in the presence of ISO (1 μM) where NPR-C can play a functional role.

Strikingly, in contrast to basal conditions, BNP and CNP decreased HR in the presence of 1 μM ISO (Fig. 3). Summary data (Fig. 3C)

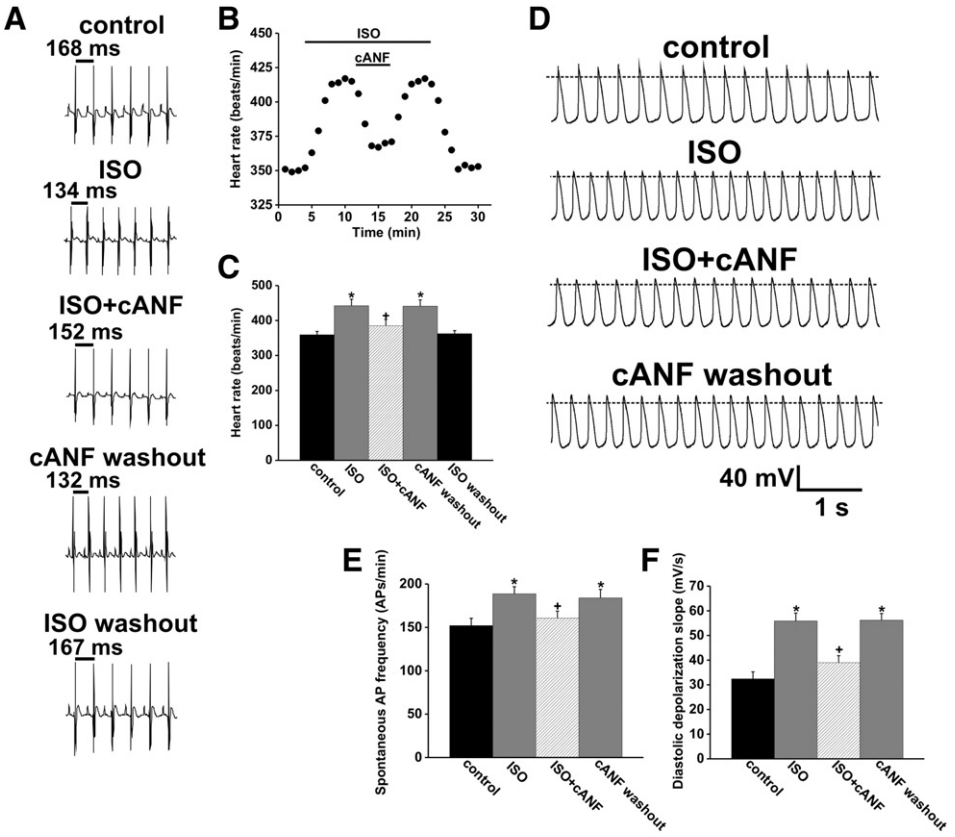


**Fig. 1.** Effects of the NPR-C agonist cANF on heart rate and sinoatrial node function in the presence of 1 μM isoproterenol. (A) Representative ECG recordings (1 s duration) from Langendorff-perfused mouse hearts in control conditions, in the presence of ISO, in the presence of ISO and cANF (500 nM), and following washout of these compounds. R–R intervals are indicated in each condition. (B) Time course of the effects of ISO and cANF on HR. (C) Summary data illustrating the effects of ISO and cANF on HR. Data are means ± SEM; $n = 5$ hearts. (D) Representative spontaneous AP recordings (5 s duration) in isolated SAN myocytes in control conditions, in the presence of ISO (1 μM), in ISO and cANF (100 nM) and following cANF washout. Dotted lines are at 0 mV. (E and F) Summary data illustrating the effects of ISO and cANF on AP frequency and DD slope in SAN myocytes. Data are means ± SEM; $n = 9$ myocytes. $^{*}P<0.05$ vs. control; $^{+}P<0.05$ vs. ISO by one way ANOVA with a Tukey posthoc test.

demonstrate that ISO increased ($P<0.05$) HR from 340 ± 14 to 395 ± 12 beats/min and that subsequent application of BNP (500 nM) decreased ($P<0.05$) HR to 367 ± 11 beats/min. These changes in HR were accompanied by changes in P wave duration and P–R interval (Supplemental Table 6). CNP (500 nM) had very similar negative chronotropic and dromotropic effects to BNP in the presence of ISO (Figs. 3D and E; Supplemental Table 7).

The effects of BNP and CNP (100 nM doses) in the presence of ISO (1 μM) were also studied in isolated SAN myocytes (Fig. 4). As expected ISO increased spontaneous AP frequency and DD slope in SAN myocytes. On average, BNP decreased ($P<0.05$) AP frequency in the presence of ISO from 202 ± 7 to 189 ± 7 APs/min and DD slope from 67.4 ± 2.5 to 53.9 ± 1.8 mV/s (Figs. 4B and C, Supplemental Table 8). Once again, CNP had very comparable effects on AP firing to BNP (Figs. 4D and E; Supplemental Table 8).

### *3.4. Effects of BNP on heart rate in the presence of 1 μM isoproterenol following NPR-A blockade*

Comparison of the effects of BNP, CNP and cANF on HR and AP firing in the presence of a 1 μM dose of ISO shows that the effects of cANF are significantly larger than BNP or CNP (Fig. 6). Specifically, the reduction in HR in Langendorff-perfused hearts elicited by cANF (57 ± 3 beats/min) was approximately 70% larger ($P<0.05$) than BNP (29 ± 4 beats/min) and CNP (31 ± 2 beats/min). Similarly, cANF reduced spontaneous AP frequency in isolated SAN myocytes by 29 ± 2 APs/min, an effect that was approximately twice as large ($P<0.05$) as that elicited by BNP (13 ± 2 APs/min) and CNP (14 ± 2 APs/min).

We hypothesized that these differences were due to the fact that cANF only activates NPR-C, whereas native peptides including BNP and CNP simultaneously activate GC-linked NPR-A and NPR-B receptors as well as NPR-C [2]. To test this hypothesis we measured the effects of BNP on HR (in the presence of 1 μM ISO) following blockade of NPR-A with A71915 (500 nM) [5,24,25].

Figs. 5(A and B) illustrate representative ECG recordings and the time course of the effects of ISO, A71915 and BNP (500 nM) on HR in Langendorff-perfused hearts. On average, HR was increased ($P<0.05$) from 345 ± 13 beats/min in control to 416 ± 12 beats/min in ISO. A71915 had no effect on HR (415 ± 13 beats/min) and application of BNP in the presence of A71915 reduced ($P<0.05$) HR to 369 ± 14 beats/min. These changes in HR were associated with corresponding changes in P wave duration and P–R interval (Supplemental Table 9).

Following NPR-A blockade the reduction in HR elicited by BNP was 46 ± 3 beats/min (Fig. 6A), which was greater ($P<0.05$) than the reduction observed without NPR-A blockade (29 ± 4 beats/min) and indistinguishable ($P = 0.26$) from the effects of cANF (57 ± 3 beats/min). These data indicate that, in the presence of 1 μM ISO, the effects of BNP (and CNP) on HR are mediated by the simultaneous activation of NPR-A (and NPR-B) as well as NPR-C and that following NPR-A blockade BNP can signal exclusively through NPR-C very similarly to cANF. The overall effect of BNP and CNP in these conditions is to decrease HR.

### *3.5. Effects of BNP on heart rate in the presence of 1 μM isoproterenol in NPR-C$^{-/-}$ mice*

To further evaluate the role of NPR-C in mediating the negative chronotropic response to BNP (and CNP) in the presence of 1 μM

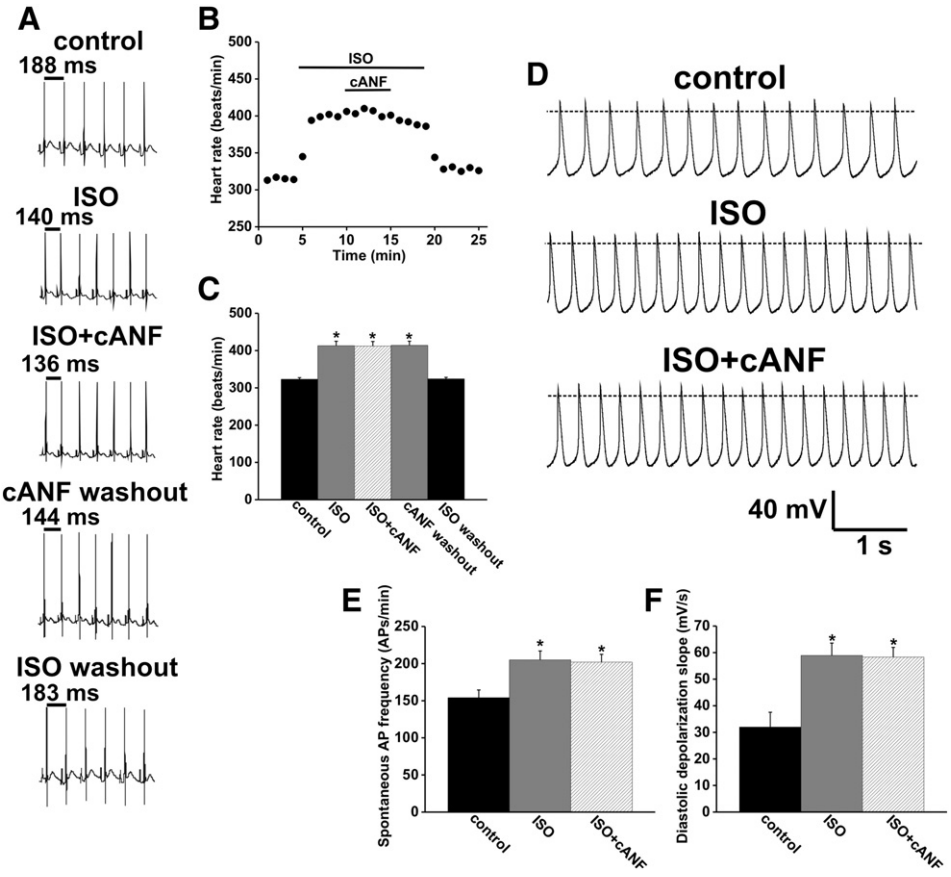


**Fig. 2.** The effects of cANF on heart rate and sinoatrial node function are completely absent in mice lacking functional NPR-C receptors (NPR-C$^{-/-}$). (A) Representative 1 s ECG recordings from Langendorff-perfused hearts illustrating the effects of ISO (1 μM) and cANF (500 nM) in NPR-C$^{-/-}$ mice. (B) Time course of the effects of ISO and cANF on HR in NPR-C$^{-/-}$ hearts. (C) Summary data illustrating that cANF has no effect on HR in the presence of ISO in NPR-C$^{-/-}$ hearts. Data are means ± SEM; $n = 5$ hearts. (D) Representative spontaneous AP recordings (5 s duration) in isolated NPR-C$^{-/-}$ SAN myocytes in control conditions, in the presence of ISO (1 μM) and in ISO and cANF (100 nM). (E and F) Summary data illustrating that cANF has no effect on AP frequency or DD slope in SAN myocytes from NPR-C$^{-/-}$ mice. Data are means ± SEM; $n = 8$ myocytes. *$P<0.05$ vs. control by one way ANOVA with a Tukey posthoc test.

ISO, we measured the effects of BNP on HR in these conditions in NPR-C$^{-/-}$ hearts (Fig. 7). We hypothesized that in the absence of NPR-C receptors BNP would only be able to signal through NPR-A and would increase, rather than decrease HR in the presence of ISO.

Figs. 7(A and B) illustrate representative ECGs and the time course of the effects of ISO (1 μM) and BNP (500 nM) on HR in Langendorff-perfused NPR-C$^{-/-}$ hearts. On average, ISO increased HR from 382 ± 1 to 445 ± 2 beats/min. In complete contrast to wildtype hearts, application of BNP further increased HR in NPR-C$^{-/-}$ hearts to 483 ± 2 beats/min. These changes in HR were associated with reductions in P wave duration and P–R interval (Supplemental Table 10).

These data definitively demonstrate a signaling role for the NPR-C receptor in the heart and clearly show that NPR-C is responsible for the negative chronotropic effect of NPs, which normally dominates the response to BNP in the presence of 1 μM ISO. Conversely, in the absence of NPR-C receptors, BNP only activates NPR-A and further increases HR. Interestingly, the magnitude of the increase in HR in the presence of 1 μM ISO (38 ± 2 beats/min) is smaller ($P<0.05$) than the increase in HR elicited by BNP in basal conditions (89 ± 6 beats/min, Supplemental Fig. S1; see Discussion).

### *3.6. Effects of BNP, CNP and cANF on heart rate and sinoatrial node function in the presence of 10 nM isoproterenol*

Given that our data show that BNP and CNP can switch from eliciting positive chronotropic effects in basal conditions (mediated by NPR-A and NPR-B) to eliciting negative chronotropic effects in the presence of 1 μM doses of ISO (mediated by NPR-C as well as NPR-A/B) we investigated when and how this switch takes place. This was done by measuring the effects of BNP, CNP and cANF on HR and SAN function in the presence of a lower dose of ISO (10 nM). Supplemental Fig. S4 illustrates the effects of BNP and CNP (500 nM) on HR in isolated hearts in these conditions. On average, HR was increased ($P<0.05$) from 343 ± 9 to 389 ± 9 beats/min in 10 nM ISO. Application of BNP further increased ($P<0.05$) HR to 429 ± 7 beats/min. The effects of CNP were very similar to BNP. Analysis of additional ECG parameters is presented in Supplemental Tables 11 and 12.

We also measured the effects of BNP (100 nM) on spontaneous AP firing in isolated SAN myocytes in the presence of 10 nM doses of ISO (Supplemental Fig. S5). Consistent with the isolated heart, AP frequency was increased ($P<0.05$) from 139 ± 5 to 165 ± 7 APs/min in 10 nM ISO. Subsequent application of BNP further increased ($P<0.05$) AP frequency to 188 ± 8 APs/min. These increases in AP frequency were associated with increases in DD slope, but no change in MDP (Supplemental Fig. S5, Supplemental Table 13).

These data show that, like in basal conditions, BNP and CNP increase HR in the presence of 10 nM ISO; however, the magnitude of these increases is smaller ($P<0.05$). In basal conditions, BNP and CNP increased HR by 89 ± 6 and 90 ± 13 beats/min, respectively; whereas in the presence of 10 nM ISO, BNP and CNP only increased HR by 41 ± 5 and 40 ± 1 beats/min (compare Supplemental Fig. S1 to Supplemental Fig. S4). Once again, we hypothesized that the basis for this difference was that, in the presence of 10 nM ISO, NPs can activate NPR-C, which elicits effects on HR in the opposite

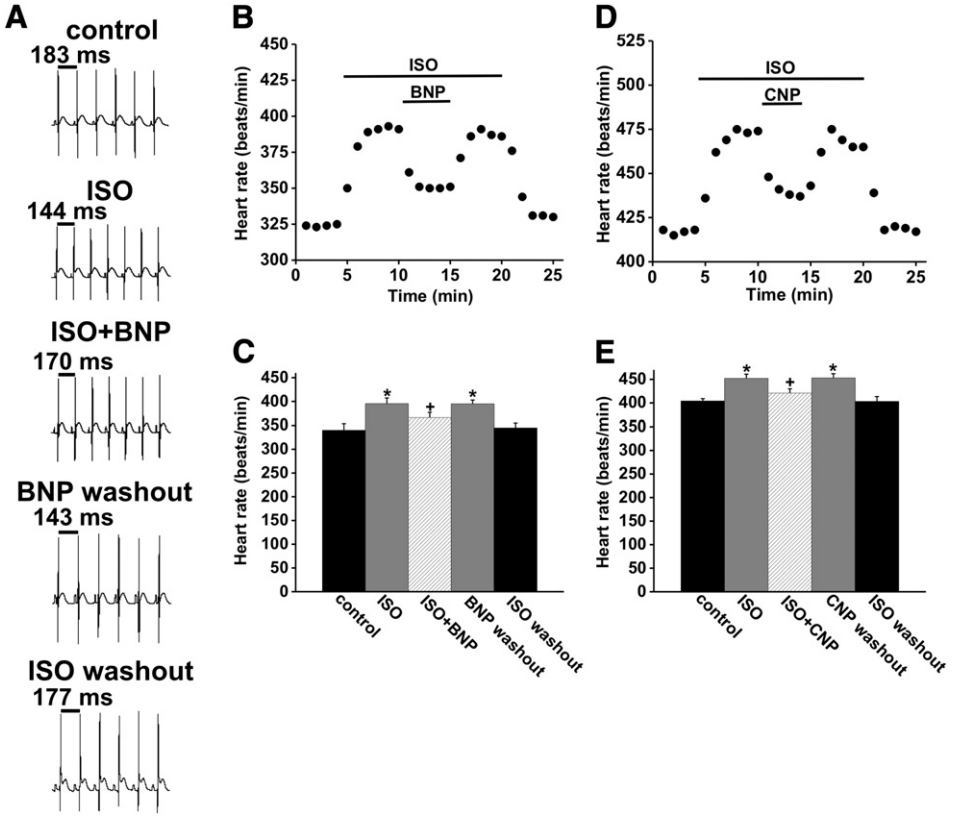


**Fig. 3.** Effects of BNP and CNP on heart rate in the presence of 1 μM isoproterenol. (A) Representative 1 s ECG recordings illustrating the effects of ISO and BNP (500 nM) on Langendorff-perfused mouse hearts. (B) Time course of the effects of ISO and BNP on HR. (C) Summary data illustrating the effects of ISO and BNP on HR. Data are means ± SEM; $n = 5$ hearts. (D) Time course of the effects of ISO and CNP (500 nM) on HR. (E) Summary data illustrating the effects of ISO and CNP on HR. Data are means ± SEM; $n = 5$ hearts. $^{*}P<0.05$ vs. control; $^{+}P<0.05$ vs. ISO by one way ANOVA with a Tukey posthoc test.

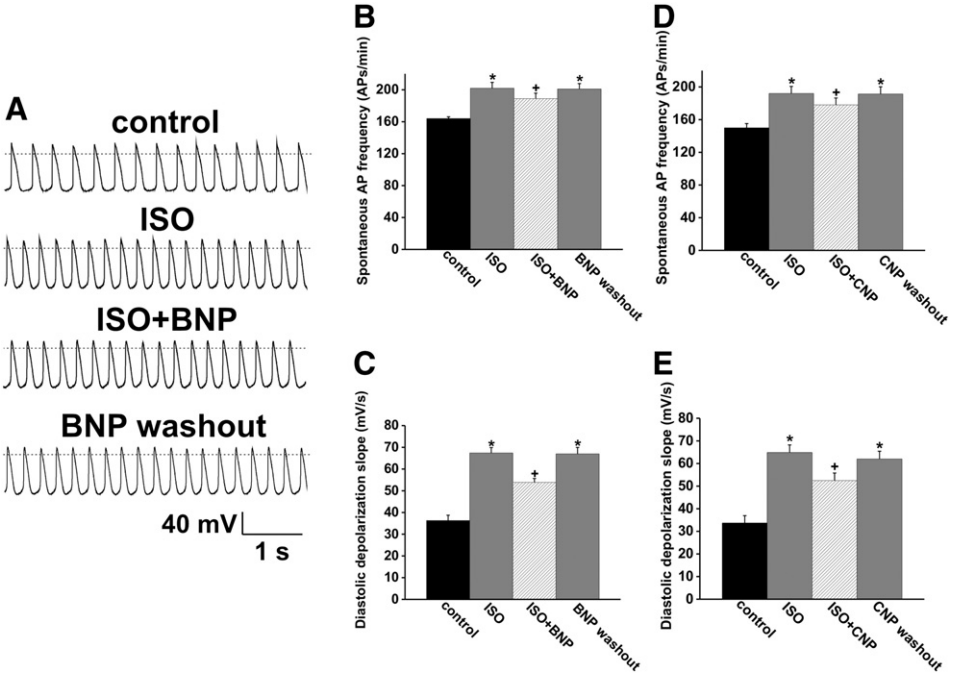


**Fig. 4.** Effects of BNP and CNP on spontaneous action potential firing in isolated mouse sinoatrial node myocytes in the presence of 1 μM isoproterenol. (A) Representative spontaneous AP recordings (5 s duration) in control conditions, in the presence of ISO, in ISO and BNP (100 nM), and following BNP washout. (B and C) Summary data illustrating the effects of ISO and BNP on AP frequency and DD slope in isolated SAN myocytes. Data are means ± SEM; $n = 6$ myocytes. (D and E) Summary data illustrating the effects of ISO and CNP (100 nM) on AP frequency and DD slope in isolated SAN myocytes. Data are means ± SEM; $n = 6$ myocytes. $^{*}P<0.05$ vs. control; $^{+}P<0.05$ vs. ISO by one way ANOVA with a Tukey posthoc test.

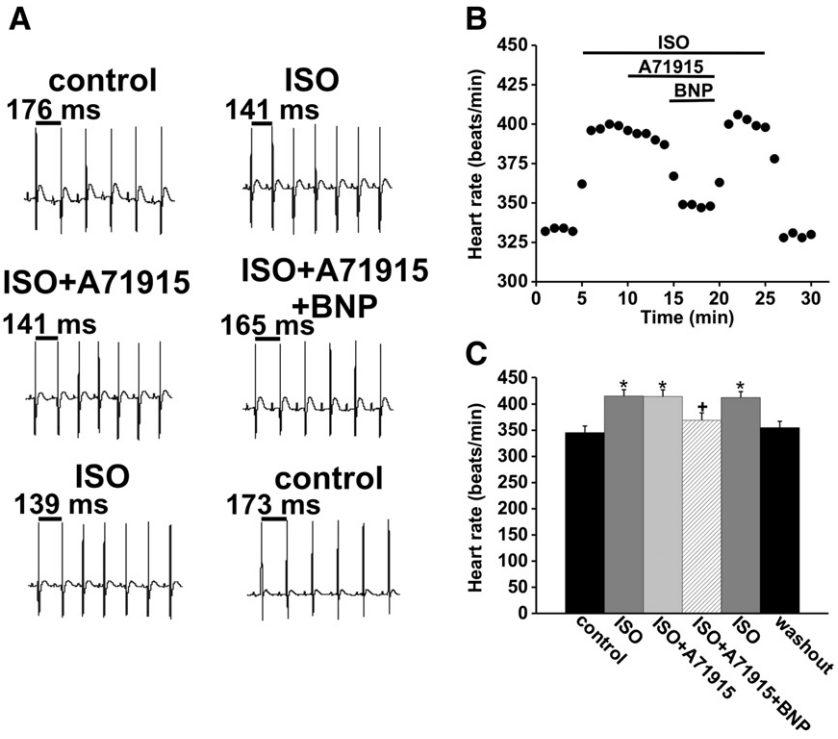


**Fig. 5.** Effects of BNP on heart rate in the presence of 1 μM isoproterenol following blockade of natriuretic peptide receptor A. (A) Representative 1 s ECG recordings illustrating the effects of ISO and BNP (500 nM) on Langendorff-perfused mouse hearts during NPR-A blockade with A71915 (500 nM). (B) Time course of the effects of ISO, A71915 and BNP on HR. (C) Summary data illustrating the effects of ISO, A71915 and BNP on HR. Data are means ± SEM; $n = 5$ hearts. *$P<0.05$ vs. control; $^{+}P<0.05$ vs. ISO + A71915 by one way ANOVA with a Tukey posthoc test.

direction to NPR-A and NPR-B. To confirm that NPR-C can be activated in these conditions, we measured the effects of selective NPR-C activation with cANF on HR and AP firing in the presence of 10 nM ISO (Supplemental Fig. S6). On average ISO (10 nM) increased ($P<0.05$) HR from 344 ± 7 to 391 ± 8 beats/min. cANF (500 nM) counteracted the effects of ISO and decreased ($P<0.05$) HR to 349 ± 9 beats/min. Associated changes in ECG intervals are shown in Supplemental Table 14.

Similarly, ISO (10 nM) increased ($P<0.05$) AP frequency in isolated SAN myocytes from 146 ± 7 to 171 ± 6 APs/min and subsequent application of cANF (100 nM) reduced ($P<0.05$) AP frequency to 157 ± 5 APs/min (Supplemental Fig. S6). These changes occurred in conjunction with changes in DD slope (31 ± 2 mV/s in control, 48.8 ± 3.4 mV/s in ISO, 38.7 ± 2.7 mV/s after cANF; Supplemental Fig. S6, Supplemental Table 15). These data show that NPR-C can be activated in the presence of 10 nM doses of ISO and that the overall effect of native NPs including BNP and CNP in these conditions is to increase HR and SAN AP frequency, albeit to a smaller extent than in basal conditions.

To definitely prove that the negative chronotropic effect mediated by NPR-C opposes the positive chronotropic effect mediated by NPR-A (and NPR-B) in 10 nM ISO we measured the effects of BNP (500 nM) on HR in these conditions using NPR-$C^{-/-}$ hearts (Supplemental Fig. S7 and Supplemental Table 16). HR was increased ($P<0.05$) from 351 ± 4 beats/min in control to 387 ± 3 beats/min in ISO and subsequent application of BNP further increased ($P<0.05$) HR to 457 ± 4 beats/min. Associated changes in ECG intervals are shown in Supplemental Table 16. The increase in HR elicited by BNP in NPR-$C^{-/-}$ hearts (70.1 ± 6.2 beats/min) was larger ($P<0.05$) than in NPR-$C^{+/+}$ hearts (40.5 ± 5.3 beats/min). Together, these data demonstrate that BNP (and CNP) activate NPR-A/B and as well as NPR-C in the presence of 10 nM ISO and that the NPR-C mediated effects oppose the NPR-A/B mediated effects.

Finally, we compared the magnitude of the effects of selective NPR-C activation with cANF (100–500 nM) on HR and AP firing in 10 nM and 1 μM doses of ISO (Supplemental Fig. S6). In the presence of 10 nM ISO cANF reduced HR by 42 ± 3 beats/min and AP frequency by 14 ± 2 AP/min. The effects of cANF were larger ($P<0.05$) in the presence of 1 μM ISO whereby HR was reduced by 57.3 ± 6 beats/min and AP frequency was reduced by 28 ± 3 AP/min. Thus, NPR-C-mediated effects in the SAN increase in magnitude as the level of β-AR activation increases, which may partially explain why NPs switch from eliciting increases in HR in basal conditions and 10 nM ISO to decreases in HR in 1 μM ISO (see Discussion).

## 4. Discussion

The NPR-C receptor has been highly controversial. Most previous studies attributing a signaling role to NPR-C have relied on pharmacological approaches and it has been suggested that proof of this phenomenon requires the demonstration that NPR-C-dependent effects are absent in mice lacking functional NPR-C receptors [2]. We have now, for the first time, provided this evidence in the heart. Our data clearly demonstrate that cANF decreases HR and SAN activity and that these effects are completely absent in NPR-$C^{-/-}$ hearts. This provides definitive proof of a signaling role for NPR-C in the heart and also confirms that cANF functions as a selective agonist of the NPR-C receptor. We further demonstrate that the negative chronotropic response elicited by BNP in the presence of 1 μM ISO is lost in NPR-$C^{-/-}$ hearts thereby proving that native NPs inhibit SAN function via NPR-C.

Having proven a signaling role for NPR-C in the SAN, we next sought to determine how NPR-C works in conjunction with NPR-A and NPR-B to determine the overall effects of NPs on chronotropic status since native NPs can simultaneously activate the GC-linked NPRs and NPR-C [2]. These interactions are very poorly understood, yet are critical to our understanding of NP signaling in the heart. Our prior work demonstrates that selective activation of NPR-C with cANF can decrease HR in the presence of 1 μM ISO [6], but in basal

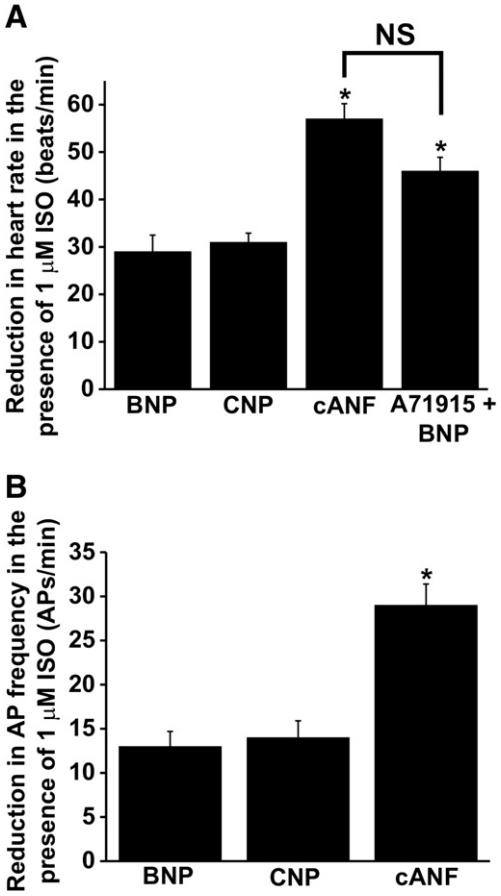


**Fig. 6.** Comparison of the effects of BNP, CNP and cANF on heart rate and spontaneous action potential frequency in the presence of a 1 μM dose of isoproterenol. (A) Average reductions in heart rate in Langendorff-perfused mouse hearts caused by BNP, CNP, cANF and BNP + A71915 (NPR-A blocker). Each peptide was applied at a concentration of 500 nM following application of ISO (1 μM). Data are means ± SEM; $n = 5$–8 hearts in each group. (B) Average reductions in AP frequency in isolated SAN myocytes caused by BNP, CNP and cANF. Each peptide was applied at a concentration of 100 nM in the presence of ISO (1 μM). Data are means ± SEM; $n = 6$ myocytes for BNP, 6 myocytes for CNP and 9 myocytes for cANF. $^{*}P<0.05$ vs. BNP and CNP; NS, not significant ($P = 0.26$) by one way ANOVA with a Tukey posthoc test.

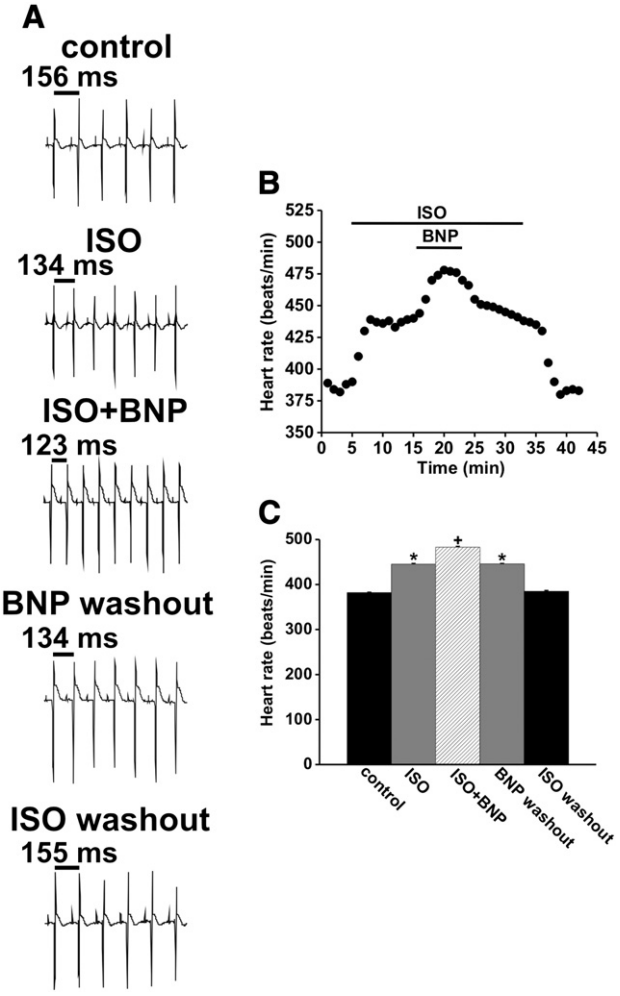


**Fig. 7.** Effects of BNP on heart rate in the presence of 1 μM isoproterenol in NPR-$C^{-/-}$ mice. (A) Representative 1 s ECG recordings illustrating the effects of ISO (1 μM) and BNP (500 nM) on Langendorff-perfused mouse hearts from NPR-$C^{-/-}$ mice. (B) Time course of the effects of ISO and BNP on HR. (C) Summary data illustrating the effects of ISO and BNP on HR. Data are means ± SEM; $n = 5$ hearts. $^{*}P<0.05$ vs. control; $^{+}P<0.05$ vs. ISO by one way ANOVA with a Tukey posthoc test.

conditions NPs, including BNP and CNP increase HR via NPR-A and NPR-B (Supplemental Figs. 1 and 2) [5]; however, it was not known how each of these mechanisms contributes to the overall effect(s) of native NPs in different physiological conditions.

Our data demonstrate that in basal conditions BNP and CNP increase HR and SAN activity exclusively via NPR-A and NPR-B activation. In the present study we show that cANF has no effect on HR or SAN AP firing in basal conditions indicating that NPR-C-dependent signaling mechanisms do not elicit chronotropic effects in this setting. Consistent with this conclusion we have shown that the increases in HR elicited by BNP and CNP in basal conditions are indistinguishable in wildtype and NPR-$C^{-/-}$ mice and are fully blocked by an NPR-A antagonist [5]. Interestingly, BNP and CNP also increase HR and AP frequency in the SAN in the presence of 10 nM doses of ISO; however, the magnitude of these increases is smaller than in basal conditions. This is at least partially because in these conditions NPR-C is also activated as evidenced by the ability of cANF to decrease HR and SAN AP frequency in the presence of 10 nM ISO. This conclusion is further strongly supported by the finding that the positive chronotropic effect of BNP in 10 nM ISO is significantly larger in NPR-$C^{-/-}$ mice compared to NPR-$C^{+/+}$ mice. These findings show that NPR-A and NPR-B mediate increases in HR and SAN activity whereas NPR-C mediates a decrease in HR and SAN activity and the two pathways can counteract each other.

We also explored the roles of each NPR subtype in the presence of high doses of ISO (1 μM). Surprisingly, in these conditions, BNP and CNP switched to causing decreases in HR and SAN AP frequency. Once again, comparison of these effects with cANF shows that cANF elicits significantly larger negative chronotropic effects than BNP and CNP (refer to Fig. 6), suggesting that the native NPs are signaling via multiple NPRs simultaneously. We confirmed that this is the case by applying BNP in the presence of A71915, a well characterized NPR-A antagonist [5,24,25]. With NPR-A blocked BNP only activates NPR-C and was able to reduce HR and SAN activity as effectively as cANF. We also measured the effects of BNP on HR (in the presence of 1 μM ISO) in NPR-$C^{-/-}$ hearts. Without NPR-C receptors, BNP can only activate NPR-A and switches back to eliciting an increase in HR. Together these data strongly support the conclusion that native NPs decrease HR via NPR-C, which clearly has a signaling role in the heart. These data further demonstrate that, in the presence of ISO, native NPs can activate multiple NPRs simultaneously and that each receptor subtype contributes to the overall effect of NPs on HR and SAN function.

The ability of BNP and CNP to switch from eliciting increases in HR and SAN activity (in basal conditions and 10 nM doses of ISO) to eliciting decreases in HR and SAN activity (in 1 μM doses of ISO) was surprising. Careful analysis of our data provides insight into the

factors that enable this switch to take place. Firstly, the magnitude of the positive chronotropic effects of BNP and CNP (mediated by NPR-A and NPR-B [5]) are more than twice as large in basal conditions (~90 beats/min) than in NPR-C$^{-/-}$ hearts in the presence of 1 μM ISO (~40 beats/min; compare Supplemental Fig. S1 to Fig. 7). This difference is also evident when comparing the reductions in HR elicited by BNP in 1 μM ISO with and without NPR-A blockade (~30 beats/min vs. ~50 beats/min; Fig. 6). The difference between these two values gives an indication of the contribution of NPR-A to the change in HR in the presence of 1 μM ISO and is comparable to the increase in HR elicited by BNP in 1 μM ISO in NPR-C$^{-/-}$ hearts. Similarly, the increase in HR elicited by BNP in the presence of 10 nM ISO in NPR-C$^{-/-}$ hearts (~70 beats/min) is comparable to the increase in HR elicited by BNP and CNP in basal conditions (~90 beats/min) and larger than the increase observed in NPR-C$^{-/-}$ hearts in the presence of 1 μM ISO (~40 beats/min). Thus, the ability of NPs to increase HR via NPR-A/B is reduced in the presence of high doses of ISO.

Secondly, the role of NPR-C, as indicated by the magnitude of the effects of cANF on HR and SAN activity, clearly increases as the concentration of ISO increases. Our data demonstrate that NPR-C plays no role in basal conditions, and that the negative chronotropic effect mediated by NPR-C is significantly larger in high (1 μM) doses of ISO compared to 10 nM doses of ISO. Together our data show that the NPR-A and NPR-B mediated effects (which are to increase SAN activity) contribute in all conditions, whereas the NPR-C mediated effects (which are to decrease SAN activity) only contribute in the setting of enhanced β-AR activation. As the concentration of ISO increases, the relative contributions of NPR-A/B decrease while the relative contribution of NPR-C increases. This may explain the ability of BNP and CNP to switch from eliciting positive chronotropic effects to negative chronotropic effect as the level of β-AR activation increases. These data also suggest that the effects of NPs in the heart *in vivo* (endogenously produced or when delivered to heart failure patients) will be dependent on the level of autonomic nervous system tone and other factors that impact the amount of adenylyl cyclase activity in the SAN.

The changes in spontaneous AP frequency elicited by BNP and CNP in SAN myocytes occurred in conjunction with changes in the DD slope. Numerous ionic currents contribute to the DD slope (a major determinant of SAN AP frequency and HR [4,26–28]). These include the hyperpolarization activated current $I_f$, a T-type $Ca^{2+}$ current ($I_{Ca,T}$), $I_{Ca,L}$, a $Na^+$–$Ca^{2+}$ exchange current ($I_{NCX}$) activated downstream of sarcoplasmic reticulum (SR) $Ca^{2+}$ release, a delayed rectifier $K^+$ current ($I_{Kr}$), as well as neuronal and cardiac $Na^+$ channels ($I_{Na}$) [4,28]. Recently it has been demonstrated that the SAN expresses the $Ca_V1.3$ form of L-type $Ca^{2+}$ channel, which activates at more negative membrane potentials than $Ca_V1.2$ enabling $I_{Ca,L}$ to contribute prominently to the DD slope (and thus HR regulation) in addition to the AP upstroke [4,29,30].

Our prior studies [5,6] have identified $I_f$ and/or $I_{Ca,L}$ as critical ionic currents modulated downstream of NPRs in SAN myocytes that affect DD slope and cause changes in HR. Activation of NPR-C is known to activate $G_i$ proteins and inhibit adenylyl cyclase activity [11,13,14]. We demonstrated that cANF as well as a synthetic $G_i$-activator peptide (corresponding to the 16 amino acid region of NPR-C that activates $G_i$) inhibited $I_{Ca,L}$ in SAN myocytes due to a reduction of cAMP dependent phosphorylation of the channel by protein kinase A (PKA) [6]. More recently we have shown that the GC-linked NPR-A and NPR-B receptors (activated by BNP and CNP) alter SAN function by stimulating $I_{Ca,L}$ and $I_f$ [5]. These ionic effects occurred due to a cGMP-dependent inhibition of phosphodiesterase 3 (PDE3), which decreases cAMP hydrolysis leading to an elevation of cAMP levels. Thus, all three NPRs have the capacity to modulate cAMP levels (and cAMP dependent ion channels) in the SAN, either due to direct alterations in adenylyl cyclase activity (NPR-C, decrease cAMP) [6,13] or via a cross-talk mechanism involving cGMP and PDE3 (NPR-A and NPR-B, increase cAMP) [5,31].

These ionic and molecular mechanisms explain how NPs can elicit positive or negative chronotropic effects in different conditions, although other cAMP-dependent currents may also contribute. For example, the SR $Ca^{2+}$ release/$I_{NCX}$ mechanism and $I_{Kr}$ have both been shown to be cAMP sensitive [28,32,33]. Also, it has recently been demonstrated that $I_{Na}$ (neuronal tetrodotoxin-sensitive and cardiac tetrodotoxin insensitive forms) contribute to SAN automaticity and HR regulation in mice [34–36] and rabbits [37,38]. Furthermore, both neuronal and cardiac $Na^+$ channels are regulated by cAMP and PKA [39–42]. Whether any of these additional cAMP-sensitive mechanisms are modulated by NPs is not known.

We have also previously demonstrated effects of NPs on cardiac fibroblasts [43]. Specifically, CNP and cANF activated a nonselective cation conductance, likely carried by transient receptor potential C (TRPC) channels, following activation of NPR-C. The SAN is characterized by a high density of fibroblasts [44], which may couple to myocytes and impact their electrical activity as has been demonstrated previously in the heart [45,46]. Further studies are needed to determine the interactions between myocytes and fibroblasts in the SAN and how they are modulated by NPs to affect electrical activity. Interestingly, it has recently been shown that TRPC channels are also present in SAN myocytes where they may contribute to regulation of spontaneous activity in the SAN [47]; however, it is unknown at present whether TRPC channels in SAN myocytes are modulated by NPs as we have shown in cardiac fibroblasts [43].

Some limitations of our study should be considered. Firstly, our ECG recordings were performed in isolated Langendorff-perfused hearts. The Langendorff preparation is well validated and provides an excellent indication of intrinsic SAN activity independent of the autonomic nervous system and circulating neurohumoral compounds that modulate HR *in vivo*. Nevertheless, the isolated heart preparation also has limitations, including variability in baseline function and heart rates lower than those seen *in vivo* [48]. Despite these limitations, the effects of BNP and CNP we observed in both isolated hearts and SAN myocytes were very comparable and clearly demonstrate the ability of NPs to modulate spontaneous SAN activity, HR and electrical conduction. In future studies it will be important to study these effects *in vivo* with an intact autonomic nervous system, which may also be affected by NPs [49,50] and contribute to the effects of NPs on HR regulation and SAN activity.

Secondly, our experiments have been performed using BNP and CNP at doses between 10 and 500 nM. These doses may be higher than those typically present in the general circulation in normal physiological conditions [2]; however, several factors must be considered. NPs are produced in the myocardium and released into the circulation in the heart thus the local NP concentrations within the heart may be much higher than those present in the general circulation. Indeed it has been hypothesized that NPs can have important autocrine/paracrine effects in the tissues they are produced in including the heart [51–54]. Furthermore, the production and the resultant circulating concentrations of NPs are profoundly elevated in heart failure [2,55]. Heart failure patients (New York Heart Association class III and IV) can have plasma BNP levels in the low nanomolar range [49,56] and with the enormous increase in NP production in the atrial and ventricular myocardium [57] it is likely that NP concentrations within the coronary circulation, including that reaching the SAN, are even higher. Interestingly, it has been shown that injection of low doses of CNP into the SAN artery in dogs increases SAN activity [58]. Furthermore, CD-NP, a chimeric NP currently in development for treatment of heart failure [17], was found to significantly elevate HR in humans by 15–30% when used at therapeutically relevant doses (10–25 ng/kg/min over 4 h by intravenous infusion) [59]. Thus, our findings in isolated heart and SAN myocytes are likely relevant *in vivo* in the setting of elevated NP levels in heart disease and/or during the use of synthetic NPs as a therapeutic intervention. Further work will be needed to understand how bolus and/or sustained infusions of NPs affect SAN activity

and electrical conduction *in vivo* and what the physiological impacts of these effects are in heart disease.

In summary, we have provided definitive proof of a signaling role for NPR-C in the heart and shown the conditions in which each NPR contributes to the regulation of HR and SAN function. Clearly, the effects of NPs in the heart are complex; however, our experiments provide critical novel insight into how the GC linked NPRs (NPR-A and NPR-B) as well as NPR-C can each contribute to the regulation of HR and SAN activity in different physiological conditions. Our data demonstrate the importance of considering the roles of each NPR subtype when interpreting the effects of NPs on cardiac function and should be considered in the context of using NPs in the treatment of cardiovascular disease.

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## Disclosure statement

None.

## Acknowledgments

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2012.08.020.

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# Natriuretic peptides regulate heart rate and sinoatrial node function by activating multiple natriuretic peptide receptors

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**Online Supplement**

## Supplemental Methods

### Animals

This study utilized male wildtype C57Bl/6 mice (Charles River) and mutant mice lacking functional NPR-C receptors (NPR-$C^{-/-}$). The NPR-$C^{-/-}$ mice were obtained from The Jackson Laboratory (strain B6;C-*Npr3*$^{lgj}$/J) and backcrossed into the C57Bl/6 line for more than 10 generations. This mouse contains a 36 base pair deletion (position 195-232) that results in the production of a nonfunctional NPR-C protein truncated by 12 amino acids in the extracellular domain [1]. All mice used in experiments were male and between the ages of 10-14 weeks. NPR-$C^{-/-}$ mice were genotyped using ear punch biopsies. All experimental procedures were in accordance with the regulations of The Canadian Council on Animal Care and were approved by the Dalhousie University animal care committee.

### Electrocardiography in isolated hearts

ECGs were recorded in isolated Langendorff-perfused hearts as we have described previously [2-4]. In brief, mice were administered a 0.2 ml intraperitoneal injection of heparin (1000 IU/ml) to prevent blood clotting. Following this, mice were anesthetized by isoflurane inhalation and then killed by cervical dislocation. The heart was excised into Tyrode's solution (37ºC) consisting of (in mmol/L) 140 NaCl, 5.4 KCl, 1.2 $KH_2PO_4$, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 5.55 glucose, and 5 HEPES, with pH adjusted to 7.4 with NaOH and cannulated via the aorta for

retrograde aortic perfusion. Hearts were perfused with oxygenated Tyrode's solution at a flow rate of 2.5 ml/min and allowed to stabilize for at least 10 min.

ECGs were acquired using needle electrodes (Grass Technologies), a Gould ACQ-7700 amplifier and the Ponemah Physiology Platform software (Data Sciences International). Standard ECG waves and intervals, including R-R interval, P wave duration, P-R interval, and Q-T interval were analyzed.

**Isolation of mouse sinoatrial node and right atrial myocytes**

The procedures for isolating single pacemaker myocytes from the mouse sinoatrial node (SAN) have been described previously [5-8] and were as follows. Mice were administered a 0.2 ml intraperitoneal injection of heparin (1000 IU/ml) to prevent blood clotting. Following this, mice were anesthetized by isoflurane inhalation and then killed by cervical dislocation. The heart was excised into Tyrode's solution (35ºC) consisting of (in mmol/L) 140 NaCl, 5.4 KCl, 1.2 $KH_2PO_4$, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 5.55 glucose, and 5 HEPES, with pH adjusted to 7.4 with NaOH. The sinoatrial node (SAN) region of the heart was isolated by separating the atria from the ventricles, cutting open the superior and inferior venae cavae, and pinning the tissue so that the crista terminalis could be identified. The SAN area is located in the intercaval region adjacent to the crista terminalis. This SAN region was cut into strips, which were transferred and rinsed in a 'low $Ca^{2+}$, $Mg^{2+}$ free' solution containing (in mmol/L) 140 NaCl, 5.4 KCl, 1.2 $KH_2PO_4$, 0.2 $CaCl_2$, 50 taurine, 18.5 glucose, 5 HEPES and 1 mg/ml bovine serum albumin (BSA), with pH adjusted to 6.9 with NaOH. SAN tissue strips were digested in 5 ml of 'low $Ca^{2+}$, $Mg^{2+}$ free' solution containing collagenase (type II, Worthington Biochemical Corporation), elastase (Worthington Biochemical Corporation) and protease (type XIV, Sigma Chemical Company) for

30 min. Then the tissue was transferred to 5 ml of modified KB solution containing (in mmol/L) 100 potassium glutamate, 10 potassium aspartate, 25 KCl, 10 $KH_2PO_4$, 2 $MgSO_4$, 20 taurine, 5 creatine, 0.5 EGTA, 20 glucose, 5 HEPES, and 0.1% BSA, with pH adjusted to 7.2 with KOH. The tissue was mechanically agitated using a wide-bore pipette. This procedure yielded individual SAN myocytes with cellular automaticity that was recovered after readapting the cells to a physiological concentration of $Ca^{2+}$.

SAN myocytes were identified by their small spindle shape and ability to beat spontaneously in the recording chamber when superfused with normal Tyrode's solution. When patch-clamped, SAN myocytes always displayed spontaneous action potentials. The capacitance of single SAN myocytes was 20 – 35 pF.

**Solutions and electrophysiological protocols**

Spontaneous action potentials (APs) were recorded using the perforated patch-clamp technique [9] on single SAN myocytes. APs were recorded at room temperature (22-23 ºC), which must be noted when comparing these data to the isolated heart ECG experiments.

For recording APs the recording chamber was superfused with a normal Tyrode's solution containing (in mmol/L) 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, and 5 glucose, with pH adjusted to 7.4 with NaOH. The pipette filling solution contained (in mmol/L) 135 KCl, 0.1 $CaCl_2$, 1 $MgCl_2$, 5 NaCl, 10 EGTA, 4 Mg-ATP, and 10 HEPES, with pH adjusted to 7.2 with KOH. Amphotericin B (200 µg/ml) was added to this pipette solution to record APs with the perforated patch clamp technique.

Micropipettes were pulled from borosilicate glass (with filament, 1.5 mm OD, 0.75 mm ID, Sutter Instrument Company) using a Flaming/Brown pipette puller (model p-87, Sutter

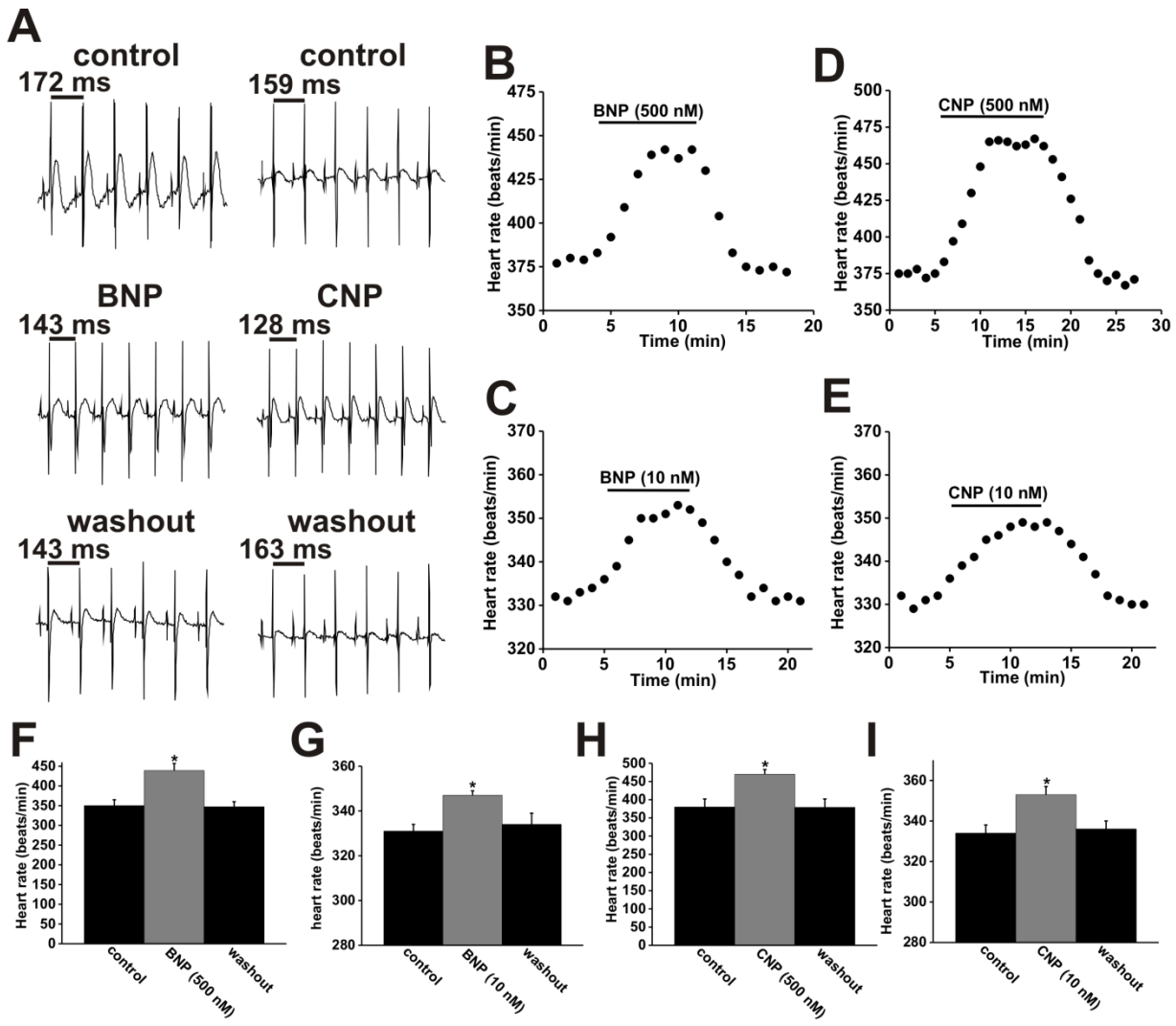
Instrument Company). The resistance of these pipettes was 4 – 8 MΩ when filled with recording solution. Micropipettes were positioned with a micromanipulator (Burleigh PCS-5000 system) mounted on the stage of an inverted microscope (Olympus IX71). Seal resistance was 2 – 15 GΩ. For perforated patch clamp experiments access resistance was monitored for the development of capacitative transients upon sealing to the cell membrane with Amphotericin B in the pipette. Typically, access resistance became less than 25 MΩ within 5 min of sealing onto the cell, which was sufficient for recording spontaneous APs in current clamp mode. Data were digitized using a Digidata 1440 and pCLAMP 10 software (Molecular Devices) and stored on computer for *post hoc* analysis.

Spontaneous AP parameters, including the maximum diastolic potential (MDP), the slope of the diastolic depolarization (DD slope), the maximum AP upstroke velocity (Vmax), the AP overshoot and the AP duration at 50% repolarization (APD50) were analyzed as described previously [6,10]. The DD slope was measured by fitting a straight line to the linear portion of this AP component [10].

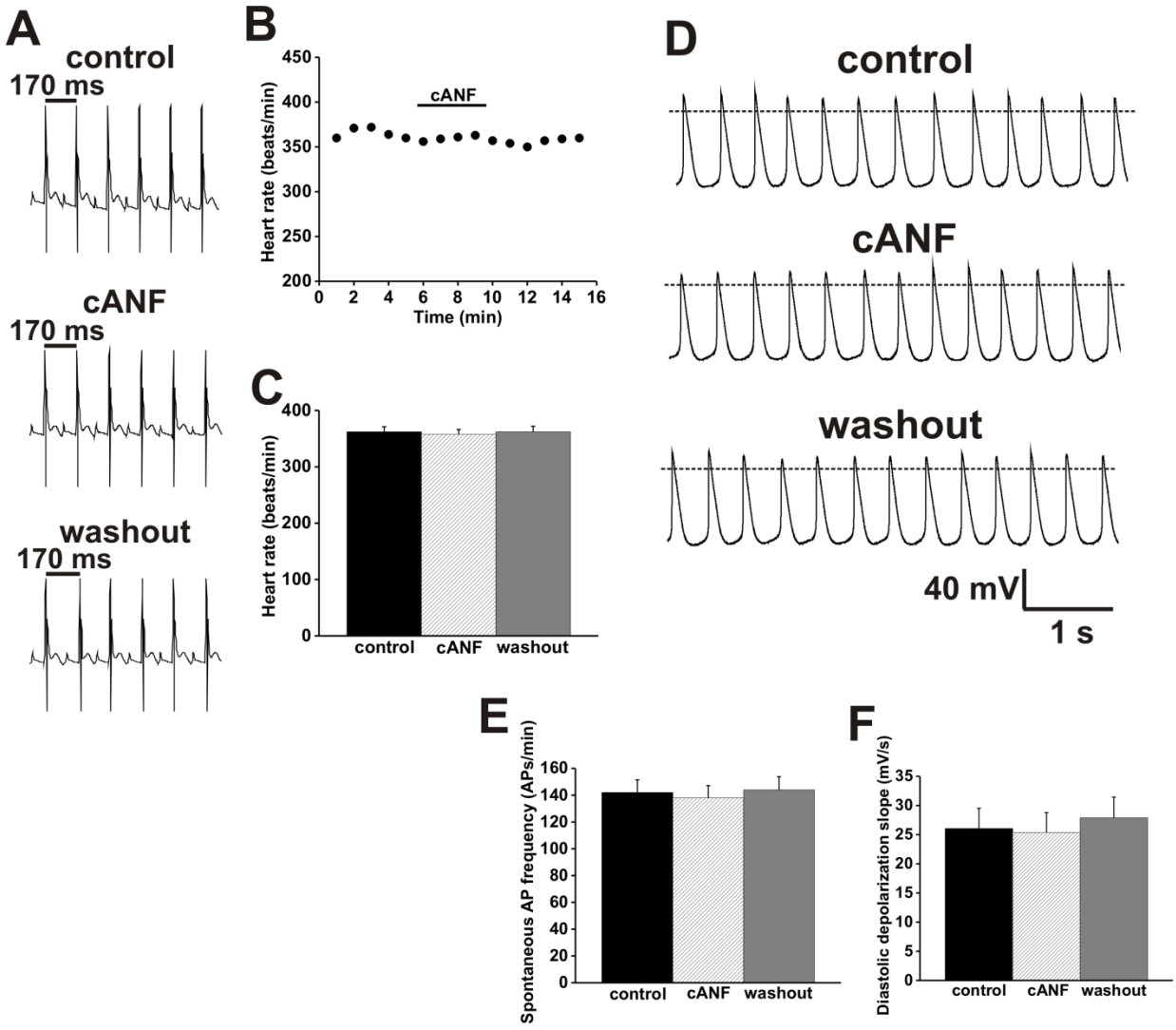
**Pharmacological compounds**

BNP, CNP, cANF and A71915 were obtained from Bachem, dissolved in water, and stored in small aliquots (-80°C) until added to Tyrode's solution for experimental use. Isoproterenol was obtained from Sigma Chemical Company and dissolved in water before being added to Tyrode's solution during experimental use.

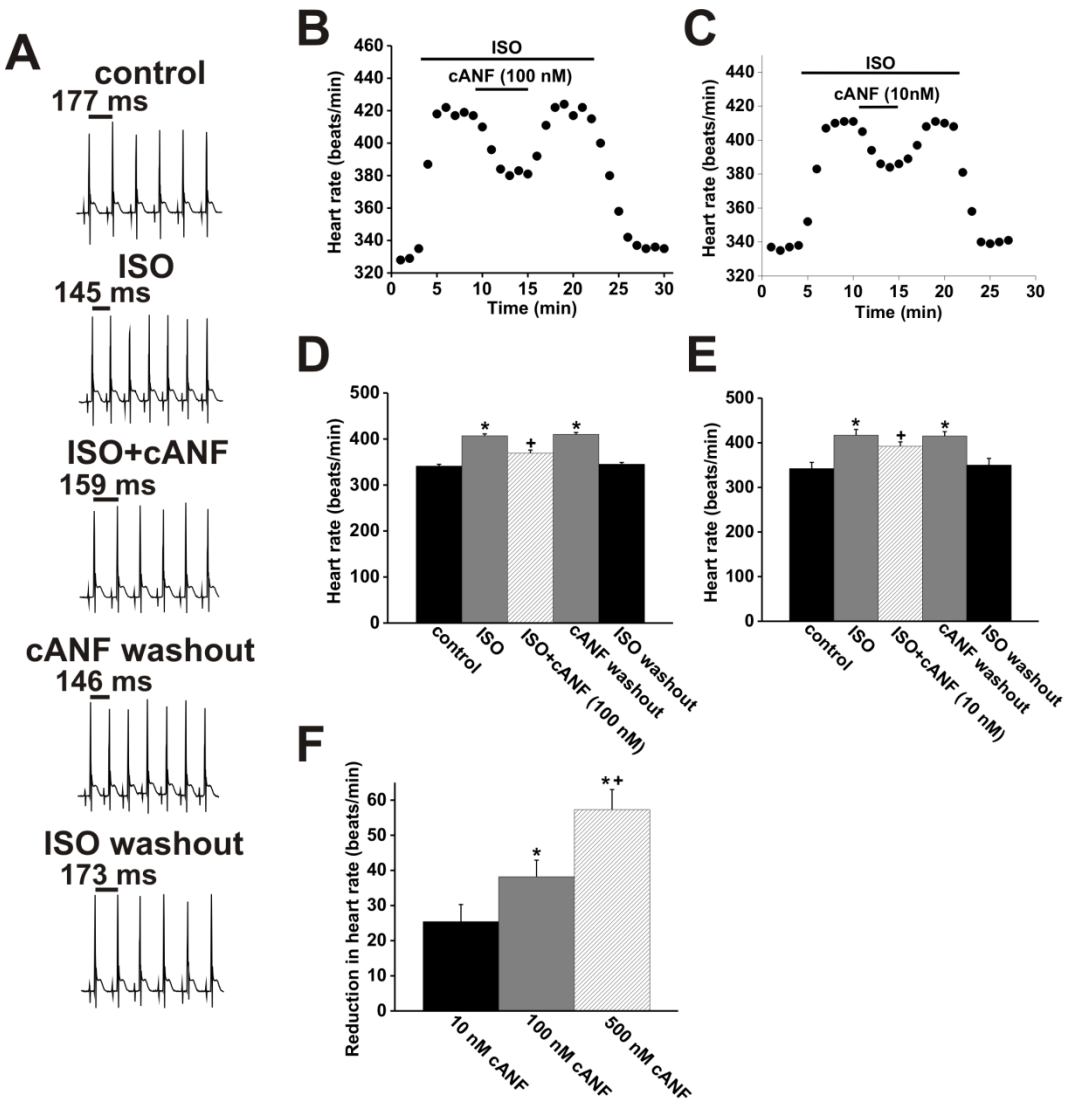
**Supplemental Results**



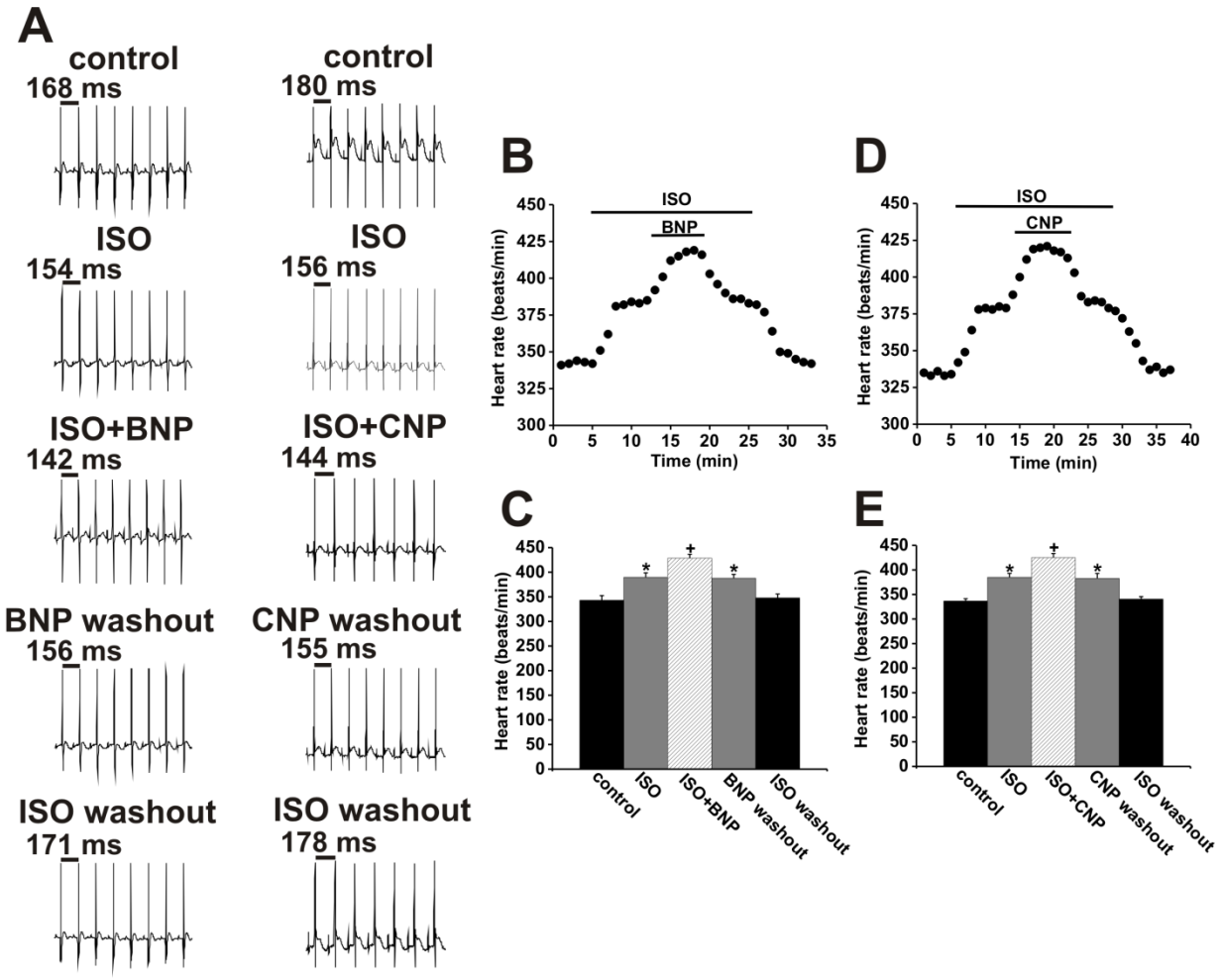

**Supplemental Figure S1:** Effects of BNP and CNP on heart rate in basal conditions. (A) Representative ECG recordings (1 s duration) illustrating the effects of BNP (500 nM) and CNP (500 nM) on Langendorff-perfused mouse hearts. R-R intervals are indicated for each condition. (B-E) Time course of the effects of BNP (500 nM and 10 nM) and CNP (500 nM and 10 nM) on HR. (F-I) Summary data illustrating effect of BNP (500 nM and 10 nM) and CNP (500 nM and 10 nM) on HR. Data are means ± SEM; $n$=5 hearts in each group. *$P$<0.05 vs. control by one way ANOVA with a Tukey posthoc test.



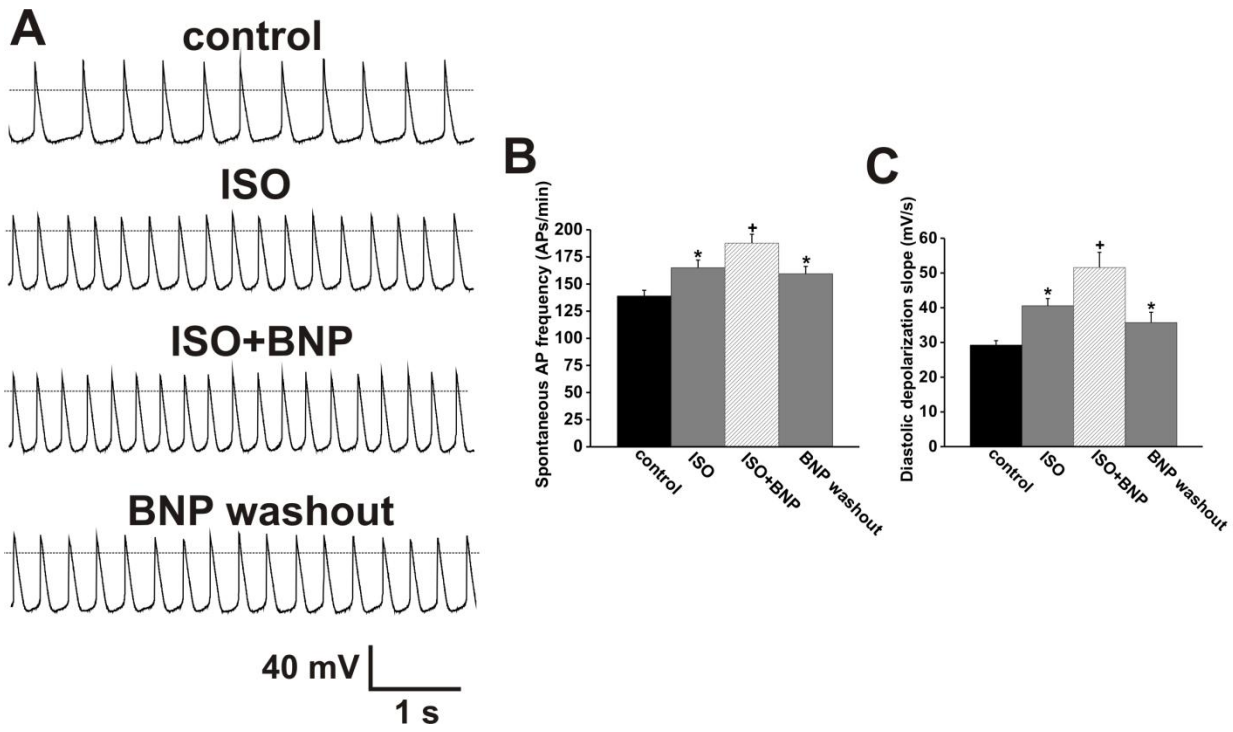

**Supplemental Figure S2:** Effects of the NPR-C agonist cANF on heart rate and sinoatrial node function in basal conditions. (A) Representative ECG recordings (1 s duration) from Langendorff-perfused mouse hearts in control conditions, in the presence of cANF (500 nM), and following washout of cANF. (B) Time course of the effects of cANF on HR in basal conditions. (C) Summary data illustrating that cANF has no effect on HR in basal conditions. Data are means ± SEM; *n*=5 hearts. (D) Representative spontaneous AP recordings (5 s duration) in isolated SAN myocytes in control conditions, in the presence of cANF (100 nM) and following cANF washout. (E and F) Summary data illustrating the lack of effect of cANF on AP frequency and DD slope in SAN myocytes in basal conditions. Data are means ± SEM; *n*=6 myocytes. Data were analyzed by one way ANOVA with a Tukey posthoc test.



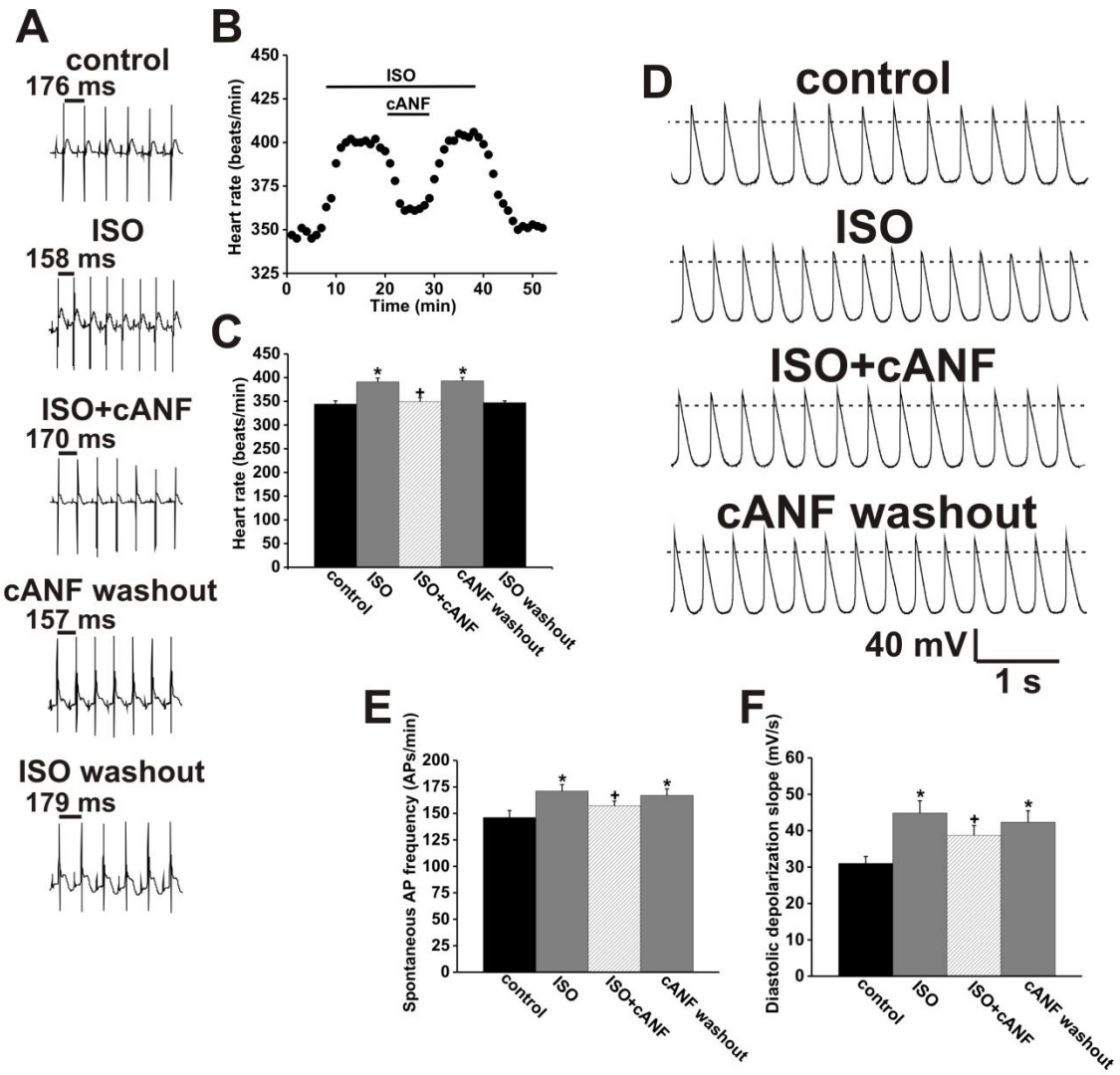

**Supplemental Figure S3:** Dose dependence of the effects of the NPR-C agonist cANF on heart rate in the presence of 1 µM isoproterenol. (A) Representative ECG recordings (1 s duration) from Langendorff-perfused mouse hearts in control conditions, in the presence of ISO, in the presence of ISO and cANF (100 nM), and following washout of these compounds. R-R intervals are indicated in each condition. (B and C) Time course of the effects of cANF (100 nM and 10 nM ) on HR. (D and E) Summary data illustrating effect of cANF (100 nM and 10 nM) on HR. Data are means ± SEM; $n$=5 hearts in each group. *$P$<0.05 vs. control; $^{+}P$<0.05 vs. ISO by one way ANOVA with a Tukey posthoc test. (F) Dose dependence of the magnitude of the reduction in HR elicited by cANF in the presence of 1 µM ISO. *$P$<0.05 vs. 10 nM cANF; $^{+}P$<0.05 vs.100 nM cANF by one way ANOVA with a Tukey posthoc test.



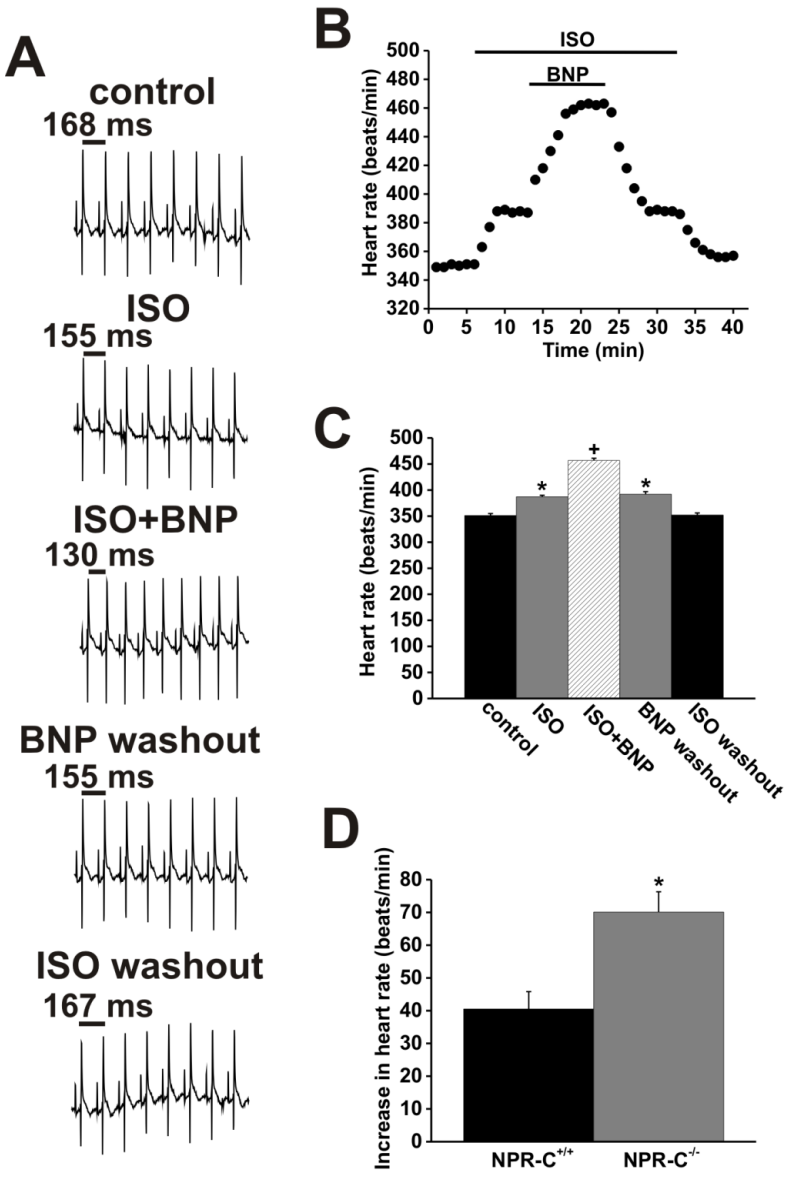

**Supplemental Figure S4:** Effects of BNP and CNP on heart rate in the presence of 10 nM isoproterenol. (A) Representative 1 s ECG recordings illustrating the effects of ISO and BNP (500 nM) or CNP (500 nM) on Langendorff-perfused mouse hearts. (B) Time course of the effects of ISO and BNP on HR. (C) Summary data illustrating the effects of ISO and BNP on HR. Data are means ± SEM; *n*=5 hearts. (D) Time course of the effects of ISO and CNP (500 nM) on HR. (E) Summary data illustrating the effects of ISO and CNP on HR. Data are means ± SEM; *n*=5 hearts. **P*<0.05 vs. control; $^{+}$*P*<0.05 vs. ISO by one way ANOVA with a Tukey posthoc test.



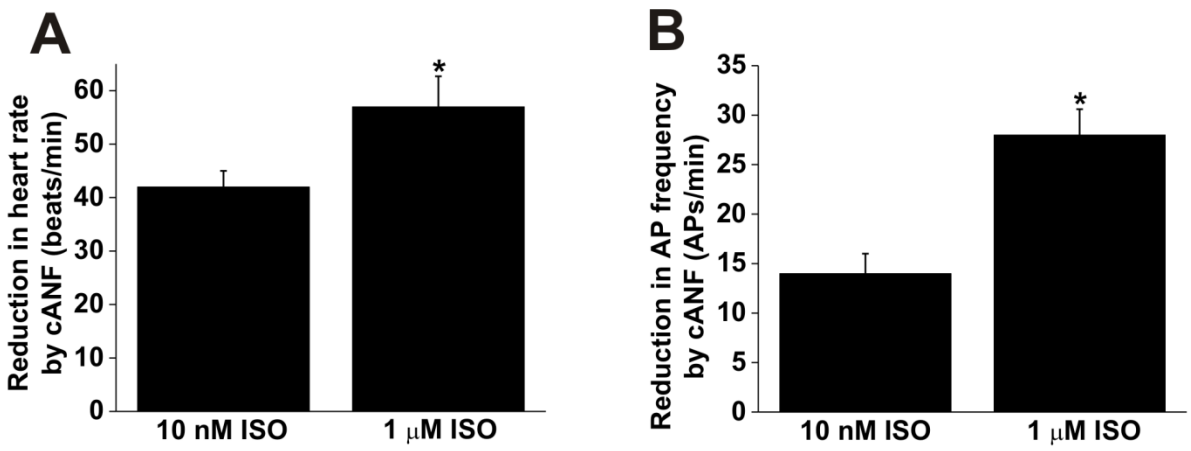

**Supplemental Figure S5:** Effects of BNP on spontaneous action potential firing in isolated mouse sinoatrial node myocytes in the presence of 10 nM isoproterenol. (A) Representative spontaneous AP recordings (5 s duration) in control conditions, in the presence of ISO, in ISO and BNP (100 nM), and following BNP washout. (B and C) Summary data illustrating the effects of ISO and BNP on AP frequency and DD slope in isolated SAN myocytes. Data are means ± SEM; $n$=6 myocytes. *$P$<0.05 vs. control; $^{+}P$<0.05 vs. ISO by one way ANOVA with a Tukey posthoc test.




**Supplemental Figure S6:** Effects of the NPR-C agonist cANF on heart rate and sinoatrial node function in the presence of 10 nM isoproterenol. (A) Representative ECG recordings (1 s duration) from Langendorff-perfused mouse hearts in control conditions, in the presence of ISO, in the presence of ISO and cANF (500 nM), and following washout of these compounds. (B) Time course of the effects of ISO and cANF on HR. (C) Summary data illustrating the effects of ISO and cANF on HR. Data are means ± SEM; *n*=5 hearts. (D) Representative spontaneous AP recordings (5 s duration) in isolated SAN myocytes in control conditions, in the presence of ISO, in ISO and cANF (100 nM) and following cANF washout. (E and F) Summary data illustrating the effects of ISO and cANF on AP frequency and DD slope in SAN myocytes. Data are means ± SEM; *n*=5 myocytes. **P*<0.05 vs. control; $^{+}$*P*<0.05 vs. ISO by one way ANOVA with a Tukey posthoc test.




**Supplemental Figure S7:** Effects of BNP on heart rate in the presence of 10 nM isoproterenol in NPR-C$^{-/-}$ mice. (A) Representative 1 s ECG recordings illustrating the effects of ISO and BNP (500 nM) on Langendorff-perfused mouse hearts from NPR-C$^{-/-}$ mice. (B) Time course of the effects of ISO and BNP on HR. (C) Summary data illustrating the effects of ISO and BNP on HR. Data are means ± SEM; $n$=4 hearts; *$P$<0.05 vs. control; $^{+}P$<0.05 vs. ISO by one way ANOVA with a Tukey posthoc test. (D) Comparison of the magnitude of the increase in HR elicited by BNP in the presence of 10 nM ISO in NPR-C$^{+/+}$ vs. NPR-C$^{-/-}$ hearts. *$P$<0.05 by Student's $t$-test.




**Supplemental Figure S8:** Comparison of the effects of cANF on heart rate and spontaneous action potential frequency in the presence of 1 μM and 10 nM doses of isoproterenol. (A) Average reductions in heart rate in Langendorff-perfused mouse hearts caused by cANF (500 nM) in the presence of 10 nM and 1 μM doses of isoproterenol. Data are means ± SEM; *n*=8 hearts in each group; **P*<0.05 vs. 10 nM ISO group by Student's *t*-test. (B) Average reductions in AP frequency in isolated SAN myocytes caused by cANF (100 nM) in the presence of 10 nM and 1 μM doses of isoproterenol. Data are means ± SEM; *n*=5 myocytes for 10 nM ISO group and 9 myocytes for 1 μM ISO group. **P*<0.05 vs. 10 nM ISO group by Student's *t*-test.

**Supplemental Table 1. Effects of cANF on ECG parameters in Langendorff-perfused hearts in basal conditions.**

| | Control | cANF | Washout |
|---|---|---|---|
| HR (beats/min) | 362±9 | 358±8 | 362±10 |
| R-R interval (ms) | 171±4 | 172±4 | 170±5 |
| P wave duration (ms) | 10.7±0.7 | 10.6±0.6 | 10.6±0.7 |
| P-R interval (ms) | 56.4±2.9 | 58.2±2.6 | 56.4±2.8 |
| Q-T interval (ms) | 62.6±1.9 | 61.8±1.3 | 61.6±1.9 |

HR, heart rate. P-R interval was measured from the start of the P wave to the peak of the QRS complex. Q-T interval was measured from the start of the QRS complex to the end of the T wave. cANF was applied at a dose of 500 nM. Data are means ± SEM; *n* = 5 hearts; cANF had no significant effects on ECG parameters in basal conditions (one way ANOVA with a Tukey posthoc test).

**Supplemental Table 2. Effects of cANF on spontaneous action potential parameters in isolated SAN myocytes in basal conditions.**

| | Control | cANF | washout |
|---|---|---|---|
| Beating rate (APs/min) | 142±9.5 | 138±9.2 | 144±9.8 |
| MDP (mV) | -65.1±4.6 | -66.3±4.6 | -64.6±4.6 |
| DD slope (mV/s) | 26±3.5 | 25.4±3.4 | 27.9±3.6 |
| $V_{max}$ (V/s) | 19.3±5.5 | 21.5±6.2 | 18.5±8.6 |
| OS (mV) | 10.7±2.4 | 11.6±2.6 | 11.7±2.9 |
| $APD_{50}$ (ms) | 56±3.3 | 57.2±3.4 | 58.6±3.6 |

MDP, maximum diastolic potential; DD slope, diastolic depolarization slope; $V_{max}$, maximum AP upstroke velocity; OS, overshoot; $APD_{50}$, AP duration at 50% repolarization; cANF was applied at 100 nM. Data are means ± SEM; *n*=6 SAN myocytes; cANF had no significant effects on spontaneous AP parameters in basal conditions (one way ANOVA with a Tukey posthoc test).

**Supplemental Table 3. Effects of cANF on ECG parameters in Langendorff-perfused hearts from NPR-C$^{+/+}$ and NPR-C$^{-/-}$ mice in the presence of 1 μM isoproterenol.**

| | NPR-C$^{+/+}$ mice | | | | NPR-C$^{-/-}$ mice | | |
|---|---|---|---|---|---|---|---|
| | Control | ISO | ISO + cANF | cANF washout | Control | ISO | ISO + cANF |
| HR (beats/min) | 359±10 | 442±19* | 385±14$^{\dagger}$ | 441±18* | 323±5 | 413±18* | 412±18* |
| R-R Interval (ms) | 169±4 | 139±6* | 157±6$^{\dagger}$ | 138±6* | 190±6 | 148±7* | 149±7* |
| P wave Duration (ms) | 10.2±0.4 | 8.3±0.4* | 9.2±0.4$^{\dagger}$ | 8.3±0.4* | 7.6±0.5 | 5.9±0.5* | 5.8±0.4* |
| P-R Interval (ms) | 55.2±2.8 | 42.7±1.8* | 50.1±1.9$^{\dagger}$ | 43.1±1.5* | 49±2.0 | 41.8±2.1* | 41.6±1.9* |
| Q-T Interval (ms) | 57.3±2.4 | 48.0±2.2* | 53.3±2.3$^{\dagger}$ | 48.2±2.0* | 69.3±1.0 | 60.8±1.0* | 60.8±0.8* |

cANF was applied at 500 nM. Data are means ± SEM; $n$ = 5 hearts in each genotype. *$P$<0.05 vs. control, $^{\dagger}P$<0.05 vs. ISO by one way ANOVA with a Tukey posthoc test. The effects of cANF were absent in NPR-C$^{-/-}$ mice.

**Supplemental Table 4. Dose dependent effects of cANF on ECG parameters in Langendorff-perfused hearts mice in the presence of 1 μM isoproterenol.**

| | 100 nM cANF group | | | | 10 nM cANF group | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | ISO | ISO + cANF | cANF washout | Control | ISO | ISO + cANF | cANF washout |
| HR (beats/min) | 341±4 | 407±4* | 369±7$^{\dagger}$ | 410±4* | 342±14 | 417±13* | 392±10$^{\dagger}$ | 415±10* |
| R-R Interval (ms) | 177±2 | 149±3* | 161±2$^{\dagger}$ | 147±3* | 176±7 | 144±4* | 154±4$^{\dagger}$ | 146±7* |
| P wave Duration (ms) | 10.6±0.4 | 7.3±0.4* | 8.7±0.6$^{\dagger}$ | 7.2±0.5* | 10.8±0.4 | 8.6±0.5* | 9.6±0.4$^{\dagger}$ | 8.7±0.6* |
| P-R Interval (ms) | 44.2±2.0 | 31±1.8* | 38.8±2.0$^{\dagger}$ | 31.4±1.9* | 49.2±2.7 | 41.0±2.3* | 45.9±2.4$^{\dagger}$ | 41.4±2.6* |
| Q-T Interval (ms) | 60.7±3.3 | 50±3.2* | 55.3±2.9$^{\dagger}$ | 50.1±3.1* | 61.2±4.3 | 52.0±4.7* | 56.4±4.9$^{\dagger}$ | 52.5±4.7* |

cANF was applied at 100 nM or 10 nM doses. Data are means ± SEM; $n$ = 5 hearts in each group. *$P$<0.05 vs. control, $^{\dagger}P$<0.05 vs. ISO by one way ANOVA with a Tukey posthoc test.

**Supplemental Table 5. Effects of cANF on spontaneous action potential parameters in isolated SAN myocytes from NPR-C$^{+/+}$ and NPR-C$^{-/-}$ mice in the presence of 1 μM isoproterenol.**

| | NPR-C$^{+/+}$ mice | | | | NPR-C$^{-/-}$ mice | | |
|---|---|---|---|---|---|---|---|
| | Control | ISO | ISO + cANF | cANF washout | Control | ISO | ISO + cANF |
| Beating rate (APs/min) | 152±8 | 189±8* | 161±8$^{\dagger}$ | 184±9* | 154±10 | 205±12* | 202±10* |
| MDP (mV) | -63.6±1.1 | -65±1.6 | -66±1.3 | -66±1.6 | -65.3±0.9 | -66.9±1.3 | -66.5±1.3 |
| DD slope (mV/s) | 32.4±2.9 | 55.9±3.2* | 38.9±2.8$^{\dagger}$ | 56.2±2.7* | 31.2±5.6 | 58.9±4.7* | 58.3±3.7* |
| $V_{max}$ (V/s) | 13.3±5.5 | 19.3±6.6 | 18.4±5.8 | 18.9±5.5 | 14.6±9.9 | 21.5±8.2 | 18.2±9.3 |
| OS (mV) | 8.2±1.4 | 10±1.2 | 10.3±1.2 | 10.8±1.6 | 9.4±4.9 | 10±4.4 | 8.8±3.9 |
| $APD_{50}$ (ms) | 52.5±4.1 | 59.4±4.2* | 55.6±3.9 | 59.4±5.5* | 37.4±2.4 | 45.8±1.8* | 44.9±3.1* |

cANF was applied at 100 nM. Data are means ± SEM; *n*=9 NPR-C$^{+/+}$ SAN myocytes and 8 NPR-C$^{-/-}$ SAN myocytes; *$P$<0.05 vs. control, $^{\dagger}P$<0.05 vs. ISO by one way ANOVA with a Tukey posthoc test. The effects of cANF were absent in NPR-C$^{-/-}$ mice.

**Supplemental Table 6. Effects of BNP on ECG parameters in Langendorff-perfused hearts in the presence of 1 μM isoproterenol.**

| | Control | ISO | ISO+BNP | BNP washout | ISO washout |
|---|---|---|---|---|---|
| HR (beats/min) | 340±14 | 395±12* | 367±11$^{\dagger}$ | 395±9* | 345±15 |
| R-R Interval (ms) | 177±7 | 152±5* | 165±6$^{\dagger}$ | 150±4* | 173±7 |
| P wave duration (ms) | 9.9±0.4 | 7.9±0.2* | 8.8±0.3$^{\dagger}$ | 7.8±0.2* | 9.9±0.4 |
| P-R Interval (ms) | 46±1.2 | 39.2±1.5* | 43.5±1.9 | 39.5±1.6* | 45.4±2.1 |
| Q-T Interval (ms) | 66±0.8 | 57.6±1.3* | 62.2±1.5$^{\dagger}$ | 57.6±1.1* | 66±0.8 |

BNP was applied at 500 nM. Data are means ± SEM; *n* = 5 hearts. *$P$<0.05 vs. control, $^{\dagger}P$<0.05 vs. ISO by one way ANOVA with a Tukey posthoc test.

**Supplemental Table 7. Effects of CNP on ECG parameters in Langendorff-perfused hearts in the presence of 1 µM isoproterenol.**

| | Control | ISO | ISO+CNP | CNP washout | ISO washout |
|---|---|---|---|---|---|
| HR (beats/min) | 354±5 | 452±9* | 421±9$^{\dagger}$ | 453±9* | 403±8 |
| R-R Interval (ms) | 151±3 | 133±2* | 144±3$^{\dagger}$ | 134±2* | 150±4 |
| P wave duration (ms) | 9.7±0.2 | 7.8±0.5* | 8.8±0.4$^{\dagger}$ | 7.8±0.4* | 9.6±0.4 |
| P-R Interval (ms) | 52.1±1.3 | 43.9±1.0* | 48.4±1.0$^{\dagger}$ | 44.0±0.9* | 52.0±1.1 |
| Q-T Interval (ms) | 58.6±1.4 | 52.2±0.9* | 55.9±1.1 | 51.9±0.7* | 58.7±1.2 |

CNP was applied at 500 nM. Data are means ± SEM; $n$ = 5 hearts. *$P$<0.05 vs. control, $^{\dagger}P$<0.05 vs. ISO by one way ANOVA with a Tukey posthoc test.

**Supplemental Table 8. Effects of BNP and CNP on spontaneous action potential parameters in isolated SAN myocytes mice in the presence of 1 µM isoproterenol.**

| | Control | ISO | ISO+BNP | control | ISO | ISO+CNP |
|---|---|---|---|---|---|---|
| Beating rate (APs/min) | 164±2 | 202±7* | 189±7$^{\dagger}$ | 150±5 | 192±8* | 178±8$^{\dagger}$ |
| MDP (mV) | -62.8±1.9 | -62.8±2.8 | -63±3.3 | -63.2±1.8 | -63.5±1.9 | -63.8±2.1 |
| DD slope (mV/s) | 36.3±2.4 | 67.4±2.5* | 53.9±1.8$^{\dagger}$ | 33.6±2.9 | 64.8±6.4* | 52.4±6.3$^{\dagger}$ |
| $V_{max}$ (V/s) | 13.6±5.1 | 15.6±4.4 | 14.6±5.4 | 12.3±7.3 | 15.2±6.9 | 13.4±6.5 |
| OS (mV) | 12.9±3.7 | 14.4±4.8 | 12.0±4.5 | 5.9±1.7 | 10.1±2.7 | 8.3±2.2 |
| $APD_{50}$ (ms) | 59.7±6.9 | 68.4±7.1* | 65.6±7.4 | 60.8±4.5 | 66.1±5.5 | 62.6±5.7* |

BNP and CNP were applied at 100 nM. Data are means ± SEM; $n$=6 SAN myocytes for the BNP and CNP groups; *$P$<0.05 vs. control, $^{\dagger}P$<0.05 vs. ISO by one way ANOVA with a Tukey posthoc test.

**Supplemental Table 9. Effects of BNP on ECG parameters in Langendorff-perfused hearts in the presence of 1 µM isoproterenol following NPR-A blockade.**

| | Control | ISO | ISO +A71915 | ISO+A71915 +BNP | BNP washout | ISO washout |
|---|---|---|---|---|---|---|
| HR (beats/min) | 345±13 | 416±12* | 415±13* | 369±14$^{\dagger}$ | 412±12* | 355±12 |
| R-R Interval (ms) | 174±6 | 147±5* | 147±5* | 164±7$^{\dagger}$ | 147±4* | 170±7 |
| P wave duration (ms) | 9.3±0.4 | 7.4±0.3* | 7.4±0.3* | 8.4±0.3$^{\dagger}$ | 7.5±0.2* | 9.3±0.3 |
| P-R Interval (ms) | 43.5±0.3 | 36.4±1.4* | 36.4±1.2* | 40.2±1.2$^{\dagger}$ | 36.8±1.3* | 44.1±0.4 |
| Q-T Interval (ms) | 64.6±1.5 | 53±1.4* | 53.6±1.2* | 58.4±1.2$^{\dagger}$ | 53.8±1.4 | 64.4±1.1 |

A71915 (NPR-A antagonist) was applied at 500 nM and BNP was applied at 500 nM. Data are means ± SEM; $n$ = 5 hearts. *$P$<0.05 vs. control, $^{\dagger}P$<0.05 vs. ISO by one way ANOVA with a Tukey posthoc test.

**Supplemental Table 10. Effects of BNP on ECG parameters in Langendorff-perfused hearts from NPR-C$^{-/-}$ mice in the presence of 1 μM isoproterenol.**

| | Control | ISO | ISO+BNP | BNP washout | ISO washout |
|---|---|---|---|---|---|
| HR (beats/min) | 382±1 | 445±2* | 483±2$^{\dagger}$ | 446±1* | 385±2 |
| R-R Interval (ms) | 157±1 | 135±1* | 124±1$^{\dagger}$ | 135±1* | 155±1 |
| P wave duration (ms) | 8.2±0.3 | 6.8±0.2* | 5.2±0.1$^{\dagger}$ | 6.6±0.2* | 8.1±0.2 |
| P-R Interval (ms) | 37.9±0.6 | 28.3±0.5* | 22.9±0.1$^{\dagger}$ | 27.2±0.2* | 36.7±0.7 |
| Q-T Interval (ms) | 65.4±0.4 | 54.1±0.1* | 36.5±0.3$^{\dagger}$ | 55.9±0.5* | 66.4±0.8 |

BNP was applied at 500 nM. Data are means ± SEM; $n$ = 5 hearts. *$P$<0.05 vs. control, $^{\dagger}P$<0.05 vs. ISO by one way ANOVA with a Tukey posthoc test.

**Supplemental Table 11. Effects of BNP on ECG parameters in Langendorff-perfused hearts in the presence of 10 nM isoproterenol.**

| | Control | ISO | ISO+BNP | BNP washout | ISO washout |
|---|---|---|---|---|---|
| HR (beats/min) | 343±9 | 389±9* | 429±7$^{\dagger}$ | 388±8* | 348±8 |
| R-R Interval (ms) | 177±5 | 155±3* | 140±2$^{\dagger}$ | 156±3* | 174±4 |
| P wave duration (ms) | 9.8±0.2 | 7.7±0.4* | 6.8±0.5$^{\dagger}$ | 7.7±0.4* | 9.7±0.2 |
| P-R Interval (ms) | 51±1.2 | 45.6±1.2* | 43.7±1.1$^{\dagger}$ | 45.7±1.1* | 50.8±1.2 |
| Q-T Interval (ms) | 61.7±1.6 | 56.9±0.9* | 55.2±0.8$^{\dagger}$ | 56.7±0.9* | 61.2±1.5 |

BNP was applied at 500 nM. Data are means ± SEM; $n$ = 5 hearts in each genotype. *$P$<0.05 vs. control, $^{\dagger}P$<0.05 vs. ISO by one way ANOVA with a Tukey posthoc test.

**Supplemental Table 12. Effects of CNP on ECG parameters in Langendorff-perfused hearts in the presence of 10 nM isoproterenol.**

| | Control | ISO | ISO+CNP | CNP washout | ISO washout |
|---|---|---|---|---|---|
| HR (beats/min) | 336±5 | 385±8* | 425±8$^{\dagger}$ | 383±10* | 340±5 |
| R-R Interval (ms) | 178±3 | 158±4* | 142±2$^{\dagger}$ | 159±4* | 175±3 |
| P wave duration (ms) | 10.2±0.4 | 8.5±0.4* | 7.4±0.4$^{\dagger}$ | 8.4±0.4* | 10.1±0.4 |
| P-R Interval (ms) | 59±1.7 | 53±1.1* | 46.8±1.8$^{\dagger}$ | 51.6±1.1* | 58.4±1.6 |
| Q-T Interval (ms) | 67.6±1.3 | 62.7±1.3* | 59.5±1.3 | 62.1±1.3* | 67.1±1.2 |

CNP was applied at 500 nM. Data are means ± SEM; $n$ = 5 hearts in each genotype. *$P$<0.05 vs. control, $^{\dagger}P$<0.05 vs. ISO by one way ANOVA with a Tukey posthoc test.

**Supplemental Table 13. Effects of BNP on spontaneous action potential parameters in isolated SAN myocytes in the presence of 10 nM isoproterenol.**

| | Control | ISO | ISO+BNP | BNP washout |
|---|---|---|---|---|
| Beating rate (APs/min) | 139±5 | 165±7* | 188±8† | 159±7 |
| MDP (mV) | -65.9±1.4 | -66.9±1.1* | -66.5±1.2† | -66.5±1.9 |
| DD slope (mV/s) | 29.2±1.3 | 40.6±2.1* | 51.6±4.4† | 35.7±2.9 |
| $V_{max}$ (V/s) | 11.5±4.8 | 14.3±8.3 | 11.9±5.6 | 11.3±6.8 |
| OS (mV) | 9.7±2.1 | 11.4±2.2 | 13.5±3.1 | 10.8±2.7 |
| $APD_{50}$ (ms) | 46.9±5.7 | 54.9±2.1* | 54.2±3.2 | 51.1±3.1 |

BNP was applied at 100 nM. Data are means ± SEM; $n$=8 SAN myocytes; *$P$<0.05 vs. control, †$P$<0.05 vs. ISO by one way ANOVA with a Tukey posthoc test.

**Supplemental Table 14. Effects of cANF on ECG parameters in Langendorff-perfused hearts in the presence of 10 nM isoproterenol.**

| | Control | ISO | ISO+cANF | cANF washout | ISO washout |
|---|---|---|---|---|---|
| HR (beats/min) | 344±7 | 391±8* | 349±9† | 393±7* | 347±8 |
| R-R Interval (ms) | 175±4 | 154±4* | 173±5† | 155±4* | 174±4 |
| P wave duration (ms) | 10.2±0.4 | 8.4±0.4* | 9.2±0.4† | 8.4±0.5* | 10.1±0.4 |
| P-R Interval (ms) | 53.6±1.6 | 47.5±1.7* | 49.7±2.3 | 47±1.9* | 52.9±1.6 |
| Q-T Interval (ms) | 66.9±1.1 | 62.3±1.5* | 64.8±1.5 | 62.4±1.5* | 66.5±1.2 |

cANF was applied at 500 nM. Data are means ± SEM; $n$ = 5 hearts. *$P$<0.05 vs. control, †$P$<0.05 vs. ISO by one way ANOVA with a Tukey posthoc test.

**Supplemental Table 15. Effects of cANF on spontaneous action potential parameters in isolated SAN myocytes in the presence of 10 nM isoproterenol.**

| | Control | ISO | ISO+cANF | cANF washout |
|---|---|---|---|---|
| Beating rate (APs/min) | 146±7 | 171±6* | 157±5$^{\dagger}$ | 167±6 |
| MDP (mV) | -64.5±1.4 | -65.8±2.3* | -65.9±2.8$^{\dagger}$ | -66.1±1.8 |
| DD slope (mV/s) | 31±2.0 | 48.8±3.4* | 38.7±2.7$^{\dagger}$ | 46.3±3.1 |
| $V_{max}$ (V/s) | 16.5±4.2 | 19.3±4.1 | 16.3±4.8 | 16.3±3.6 |
| OS (mV) | 7.4±3.2 | 7.9±1.6 | 6.7±2.0 | 8.4±1.1 |
| $APD_{50}$ (ms) | 54.7±7.4 | 62.6±6.5* | 60.2±6.9 | 58.4±6.3 |

cANF was applied at 100 nM. Data are means ± SEM; *n*=5 SAN myocytes; **P*<0.05 vs. control, $^{\dagger}$*P*<0.05 vs. ISO by one way ANOVA with a Tukey posthoc test.

**Supplemental Table 16. Effects of BNP on ECG parameters in Langendorff-perfused hearts from NPR-C$^{-/-}$ mice in the presence of 10 nM isoproterenol.**

| | Control | ISO | ISO+BNP | BNP washout | ISO washout |
|---|---|---|---|---|---|
| HR (beats/min) | 351±4 | 387±3* | 457±4$^{\dagger}$ | 392±5* | 352±4 |
| R-R Interval (ms) | 171±2 | 155±1* | 131±1$^{\dagger}$ | 153±2* | 171±2 |
| P wave duration (ms) | 10.9±0.7 | 9.1±0.7* | 7.1±0.8$^{\dagger}$ | 8.9±0.7* | 11.0±0.5 |
| P-R Interval (ms) | 50.8±1.1 | 45.9±0.7* | 41.9±0.8$^{\dagger}$ | 45.5±0.7* | 50.6±1.1 |
| Q-T Interval (ms) | 61.5±1.7 | 56.4±1.5* | 52.1±1.9$^{\dagger}$ | 55.8±1.0* | 61.8±1.7 |

BNP was applied at 500 nM. Data are means ± SEM; *n* = 4 hearts in each genotype. **P*<0.05 vs. control, $^{\dagger}$*P*<0.05 vs. ISO by one way ANOVA with a Tukey posthoc test.

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