NATRIURETIC PEPTIDES MODULATE HEART RATE, ELECTRICAL CONDUCTION AND ARRHYTHMOGENSIS *IN VIVO*

by

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Submitted in partial fulfillment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
June 2013

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ABSTRACT

Natriuretic peptides (NPs) are a family of cardioprotective hormones including B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). NPs elicit their effects via three receptors denoted NPR-A (binds BNP), NPR-B (binds CNP) and NPR-C (binds all NPs). In this study we performed electrocardiograms (ECG) and programmed stimulation protocols to study the effects of BNP, CNP and their NPRs on heart rate (HR), electrical conduction, and susceptibility to atrial fibrillation (AF). Acute injection of BNP and CNP increased HR in wildtype animals, whereas selective activation of NPR-C decreased HR. No differences in HR, sinus node function or blood pressure were observed in NPR-$C^{-/-}$ mice compared to wildtype (NPR-$C^{+/+}$) and heterozygote (NPR-$C^{+/-}$) littermates. However, NPR-$C^{-/-}$ mice are more susceptible to AF compared to wildtype controls. These experiments provide novel insight into the direct electrophysiological effects of NPs and their receptors in the heart.

LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Percentage
°C	Degrees Celsius
β-AR	β-adrenergic receptor
ACh	Acetylcholine
AERP	Atrial effective refractory period
AF	Atrial fibrillation
ANF	Atrial natriuretic factor
ANP	Atrial natriuretic peptide
ANS	Autonomic nervous system
AP	Action potential
ATP	Adenosine triphosphate
AVN	Atrioventricular node
AVNERP	Atrioventricular node effective refractory period
BNP	B-type natriuretic peptide
cAMP	Cyclic adenosine monophosphate
cANF	Cys^{18}-atrial natriuretic factor
cGMP	Cyclic guanosine monophosphate
CHF	Congestive heart failure
CNG	Cyclic nucleotide-gated ion channel
CNP	C-type natriuretic peptide
CNS	Central nervous system
COOH	Carboxyl
cSNRT	Corrected sinus node recovery time
DAG	Diacylglycerol
DNP	Dendroaspis natriuretic peptide
ECD	Extracellular domain

ECG	Electrocardiogram
EDHF	Endothelium derived hyperpolarizing factor
fmol	Femtomole
g	Gram
GC	Guanylyl cyclase
GCD	Guanylyl cyclase domain
G_i	Inhibitory G protein
GIRK	G protein inward rectifier potassium channel
GPCR	G protein coupled receptor
G_s	Stimulatory G protein
GTP	Guanosine triphosphate
HCN	Hyperpolarization-activated cyclic-nucleotide gated
HR	Heart rate
Hz	Hertz
$I_{Ca,L}$	L-type calcium current
$I_{Ca,T}$	T-type calcium current
IDE	Insulin-degrading enzyme
I_f	Hyperpolarization-activated “funny” current
$I_{K,1}$	Inward rectifier potassium current
I_K	Delayed rectifier potassium current
$I_{K,Ach}$	Acetylcholine-activated inward rectifier potassium current
I_{Na}	Inward sodium current
I_{NCX}	Na^+-Ca^{2+} exchange current
IP	Intraperitoneal
IP_3	Inositol triphosphate
ISO	Isoproterenol
IV	Intravenous

kg	Kilogram
KHD	Kinase homology domain
L	Litre
LBD	Ligand binding domain
mA	Milliampere
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
MDP	Maximum diastolic potential
mg	Milligram
min	Minute
ml	Millilitre
mmHg	Millimetre of mercury
MMP	Matrix metalloproteinases
ms	Millisecond
NH_2	Amino
nM	Nanomolar
NP	Natriuretic peptide
NPR	Natriuretic peptide receptor
NPR-A	Natriuretic peptide receptor A
NPR-B	Natriuretic peptide receptor B
NPR-C	Natriuretic peptide receptor C
PDE	Phosphodiesterase
PIP_2	Phosphatidyl inositol bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLCβ	β isoform of phospholipase C

pmol	Picomole
PSNS	Parasympathetic nervous system
RMP	Resting membrane potential
s	Second
SAN	Sinoatrial node
SNRT	Sinus node recovery time
$SNRT_{100}$	Sinus node recovery time with a basic cycle length of 100 ms
SNS	Sympathetic nervous system
SR	Sarcoplasmic reticulum
µg	Microgram
µl	Microlitre
uM	Micromolar
VEGF	Vascular endothelial growth factor
VSM	Vacular smooth muscle

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisor, Dr. Robert Rose for giving me the opportunity to complete my graduate degree in such a high quality research laboratory. Your guidance and support have been greatly appreciated and it has truly been an honour to work with such a passionate and knowledgeable scientist. You have helped me acquire a wealth of knowledge related to cardiac electrophysiology, taught me to think more critically and motivated me to become a better student and scientist. Thank you Rob for such a "phosphorylating" experience!

I would also like to extend gratitude to members of my supervisory committee; Dr. Susan Howlett and Dr. Younes Anini for their encouragement, guidance and support over the course of my research project. Sincere thanks to our graduate coordinator Dr. Elizabeth Cowely for her assistance and advice throughout my graduate degree.

To my fellow members of the Rose Lab both past and present, thank you for your friendship, encouragement and support both in an out of the lab. You have helped make this experience a truly memorable one and I wish you all the best in your future endeavours. A special thanks to Dr. Rui Hua and Dr. Emmanuel Egom for their advice and assistance throughout my research project and to Purnima Narayanan for her patience, guidance and technical support.

Finally I would like to thank my family and friends for their unwavering love and support. Mom and Dad, I would not be where I am today with you. Thank you for always supporting me while I pursue my dreams, and for always being there for me throughout the good times and bad. Words cannot express my gratitude for everything you both have done for me.

CHAPTER 1: INTRODUCTION

1.1 Overview

Regulation of the electrical activity of the heart is a complex process that involves both neural and hormonal control. Heart rate (HR) is a major determinant of cardiac output and therefore plays a critical role in maintaining cardiac performance in both physiological and pathophysiological conditions. The spontaneous generation of electrical impulses in the sinoatrial node (SAN) and the coordinated conduction of these impulses throughout the conduction system are necessary for maintaining automaticity and rhythmicity in the heart. There are several regulators of heart rate, most notably the autonomic nervous system (ANS). Recently, we have shown that natriuretic peptides (NPs), a family of cardioprotective peptide hormones produced in the heart, regulate heart rate, sinoatrial node function and electrical conduction *in vitro* (Azer *et al.* 2012; Springer *et al.* 2012). Although significant progress has been made in this area of research, the electrophysiological effects of NPs *in vivo* are not well understood and thus this was the main focus of the studies described in this thesis. Here, we have recorded electrocardiograms (ECG) in mice to study the effects of B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) as well as their receptors on heart rate and electrical conduction in the presence of an intact nervous system. We have also performed surface and intracardiac ECGs in conjunction with standard programmed stimulation protocols to characterize the cardiac electrophysiological phenotype of mice lacking functional natriuretic peptide receptor C (NPR-C).

1.2 Heart Rate and Electrical Conduction

In higher vertebrates, the heart exhibits a unique property of generating spontaneous electrical impulses that dictate the frequency of contraction of the heart under normal physiological conditions. This automaticity is generated by a specialized population of cells that have low contractility but exhibit periodical electrical oscillations (Mangoni and Nargeot, 2008). These specialized cells are found in the SAN, the atrioventricular node (AVN), and the Purkinje fibres. While all of these specialized cells exhibit properties of automaticity, the SAN acts as the physiological or leading pacemaker of the heart in normal conditions and will be further discussed in the next section.

1.2.1 The Sinoatrial Node and Cardiac Automaticity

The SAN is a structurally heterogeneous region located at the junction of the superior vena cava and the right atrium, and adjacent to the crista terminalis (Mangoni and Nargeot, 2008; Monfredi *et al.* 2010). Unlike the working myocardium, pacemaker cells in the SAN do not have a stable resting membrane potential (RMP). Instead SAN cells exhibit a gradual and spontaneous depolarization in the diastolic phase which is referred to as a diastolic depolarization or pacemaker potential (Mangoni and Nargeot, 2008; Monfredi *et al.* 2010). This spontaneous depolarization forms the basis of automaticity in the SAN and involves several ionic currents including the inward hyperpolarization-activated "funny" current (I_f) carried by hyperpolarization-activated cyclic-nucleotide gated (HCN) channels, T- and L-type Ca^{2+} currents ($I_{Ca,T}$ and $I_{Ca,L}$), and the inward Na^{+}-Ca^{2+} exchange current (I_{NCX}) which is driven by Ca^{2+} release from the

sarcoplasmic reticulum (SR) (Mangoni and Nargeot, 2008; Monfredi *et al.* 2010). The lack of the inward rectifier K^+ current ($I_{K,1}$) in the SAN and the decay of the delayed rectifier K^+ current (I_K) following an action potential (AP) also facilitate the generation of the pacemaker potential (Monfredi *et al.* 2010). The lack of $I_{K,1}$ in the SAN also accounts for a more depolarized maximum diastolic potential (MDP) in SAN cells compared to the RMP of the working myocardium (Monfredi *et al.* 2010). Similar to non-automatic cells, once the membrane potential in SAN cells reaches threshold, an AP is triggered. However, the rate of AP depolarization in SAN cells is slower than that of the working myocardium as SAN cells have little sodium current (I_{Na}) that is responsible for the AP upstroke in most excitable cells (Mangoni and Nargeot, 2008; Monfredi *et al.* 2010). Instead, the depolarization phase of the AP in SAN cells is due to $I_{Ca,L}$ (Mangoni and Nargeot, 2008; Monfredi *et al.* 2010). While the SAN is considered the "natural" pacemaker that sets the heart rate, the frequency of electrical impulses can be modulated by either altering the rate of the diastolic depolarization and/or the maximum diastolic potential. This will be discussed further in the autonomic regulation of heart rate below.

1.2.2 Electrical Conduction and ECGs

The SAN acts as the pacemaker of the heart initiating electrical impulses to stimulate contractile cells of the working myocardium. Rhythmic and synchronized contraction of the atria and ventricles is essential for the maintenance of cardiac output and is accomplished through a specialized cardiac conduction system. This system consists of three main parts: the SAN, the AVN and the His-Purkinje system (Boyett, 2009). Following their generation in the SAN, electrical impulses spread throughout the atria, exciting the contractile atrial syncitium and sending a wave of depolarization to the

AVN. The AVN is located near the base of the right atrium and conducts the electrical impulse from the atria to the ventricles. Conduction velocity through the AVN is slow, allowing the atria to pump blood into the ventricle prior to ventricular systole. The atrioventricular bundle (or the Bundle of His), bundle branches and Purkinje fibres make up the His-Purkinje system and conduct the electrical impulse throughout the ventricles to facilitate a coordinated ventricular contraction (Boyett, 2009).

Abnormalities in the initiation and/or conduction of electrical impulses throughout the heart can lead to the generation of cardiac arrhythmias; therefore recording the electrical activity of the heart is an invaluable research and clinical tool. An ECG records the electrical activity generated by the working myocardium using electrodes placed on the surface of the body. The electrical events generated by the specialized cells of the conduction system are too small to be recorded by an ECG, however, based on recordings from the atria and ventricles one can assess the rate, rhythm and conduction of electrical impulses throughout the heart (Becker, 2005). In general a typical ECG trace consists of three deflections called the P wave, QRS complex and T wave (see Figure 3 on page 34). The P wave represents depolarization of the atrial myocardium; the QRS complex represents depolarization of the ventricles and the T wave represents repolarization of the ventricles. The intervals between the deflections also provide essential information about electrical conduction through the heart. The PR interval represents conduction through the AV node and the QT interval is a measure of conduction through, and subsequent repolarization of the ventricles.

1.2.3 *Heart Rate Regulation*

The SAN is the intrinsic pacemaker of the heart, however several inputs (neural and hormonal) act on the SAN to modulate heart rate. The SAN is under tonic control of both divisions of the autonomic nervous system and autonomic regulation of SAN activity has been well documented as discussed below.

1.2.3.1 Autonomic Regulation of SAN Activity

The autonomic nervous system (ANS) is a critical regulator of heart rate and consists of two major divisions; the sympathetic nervous system (SNS) and the parasympathetic nervous system (PSNS). Input from the SNS on the SAN enhances automaticity (and thus heart rate) while input from the PSNS has opposite effects. Sympathetic nerve fibres in the heart release catecholamines (norepinephrine and epinephrine) that act, at least in part, on β-adrenergic receptors (β-AR) in the SAN (Mangoni and Nargeot, 2008). Activation of β-ARs is coupled to the activation of stimulatory heterotrimeric G-proteins (G_s). Upon activation, the α-subunit of G_s activates the enzyme adenylyl cyclase which catalyzes the conversion of adenosine triphosphate (ATP) into the second messenger cyclic adenosine monophosphate (cAMP). cAMP increases heart rate by binding directly to HCN channels in the SAN, increasing I_f and the slope of the diastolic depolarization (Mangoni and Nargeot, 2008). cAMP also indirectly increases heart rate by activating protein kinase A (PKA). PKA mediates the phosphorylation of HCN channels, voltage gated Ca^{2+} channels and ryanodine receptors on the SR to increase I_f, $I_{Ca,L}$, SR Ca^{2+} release and I_{NCX} respectively (Mangoni and Nargeot, 2008).

Parasympathetic regulation of heart rate is mediated by binding of the neurotransmitter acetylcholine (ACh) to the muscarinic M_2 receptor. The M_2 receptor is coupled to the heterotrimeric inhibitory G protein (G_i). The α-subunit associated with G_i inhibits the activity of adenylyl cyclase and decreases production of cAMP. This leads to a decrease in I_f, $I_{Ca,\,L}$ and $I_{NCX,}$ thereby reducing the slope of the diastolic depolarization and decreasing heart rate (Mangoni and Nargeot, 2008). The βγ subunit of G_i proteins associated with M_2 receptors also activates the ACh-sensitive potassium current, $I_{K,ACh}$ which is carried by G protein inward rectifier potassium (GIRK) channels (Mangoni and Nargeot, 2008). Activation of $I_{K,ACh}$ increases the repolarizing current during diastole consequently hyperpolarizing the MDP. Therefore heart rate is slowed due to prolongation of the time required to reach threshold.

In addition to the ANS, a number of peptides including angiotensin II and vasoactive intestinal peptide have also been shown to modulate heart rate either by direct action on the SAN or through interactions with the ANS (Beaulieu and Lambert, 1998). Another group of peptides that have such effects is the natriuretic peptides, which are the focus of my research project and will be discussed in greater detail below.

1.3 Natriuretic Peptides

1.3.1 Discovery and Structure

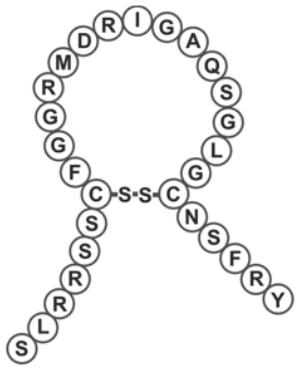
Natriuretic peptides (NPs) are a family of structurally related but genetically distinct peptide hormones that regulate blood pressure and blood volume in humans and nonhuman vertebrates (Martinez-Rumayor *et al.* 2008). As their name suggests, NPs

maintain cardiovascular homeostasis by promoting natriuresis, diuresis and vasodilation (McDowell *et al.* 1995; Levin *et al.* 1998). The field of natriuretic peptides was pioneered by de Bold and colleagues in 1981 with the landmark discovery that infusion of atrial extracts had potent natriuretic and diuretic effects in rats (de Bold *et al.* 1981). This discovery led to the isolation of atrial natriuretic peptide (ANP; also referred to as atrial natriuretic factor, ANF) and established the role of the heart as an endocrine organ (de Bold, 1985). Subsequent isolation of B-type natriuretic peptide (BNP), originally named brain natriuretic peptide (Sudoh *et al.* 1988) and C-type natriuretic peptide (CNP) (Sudoh *et al.* 1990) from porcine brain extracts was based on their potent relaxant effect on smooth muscle. Recently, a fourth NP termed Dendroaspis natriuretic peptide (DNP) was isolated from the venom of the green mamba snake (*Dendropaspis angusticeps)* (Schweitz *et al.* 1992). The physiological role of this 38-amino acid peptide in mammals, including humans, is still under investigation and will not be discussed further.

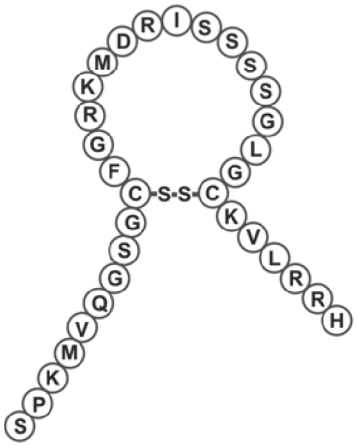
All NPs are structurally similar and contain the highly conserved sequence CFGXXDRIXXXXGLGC (see Figure 1) where X is any amino acid (Potter *et al*. 2006). A disulfide-linkage between the flanking cysteines forms a 17 amino acid ring that is essential for biological activity (Misono *et al.* 1984). The length and amino acid composition of the amino (NH_2)-terminal and carboxyl (COOH)-terminal amino acid chains vary between members of the NP family, giving each peptide its unique properties. CNP is highly homologous to ANP but lacks a COOH-terminus tail. BNP exhibits the greatest interspecies variability in primary structure while CNP is the most evolutionary conserved NP (Pandey, 2005).

Figure 1.

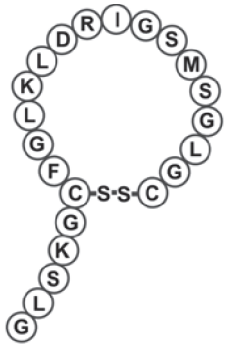
Schematic illustrating the amino acid sequence and ring structure of natriuretic peptides. ANP: Atrial natriuretic peptide; BNP: B-type natriuretic peptide; CNP: C-type natriuretic peptide; DNP: Dendroaspis natriuretic peptide.



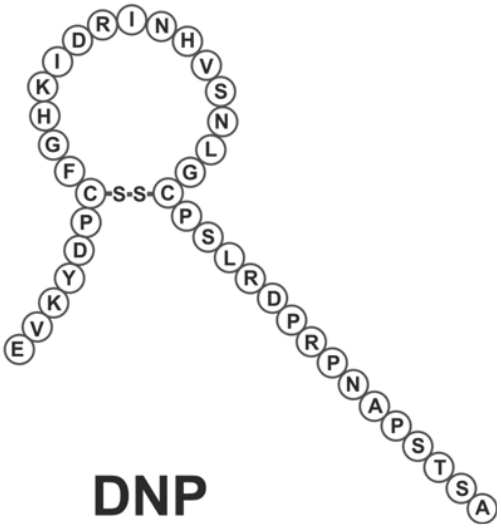
ANP



BNP



CNP



DNP

Figure 1.

While NPs share a highly homologous structure, all NPs are genetically distinct. CNP is the most ancient NP and evolutionary analysis has revealed that ANP and BNP evolved from a CNP duplication event (Inoue *et al.* 2003). The gene encoding human ANP (*NPPA)* which is located on the short arm of chromosome 1 (1p36.21) is only 8 kb downstream of *NPPB*, the gene encoding BNP (1p36.2) (Tamura *et al.* 1996; Potter *et al.* 2006). The gene that encodes human CNP (*NPPC*) is located on chromosome 2 (2q24-qter) (Ogawa *et al.* 1994b).

1.3.2 Synthesis, Posttranslational Processing and Circulating Levels

All natriuretic peptides are synthesized as preprohormones that undergo posttranslational processing to produce the active peptide. ANP is synthesized in atrial granules, first as the 151-amino acid prepro-ANP (Potter *et al.* 2006). Cleavage of the NH_2- terminal signal sequence produces the 126-amino acid pro-ANP which is the predominant form of ANP stored in atrial granules (Inagami, 1989). The biologically active form of ANP is produced upon secretion from atrial myocytes through proteolytic cleavage of pro-ANP by corin, a transmembrane cardiac serine protease (Yan *et al.* 2000; Wu *et al.* 2002; Chan *et al.* 2005). Corin is highly expressed on the surface of atrial cardiomyocytes (Hooper *et al.* 2000) and cleaves pro-ANP into the bioactive 28 amino acid ANP (residues 99-126) and the inactive NH_2-terminal pro-ANP (residues 1-98). Alternative processing of pro-ANP in the kidney produces a 32 amino acid protein called urodilatin which was purified from human urine and acts exclusively in the kidney to elicit natriuretic and diuretic effects (Schulz-Knappe *et al.* 1988; Goetz, 1991; Forssmann *et al.* 1998). Under physiological conditions, ANP is primarily expressed and stored in atrial granules; however it is also expressed at much lower levels in the ventricles (de

Bold *et al.* 2001). Interestingly, production of ANP by the ventricles is increased in neonates (Cameron *et al.* 1996) and in patients with cardiovascular diseases, including cardiac hypertrophy and heart failure (Saito *et al.* 1989; Takemura *et al.*1991; Yasue *et al.* 1989). While ANP is continuously secreted from the heart, mechanical and neuroendocrine stimuli can increase the rate of secretion (de Bold *et al.* 1996). Atrial distension due to increased intravascular volume has been identified as the primary stimulus for acute ANP secretion (Dietz, 1984; Lang *et al.* 1985; Bilder *et al.*1986; de Bold *et al.* 1986; Edwards *et al.* 1988). Several additional stimuli have been identified which induce ANP secretion including; water immersion (Ogihara *et al.* 1986) head down tilt (Hollister *et al.* 1986), vasopressin (Manning *et al.* 1985; Zongazo *et al.* 1991), angiotensin II (Soualmia *et al.* 1997), endothelin (Stasch *et al.* 1989; Gardner *et al.* 1991), acetylcholine and α- and β- adrenergic agonists (Schiebinger *et al.* 1987; Shields *et al.* 1989). Following secretion into the coronary sinus, ANP travels to various target tissues and exerts its effects. Circulating levels of ANP are relatively low (10 fmol/ml), however they are greater than that of other NPs (Mukoyama *et al.* 1991). Plasma ANP levels are increased 10-30 fold in patients with congestive heart failure (CHF) and circulating ANP levels positively correlate with the severity of cardiac failure (Burnett *et al.* 1986; Cody *et al.* 1986).

BNP was first purified from extracts from porcine brain and as such was termed brain natriuretic peptide. Later, it was shown that BNP is primarily synthesized and secreted from the ventricles (Minamino *et al.*1988; Hosoda *et al.* 1991; Mukoyama *et al.* 1991; Ogawa *et al.* 1991), particularly the left ventricle in humans (Mukoyama *et al.* 1990; Yoshimura *et al.* 1993); thus it is now referred to as B-type natriuretic peptide. In

addition to the heart, BNP is also expressed at lower concentrations in the central nervous system (CNS), lung, thyroid, adrenal glands, kidney, spleen, small intestine, ovary, uterus, and striated muscle (Semenov and Seferian, 2011). Human BNP is a 32 amino acid peptide that is synthesized as a 134-amino acid preprohormone (Kambayashi *et al.*1990). Cleavage of the signal sequence produces a 108- amino acid prohormone that is further cleaved to form the inactive 76-amino acid (residues 1-76) NH_2-terminal fragment (NT-proBNP) and the bioactive 32-amino acid (residues 77-108) COOH-terminal fragment (Potter *et al.* 2006). Both fragments have been shown to circulate in the plasma (Hunt *et al.* 1995). In vitro studies have suggested that pro-BNP may be cleaved by both corin (Semenov *et al.* 2010) and the intracellular serine endoprotease furin (Sawada *et al.* 1997) to form the circulating BNP fragments. Unlike ANP which is produced by cleavage of pro-ANP during secretion, processing of pro-BNP into BNP-32 is thought to occur intracellularly and prior to secretion into the circulation (Pandey 2005, Semenov and Seferian, 2011). While the majority of BNP is found in the ventricles, low levels of BNP are stored with ANP in atrial granules (Thibault *et al.* 1992; de Bold *et al.* 1996). Like ANP, BNP is constitutively secreted into the circulation; however expression and secretion of BNP by the ventricles can be increased in response to various mechanical and hormonal stimuli, particularly in the setting of heart disease. Transcription of BNP is increased in response to ventricular-load induced stretch which is associated with several cardiac pathologies including heart failure, myocardial injury and cardiomyopathies (Magga *et al.* 1997; Tokola *et al.* 2001). There is currently an abundance of evidence to show that BNP release is closely related to the severity of left ventricular dysfunction and as such BNP is commonly used as both a diagnostic and prognostic marker of heart

failure (Richards, 2004). In addition to mechanical stimuli, several hormones and cytokines including endothelin-1, angiotensin II, interleukins and adrenergic agonists have been shown to induce transcription of BNP (Semenov and Seferian, 2011). Several transcription factors have been shown to regulate both basal and inducible transcription of BNP including members of the GATA 4 family, muscle-CAT-binding proteins, and activating protein-1/cAMP-response element binding proteins (Thuerauf *et al.* 1994; He *et al.* 2002; LaPointe, 2005). Circulating levels of BNP are on the order of 1 fmol/ml in healthy individuals however like ANP, plasma levels of BNP are profoundly elevated in patients with heart disease (Mukoyama *et al.* 1991). In patients with CHF, BNP levels are elevated between 200-300 fold compared to normal subjects (Potter *et al.* 2006) and this increase in BNP is 10-50 times greater than the increase in ANP levels (Mukoyama *et al*. 1991).

Unlike ANP and BNP, CNP does not appear to behave as a circulating hormone. Circulating levels of CNP are very low thus, CNP is considered to act in an autocrine and paracrine manner rather than as a typical endocrine hormone (Igaki *et al.* 1996; Pandey, 2005). Like BNP, CNP was originally isolated from the brain and it is the most highly expressed NP in the CNS (Ogawa *et al.* 1992). Outside of the CNS, CNP is highly expressed in endothelial cells (Suga *et al.* 1992; Stingo *et al.* 1992) and chondrocytes (Hagiwara *et al.* 1994) while low levels are found in the heart, immune system (Vollmar *et al.* 1993) and male and female reproductive organs (Walther *et al.* 2004; Xia *et al.* 2007; Zhang *et al.* 2010). CNP is not stored in granules but like other NPs, is produced from a larger precursor protein (Lumsden *et al*. 2010). The gene that encodes human CNP produces the 126-amino acid prepro-CNP. Upon cleavage of the 23 amino acid

signal sequence by signal peptidases (Tawaragi *et al.* 1991), a 103-amino acid NH_2-terminal pro-CNP (NT-proCNP) is formed. It has been demonstrated *in vitro*, that processing of pro-CNP to the 53 amino acid COOH-terminal biologically active form of CNP (CNP-53) is mediated by furin (Wu *et al.* 2003). CNP-53 is the major active form of CNP at the tissue level (Brown *et al.* 1997); however a shorter 22 amino acid form (CNP-22) is the dominant form of CNP found in the circulation (Potter *et al.* 2009). The protease responsible for the production of CNP-22 from CNP-53 is still unknown. In endothelial cells, secretion of CNP is upregulated by sheer stress (Chun *et al.* 1997), and several cytokines and growth factors including: tumor necrosis factor-α (Suga *et al.* 1993), transforming growth factor-β (Suga *et al.* 1992b; Suga *et al.* 1993), and interleukin-6 (Suga *et al.* 1993). Interestingly, ANP and to a lesser extent BNP, were shown to increase expression and secretion of CNP from the vasculature (Nazario *et al.* 1995). Insulin (Igaki *et al.* 1996) and vascular endothelial growth factor (VEGF) (Doi *et al.* 1996) have been shown to suppress CNP secretion from the endothelium. CNP secretion in vascular smooth muscle is also increased in response to vascular injury (Brown *et al.* 1997). Normal circulating levels of both forms of CNP are approximately 1 fmol/ml and levels are increased in patients with CHF (Kalra *et al.* 2003; Del Ry *et al.* 2005; Charles *et al.* 2006) but to a lesser degree compared to ANP and BNP (Wei *et al.* 1993).

1.3.3 Metabolism and Degradation

Upon release into the circulation, NPs are rapidly cleared from the body resulting in short biological half-lives. Human BNP has the longest plasma half-life of

approximately 20 min (Holmes *et al.* 1993) while ANP and CNP have shorter half-lives of approximately 2-3 min (Nakao *et al.* 1986; Yandle *et al.* 1986; Hunt *et al.* 1994). There are two processes responsible for NP degradation: receptor mediated internalization followed by lyzosomal degradation and NP degradation by extracellular proteases (Potter, 2011).

The first mechanism of NP degradation involves internalization of the peptide-receptor complex followed by hydrolytic degradation in the lysosome. Historically, one of the natriuretic peptides receptors (NPRs; addressed in more detail below) denoted NPR-C was classified as the 'clearance receptor' responsible for mediating this process. This NPR-C receptor was thought to lack a signaling function (Maack *et al.* 1987), which appears to be the basis for ascribing only a 'clearance' function to this NPR. Experiments utilizing a selective agonist of NPR-C and NPR-C null animals have demonstrated a prolongation of the half-life of ANP and no change in the total ANP concentration which provided further support for the role of NPR-C as a clearance receptor (Maack *et al.* 1987, Matsukawa *et al.* 1999). The mechanics of NPR-C-mediated NP degradation include internalization of the peptide-receptor complex, lysosomal ligand hydrolysis and recycling of a portion of the free receptor population back to the plasma membrane (Potter, 2011). It is speculated that this internalization of the peptide-receptor complex occurs through a clathrin-dependent mechanism; however, NPR-C lacks cytoplasmic internalization motifs which are commonly found on other receptors that undergo internalization in a clathrin-dependent manner (Potter, 2011). More importantly, there is now substantial evidence indicating that NPR-C is not simply a clearance receptor, but rather is coupled to the activation of inhibitory G proteins (discussed further below). Thus

while internalization of NPR-C receptors may lead to removal of NPs from the circulation, this coincides with the activation of intracellular signaling pathways.

The second mechanism of NP degradation is through enzymatic degradation by extracellular proteases. The protease most commonly associated with NP degradation is a zinc-containing, membrane bound ectoenzyme called neprilysin (also referred to as neutral endoprotease 24.11, enkephalinase, common acute lymphoblastic leukemia antigen and CD10) (Malito *et al.* 2008) that cleaves substrates on the amino side of hydrophobic residues. Multiple neprilysin cleavage sites have been identified in natriuretic peptides, however the primary cleavage site in ANP and CNP is between the cysteine and phenylalanine residues that are part of the conserved ring structure (Cys7-Phe8) (Kenny *et al.* 1993; Watanabe *et al.* 1997). Cleavage at this location opens the ring structure and inactivates the peptide. Neprilysin has been shown to effectively cleave ANP and CNP; however degradation of BNP by neprilysin occurs at low rates and appears to be species dependent (Potter, 2011). While human BNP contains the conserved cysteine and phenylalanine residues (Cys10-Phe11), neprilysin does not act at that position (Norman *et al.* 1991; Watanabe *et al.* 1997) suggesting that BNP degradation in humans occurs in a neprilysin-independent manner. A sequential cleavage method was proposed by Pankow *et al.* (2007) in which BNP is first cleaved by the metalloprotease meprin A which facilitates subsequent ring cleavage of BNP by neprilysin. Meprin A has been shown to effectively cleave mouse BNP, rat BNP and porcine BNP (Pankow *et al.* 2007), however human BNP lacks the meprin A cleavage site and therefore human BNP is not cleaved by this mechanism (Dickey and Potter, 2010). Interestingly, degradation of human BNP was inhibited in the presence of

leupeptin, a serine protease inhibitor; however the specific protease inhibited by leupeptin remains unknown (Dickey and Potter, 2010). In addition to neprilysin, human ANP and CNP are also cleaved by insulin-degrading enzyme (IDE), a zinc metalloprotease found on the cell membrane and in cytoplasmic fractions (Duckworth *et al.* 1998; Malito *et al.* 2008; Ralat *et al.* 2011). Like neprilysin, BNP is a poor substrate for IDE and IDE does not appear to play a significant role in degradation of human BNP (Potter, 2011).

1.4 Natriuretic Peptide Receptors

The actions of NPs throughout the body are both diverse and complex. This stems from the fact that NPs elicit their effects by binding to one of three membrane bound natriuretic peptide receptors (NPRs) denoted NPR-A, NPR-B and NPR-C (Figure 2). ANP and BNP are selective ligands for NPR-A, CNP is selective for NPR-B and all three NPs bind NPR-C (Bennett *et al*. 1991; Koller *et al.* 1991; Suga *et al.* 1992a). NPR-A (GC-A) and NPR-B (GC-B) are guanylyl cyclase (GC) linked receptors with the same overall topology; as such they will be discussed together in the next section. NPR-C is not linked to a guanylyl cyclase enzyme; however it has been shown that the intracellular domain is coupled to the activation of G_i proteins (see discussion below). A summary of the natriuretic peptide receptors and their respective ligands is shown in Figure 2.

1.4.1 NPR-A/NPR-B

NPR-A and NPR-B are found in many different tissues which contributes to the diverse effects of NPs. NPR-A is highly expressed in the heart, kidney, brain, vascular smooth muscle (VSM), adipose, adrenal, testis and lung (Lowe *et al.* 1989; Wilcox *et al.*

1991; Nagase *et al.* 1997; Goy *et al.* 2001; Muller *et al.* 2004). ANP and BNP are both ligands for NPR-A however this receptor has a 1000 times greater affinity for ANP compared to BNP (Schultz *et al.* 1989). NPR-B and its mRNA are expressed in the bone, brain, fibroblasts, heart, kidney, liver, lung, uterus and VSM (Chrisman *et al.* 1993; Langub *et al.* 1995; Herman *et al.* 1996; Nagase *et al.* 1997; Chrisman and Garbers, 1999; Bryan *et al.* 2006; Dickey *et al.* 2007). CNP is the selective agonist for NPR-B at physiological conditions and the affinity of NPR-B for CNP is 50-500 fold greater than the other NPs (Koller *et al.* 1991; Suga *et al.* 1992a). As previously stated, both NPR-A and NPR-B are members of the guanylyl cyclase receptor family which mediate the conversion of guanosine triphosphate (GTP) into the second messenger cyclic 3',5'-guanosine monophosphate (cGMP) (Schultz and Waldman, 1999; Misono, 2002; Tremblay *et al*. 2002). Like other membrane bound GCs, NPR-A and NPR-B share a unique topology. All members contain a large extracellular ligand binding domain (approximately 450 amino acids) connected to the intracellular domain by a single transmembrane spanning region consisting of 20-25 hydrophobic residues (Potter *et al.* 2009). The intracellular domain consists of a protein kinase homology domain (KHD), an amphipathic hinge region and a guanylyl cyclase domain (GCD) (Misono *et al.* 2011). The extracellular domain (ECD) contains multiple intramolecular disulfide bonds in addition to several N-linked glycosylation sites which are necessary for proper folding and localization of the receptor to the cell membrane (Miyagi *et al.* 2000). The ECD also contains a conserved chloride-binding site and it has been shown that ANP binding to NPR-A occurs in a chloride concentration dependent fashion (Misono, 2000). The role of the KHD is not well understood; however, it is required for ligand-dependent receptor

Figure 2.

Schematic representation of natriuretic peptides receptors (NPRs) and their downstream signaling pathways. NPR-A,-B,-C: natriuretic peptide receptor type A, B, C; ANP: atrial natriuretic peptide; BNP: brain-type natriuretic peptide; CNP: C-type natriuretic peptide; DNP: Dendroaspis natriuretic peptide; cANF: selective natriuretic peptide type C receptor agonist, cGMP: cyclic guanosine monophosphate; G_i: inhibitory G-protein.

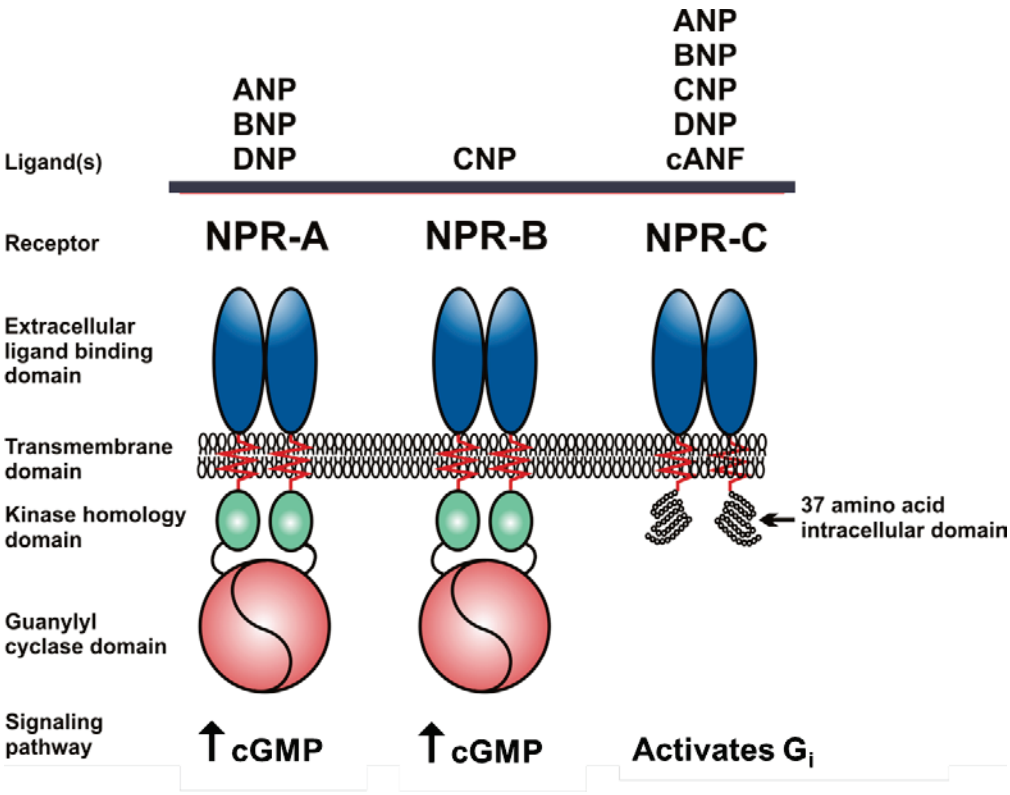

Ligand(s)
ANP
BNP
DNP
CNP
ANP
BNP
CNP
DNP
cANF
Receptor
NPR-A
NPR-B
NPR-C
Extracellular ligand binding domain
Transmembrane domain
Kinase homology domain
37 amino acid intracellular domain
Guanylyl cyclase domain
Signaling pathway
cGMP
cGMP
Activates G_i

Figure 2.

activation and is thought to act as a repressor of the catalytic GC domain in the absence of the ligand (Chinkers and Garber, 1989; Koller *et al.* 1992; Duda *et al*. 1993). In the presence of a ligand, ATP has been shown to act as a positive allosteric regulator of the KHD which facilitates GC stimulation by NPs (Kurose *et al.* 1987; Chinkers *et al.* 1991; Larose *et al.* 1991; Wong *et al.* 1995; Foster and Garbers, 1998). Like other GCs, NPR-A and NPR-B exist as homodimers in their native state and bind NPs with a ratio of 2:1 (Misono *et al.* 2011). The coiled-coil hinge region connects the KHD and GCD and is required for receptor dimerization and GC activation (Wilson and Chinkers, 1995; Potter and Hunter, 2001). The COOH-terminal region of the receptor is the GC enzyme that is responsible for the conversion of GTP to cGMP. A possible mechanism for activation of GC has been proposed by Misono and colleagues who have shown that ligand binding induces rotation of the ECD of the dimerized receptor. Rotation of the ECD reorients the GC domains into the active conformation enabling GC catalysis (Ogawa *et al.* 2004; Qiu *et al.* 2004; Misono *et al.* 2005). While Pandey and colleagues have reported internalization of ligand-receptor complexes following chronic NP exposure *in vitro* (Pandey *et al.* 1986; Pandey, 1993; Pandey *et al.* 2000; Pandey *et al.* 2002), the mechanism behind ligand-dependent internalization and desensitization of NPRs is still under debate. NPR-A and NPR-B are phosphorylated on serine and threonine residues in the KHD domain in basal conditions and it has been suggested that dephosphorylation by protein kinase C (PKC) mediates desensitization of these receptors (Potter and Garbers, 1992; Potter and Garbers, 1994). In contrast, Duda *et al.* (1990) suggested that desensitization of NPRs is correlated with the level of receptor phosphorylation which is

consistent with the mechanism of desensitization of other G-protein-coupled receptors (GPCRs).

1.4.2 NPR-C

NPR-C is the most abundantly expressed NPR and is widely distributed in several tissues and cell types including cardiac myocytes, cardiac fibroblasts, purkinje fibres of the cardiac conduction system, VSM, platetelets, glomeruli, collecting duct, pituitary glands, the cerebral cortex, brain striatum, hypothalamus, adrenal glands, cilary process of the eye, bone and chondrocytes (Anand-Srivastava, 2005; Rose and Giles, 2008). In humans and rats, NPR-C can bind all NPs with the following affinity: ANP≥CNP>BNP (Bennett *et al.* 1991; Suga *et al.* 1992a). Similar to the GC-linked NPRs, NPR-C has a large extracellular ligand binding domain and a single transmembrane spanning region (Porter *et al.* 1990). However, unlike NPR-A and NPR-B, NPR-C is not a guanylyl cyclase linked receptor and contains only a short 37 amino acid intracellular domain (Figure 2). NPR-C is a disulfide-linked homodimer and binds NPs with the ligand to receptor stoichiometry of 1:2 (Anand-Srivastava, 2005). As mentioned previously, NPR-C was commonly referred to as a clearance receptor which lacked a signaling function and whose role was to control the circulating levels of NPs. However, more recently it has been demonstrated that NPR-C is coupled to the inhibition of adenylyl cyclase through activation of G_i proteins (Anand-Srivastava *et al.* 1987; Anand-Srivastava *et al.* 1990; Anand-Srivastava *et al.* 1996). Several G_i activator sequences have been identified within the intracellular domain of NPR-C that inhibits adenylyl cyclase activity in a GTP-dependent manner (Murthy and Makhlouf, 1999; Pagano and Anand-Srivastava, 2001). These sequences are characterized by the presence of two NH_2-terminal basic residues

and a COOH-terminal BBXXB motif where B and X are basic and non-basic resides respectively (Okamoto *et al.* 1990). Zhou *et al*. (2003) demonstrated that a 17 amino acid sequence in the middle of the intracellular domain containing two NH_2-terminal arginine residues ($R^{469}R^{470}$) and a basic COOH-terminal motif ($H^{481}RELR^{486}$) is both sufficient and required for activation of G_i. Currently, several physiological functions involving the inhibition of adenylyl cyclase/cAMP signal transduction system downstream of NPR-C have been identified (as reviewed in Anand-Srivastava, 2005 and Rose and Giles, 2008). In addition to the inhibitory effects on adenylyl cyclase, NPR-C can also activate the β isoform of phospholipase C (PLCβ) through G_i (Pagano and Anand-Srivastava, 2001). Activation of PLCβ leads to the generation of inositol triphosphate (IP_3) and diacylglycerol (DAG) from phosphatidyl inositol bisphosphate (PIP_2) which mediate Ca^{2+} mobilization and activation of PKC respectively.

1.5 Downstream Signaling Pathways of Natriuretic Peptides

The physiological effects of natriuretic peptides are mediated by the second messengers cGMP and cAMP, which are important regulators of cardiac function (Fischmeister *et al*. 2006). Elevation of cGMP in response to activation of GC-linked receptors modulates the activity of several downstream proteins including cGMP-dependent protein kinases (PKG), cGMP binding phosphodiesterases (PDEs) and cyclic nucleotide-gated (CNG) ion channels (Baxter, 2004; Potter *et al.* 2006). These downstream targets are differentially expressed and mediate various physiological effects of natriuretic peptides in different tisuses. There are two types of PKG (a serine threonine kinase) that are denoted PKG I and PKG II (Baxter, 2004). PKG I is a cystolic kinase

expressed in platelets, smooth muscle, cardiomyocytes and the brain while PKG II is a membrane bound kinase expressed in the intestine, kidney, brain, chrondrocytes and bone (Smolenski *et al.* 1998).

Cyclic nucleotide PDEs are enzymes that regulate cyclic nucleotide signaling by degrading cyclic nucleotides into the inactive 5'-nucleotide monophosphates (Potter *et al.* 2006). There are 11 different families of PDEs that are categorized based on their specificity for cGMP and/or cAMP (Beavo, 1995). Multiple isoforms that are both region and species specific exist within each PDE family. In the heart, expression of 7 PDE families has been documented (Zaccolo and Movsesian, 2007). PDE1, PDE2 and PDE3 are dual-specificity enzymes that hydrolyze both cAMP and cGMP; PDE4 and PDE8 hydrolyze cAMP only; and PDE5 and PDE9 selectively hydrolyze cGMP (Zaccolo and Movsesian, 2007). While cGMP is a substrate for PDEs, it also acts as an allosteric regulator of some PDE families (Omori and Kotera, 2007). This allosteric regulation of PDEs by cGMP is complex and creates an important cross-talk between the cGMP and cAMP signaling pathways. PDE2 is activated by cGMP thereby decreasing intracellular cAMP levels, while cGMP inhibits PDE3 activity effectively increasing cAMP concentrations (Omori and Kotera, 2007; Zaccolo and Movsesian, 2007). While these effects may seem contradictory, the sensitivity of PDE3 to cGMP is much greater than that of PDE2 (Zaccolo and Movsesian, 2007). In addition, it is now thought that different PDEs have a unique temporal and spatial localization within myocytes resulting in distinct subcellular microdomains for each PDE (Movsesian, 2002; Maurice et al. 2003; Fischmeister *et al.* 2006; Zaccolo and Movsesian, 2007; Zaccolo, 2011). PDE5 is also activated by cGMP which acts in a feed-forward mechanism to increase cGMP

degradation (Omori and Kotera, 2007; Zaccolo and Movsesian, 2007). The role of PDE5 in the heart under physiological conditions is still under debate, however evidence suggesting enhanced activity of PDE5 in cardiac hypertrophy and heart failure has recently been presented (Zhang and Kass, 2011) Additionally, the major downstream target of cAMP is PKA which also regulates the activity of PDE1, PDE3, PDE4 and PDE5 (Omori and Kotera, 2007). Further adding to the complexity of NP signaling is the inhibition of adenylyl cyclase activity mediated by NPR-C which acts to decrease intracellular levels of cAMP.

Finally, cGMP also regulates a family of non-selective cation channels called cyclic nucleotide-gated (CNG) ion channels. These channels contain a COOH-terminal cyclic nucleotide-binding domain that binds cAMP or cGMP (Potter *et al.* 2006). While it has been shown that CNG channels act as a downstream target of cGMP, currently no natriuretic peptide functions appear to involve the activation of CNG channels (Potter *et al.* 2006).

1.6 Cardiovascular Effects of Natriuretic Peptides

As their name suggests, natriuretic peptides are well known for their natriuretic and diuretic effects on the kidney. However, due to the diverse expression of natriuretic peptides and their receptors, several physiological effects in mulitple organ systems have been attributed to the action of these peptides (Potter *et al.* 2006; Pandey, 2005). The use of genetically engineered animals in which expression of a NP or NPR has been altered has been paramount in identifying several physiological effects of NPs. However, since

our interest is specifically focused on the effects of NPs on cardiac electrophysiology, only effects of NPs on cardiac function will be discussed below.

1.6.1 Chronotropic Effects of NPs

A direct chronotropic effect of NPs was first proposed by Ackermann *et al.* (1984) who demonstrated that infusion of atrial extracts in rats depressed heart rate. Infusion of ANP in rats and dogs produced similar results further suggesting a negative chronotropic effect of ANP (Hirata *et al.* 1985; Ackermann 1986; Gellai *et al.* 1986; Kleinert *et al.* 1986; Allen and Gellai, 1987; Lambert *et al.*1994). In contrast to these findings, it has also been reported by several groups that ANP has little effect on heart rate and sinus node activity in human, canine, rat, and guinea pig models (Bie *et al.* 1988; Shen *et al.* 1991; Woods *et al*. 1989). Recently, Herring *et al.* (2001) demonstrated an increase in heart rate of spontaneously beating guinea pig atrial preparations in response to high doses (100-500 nM) of ANP and suggested that the dose of ANP used in previous studies that demonstrated no change in heart rate was too low to observe an effect. Interestingly, opposing effects of CNP on heart rate were also proposed however the effects appeared to be mediated by different NPRs. Initially, Beaulieu *et al.* (1996) observed a direct positive chronotropic effect of CNP in anesthetized dogs and isolated right atrial preparations. Similar findings were observed by Hirose *et al.* (1998) and Herring *et al.* (2001) who demonstrated that the positive chronotropic effect of CNP was mediated through the guanylyl cyclase linked NPR-B. In contrast, Rose *et al.* (2004) demonstrated a decrease in $I_{Ca,L}$ and spontaneous AP frequency in SAN myocytes in response to CNP and the selective NPR-C agonist cys^{18}-atrial natriuretic factor (cANF). cANF is a synthetic NP with a partial ring deletion that has no capacity to alter guanylyl

cyclase activity via NPR-A/B, but effectively reduces cAMP via NPR-C (Anand-Srivastava *et al.* 1990). These effects on $I_{Ca,L}$ and spontaneous AP frequency were mediated by activation of NPR-C and were only observed in the presence of a saturating dose of the β-adrenergic receptor agonist isoproterenol (ISO) suggesting the level of sympathetic tone affects the chronotropic response of NPs. Recently the Rose laboratory has shown that BNP and CNP dose-dependently increased heart rate in an isolated heart model. This increase in heart rate was associated with increased conduction through the atria and AVN as indicated by a shortened P wave duration and PR interval respectively (Springer *et al.* 2012). Additionally, BNP and CNP increased the spontaneous AP frequency in isolated SAN myocytes in association with increases in both $I_{Ca,L}$ and I_f and this effect was mediated by NPR-A and NPR-B respectively (Springer *et al.* 2012). Interestingly, we have also shown that the chronotropic effects of NPs are complex and condition specific as BNP and CNP can activate NPR-C to inhibit adenylyl cyclase activity and decrease heart rate in the presence of β-adrenergic stimulation (Azer *et al.* 2012). Thus, our recent findings indicate that NPs can either increase or decrease heart rate depending on experimental conditions and which NPRs are activated in these conditions.

Recently, a heterozygous frameshift mutation causing a 12 amino acid extension of ANP has been associated with patients with familial atrial fibrillation (AF), however the direct mechanism by which the mutant form of ANP leads to AF is still not well understood (Hodgson-Zingman *et al.* 2008).

Other Cardiac Effects of NPs

An NPR-A dependent decrease in blood pressure is well documented in the literature. Studies utilizing mice with targeted deletion of ANP or NPR-A results in blood pressures that are 20-40 mmHg higher than normal (Lopez *et al.* 1995; John *et al.* 1996; Oliver *et al.* 1997) while animals that overexpress ANP or BNP are hypotensive with blood pressures 20-30 mmHg lower than normal (Steinhelper *et al.* 1990; Ogawa *et al.* 1994a). ANP mediates a decrease in blood pressure through natriuresis, diuresis, vasorelaxation, increasing endothelium permeability and intravascular volume and through antagonism of the renin-angiotensin system (Potter *et al.* 2009). Interestingly, Mastukawa et al. (1999) reported that mice lacking NPR-C are slightly hypotensive; however the exact mechanism behind this observation is not well understood and experiments conducted in the present study do not support a change in blood pressure in NPR-$C^{-/-}$ mice (see discussion).

In addition to the effects on blood pressure, NPs are actively involved in cardiac remodeling and have antihypertrophic and antifibrotic effects (D`Souza *et al*. 2004; Nishikimi *et al*. 2006; Calvieri *et al*. 2011). These cardioprotective effects of NPs can have major implications in several cardiac diseases including heart failure and cardiac arrhythmias like atrial fibrillation. Mice lacking ANP or NPR-A have enlarged hearts (Steinhelper *et al.* 1990; Kishimoto *et al.* 2001; Knowles *et al.* 2001) while mice that overexpress ANP have smaller hearts than normal (Barbee *et al.* 1994). In contrast, mice lacking BNP do not exhibit cardiac hypertrophy; however there is an increase in ventricular fibrosis that is augmented by pressure overload (Tamura *et al.* 2000). The mechanism of the fibrotic effects of BNP is controversial. BNP has been shown to inhibit

proliferation of cardiac fibroblasts *in vitro* and it is suggested that this effect is mediated by inhibition of mitogen-activated protein kinase (MAPK) or matrix metalloproteinases (MMPs) (Takahashi *et al.* 2003; Kapoun *et al.* 2004). Recently, evidence for a role of the CNP in cardiac remodeling has also been proposed. CNP was shown to downregulate the expression of many hypertrophic genes/transcription factors and displayed more potent antihypertrophic effects than ANP and BNP in cultured cardiomyocytes (Rosenkranz *et al.* 2003; Tokudome *et al.* 2004). Recently, Horio *et al.* (2003) showed that CNP was secreted from cardiac fibroblasts and inhibited collagen synthesis in fibroblasts in a cGMP-dependent manner. Additionally CNP infusion reduced cardiac remodeling in an experimental model of myocardial infarction in rats (Soeki *et al.* 2005). In addition to antiproliferative effects on cardiac fibroblasts, ANP and CNP have been shown to mediate similar effects in vascular smooth muscles cells and could play a role in vascular remodeling (Furuya *et al.* 1991; Hutchinson *et al.* 1997).

Unlike ANP and BNP, CNP does not induce natriuresis and diuresis and instead acts locally as a paracrine/autocrine regulator. CNP has a prominent role in regulating vascular tone and has been shown to be a potent arterial and veno-dilator (Lumsden *et al.* 2010). CNP has been described as an endothelium-derived hyperpolarizing factor (EDHF) (Chauhan *et al.* 2003) and evidence suggests that the arterial vasodilating effects of CNP are mediated by NPR-C while venodilation appears to be mediated by NPR-B (Chauhan *et al.* 2003; Madhini *et al.* 2003; Villar *et al.* 2007).

1.7 Objectives

Natriuretic peptide signaling in the heart is complex and involves multiple receptors and signaling pathways. Previously we have shown that BNP and CNP can increase heart rate and electrical conduction in basal conditions through activation of NPR-A and NPR-B respectively (Springer *et al.* 2012). An increase in cGMP leads to inhibition of PDE3 and in turn increases intracellular cAMP. However, we have also shown that after β-AR stimulation with ISO, activation of NPR-C mediates a decrease in heart rate through inhibition of adenylyl cyclase activity (Azer *et al.* 2012). The roles of these opposing pathways in mediating the effects of NPs on heart rate and electrical conduction *in vivo* with all the regulatory mechanisms intact are not well understood. Therefore the objective of the present study was to further investigate the effects of NPs and different NPRs on heart rate and electrical conduction *in vivo.* Both BNP and CNP were used as agonists so that we could assess the roles of all three NPRs. Additionally, our second objective was to characterize the cardiac function and electrophysiological properties of mice that lack functional NPR-C receptors (NPR-$C^{-/-}$). Heart rate, sinus node function, conduction through the atria and susceptibility to AF were evaluated in mice lacking functional NPR-C receptors and compared to that of wildtype (NPR-$C^{+/+}$) and heterozygous (NPR-$C^{+/-}$) littermates. It is well documented in the literature that NPs decrease blood pressure, a measure of cardiac performance, through their natriuretic and diuretic effects. Blood pressure can modulate heart rate through activation of the baroreceptor reflex system, therefore blood pressure in NPR-$C^{-/-}$ mice was measured and compared to that of NPR-$C^{+/+}$ and NPR-$C^{+/-}$ littermates to evaluate any secondary effects of blood pressure on heart rate. As previously mentioned, a mutation in the natriuretic

peptide system has been associated with a familiar form of AF (Hodgson-Zingman *et al.* 2008). This finding, in addition to our previous data suggests that natriuretic peptides modulate electrical conduction through the heart and provides the basis of studying the effects of NPs on the susceptibility to atrial arrhythmias.

1.8 Hypotheses

In accordance with previous studies *in vitro* we hypothesize that acute injection of BNP and CNP will increase heart rate and electrical conduction *in vivo.*We have previously shown that activation of NPR-C by its selective agonist cANF decreases heart rate and electrical conduction following stimulation of the β-AR receptors with ISO. Since all mammels have some level of sympathetic tone we hypothesize that stimulation of NPR-C by cANF *in vivo* will decrease heart rate and electrical conduction without prior stimulation of adenylyl cyclase activity. As already described, we have shown that NPs modulate heart rate and atrial electrical conduction in isolated hearts and that this effect involves NPR-C. Therefore we hypothesize that heart rate, sinus node function and susceptibility to atrial arrhythmias will be altered in mice lacking functional NPR-C receptors. Finally we hypothesize that in the absence of NPR-C, blood pressure will be reduced due to enhanced activity of endogenous natriuretic peptides.

CHAPTER 2: MATERIALS AND METHODS

2.1 Experimental Animals

2.1.1 C57BL/6 Mice

All mice used to study the effects of acute injection of natriuretic peptides on heart rate and electrical conduction were male wildtype C57BL/6 (Charles River Laboratories) mice between the ages of 10-15 weeks. This study used only male mice in order to eliminate any inherent sex differences due to the estrous cycle. These mice weighed between 20-35g and were housed at a maximum of five mice per cage at room temperature in the Carlton Animal Care facility at Dalhousie University. All mice were maintained on a 12-hour light/dark cycle and were fed standard rodent chow which was provided *ad libitum.* All experimental protocols were in accordance with the regulations of The Canadian Council on Animal Care and were approved by Dalhousie University.

2.1.2 NPR-C Mutants

To establish the role of NPR-C on heart rate and cardiac electrophysiological parameters, a mutant mouse lacking functional NPR-C receptors (NPR-$C^{-/-}$) was used (Jaubert *et al.* 1999). These mice were obtained from the Jackson Laboratory (strain B6;C-Npr3^{lgj}/J) and backcrossed into a C57BL/6 background for more than 10 generations. A 36 base pair deletion at position 195-232 on chromosome 15 produces a non-functional NPR-C protein that is truncated by 12 amino acids in the extracellular domain (Jaubert *et al.* 1999). NPR-C mice were genotyped using ear punch biopsies and housed in identical living conditions to C57BL/6 mice. All NPR-C mice used in experiments were male and between the ages of 9-18 weeks.

2.2 Pharmacological Compounds and Delivery Methods

BNP-32 (rat) trifluoroacetate, CNP-32-53, (human, porcine, rat) and (Cys^{18})-Atrial Natriuretic Factor (4-18) amide (cANF; rat) were obtained from Bachem, dissolved in double distilled water to produce 100 μM stock solutions and stored at -80°C until time of use. At the time of injection, saline (0.9% NaCl) was added to the peptide solution to bring the final volume to 100 μl.

Atropine sulfate salt hydrate and propranolol hydrochloride (Sigma-Aldrich) were utilized as antagonists of muscarinic acetylcholine receptors and β-adrenergic receptors respectively. Fresh stock solutions of 1 mg/ml were made daily by dissolving atropine and propranolol in double distilled water.

Table 1. Dose and delivery method of pharmacological compounds used for acute NP injections.

Pharmacological Compound	Dose	Mode of Delivery
CNP	10 μg/kg & 100 μg/kg	Intravenous (IV) via tail vein
BNP	100 μg/kg	IV via tail vein
cANF	100 μg/kg	IV via jugular vein
Atropine & Propranolol	10 mg/kg	Intraperitoneal (IP)

2.3 Experimental Approaches

2.3.1 Surface and Intracardiac ECG Recordings

Mice were anesthetized (2-2.5% isoflurane inhalation in 0.9 L/min O_2) and placed on a heating pad to maintain normal body temperature (37 ± 1 °C) which was monitored

using a rectal thermometer. Surface ECGs were recorded using three 30 gauge subdermal needle electrodes (Grass Technologies) inserted in a lead II position. For intracardiac ECGs a 1.1 French octapolar electrode electrophysiology catheter (Transonic Scisense Inc, London ON) was inserted into the right jugular vein of anesthetized mice (3% isoflurane in 1.0 L/min O_2) and advanced through the right atrium into the right ventricle via the tricuspid valve. All surface and intracardiac ECG signals were recorded, amplified and filtered between 0.3 Hz and 300 Hz using a Gould ACQ-7700 amplifier and the Ponemah Physiology Platform software (version 4.60, Data Sciences International). Standard ECG parameters including P wave duration, PR interval, QRS complex duration, QT interval and R-R interval were analyzed from surface ECG recordings. PR interval was measured from the start of the P wave to the peak of the R wave. QT interval was measured from the start of the QRS wave to the end of the T wave. Figure 3 illustrates a representative mouse ECG trace with waves and intervals labelled. The QT interval was corrected for heart rate according to Bazett`s formula: $QTc = QT/\sqrt{RR}$ (Bazett, 1920).

To measure intracardiac ECG parameters including sinus node recovery time (SNRT), atrial effective refractory period (AERP) and atrioventricular node effective refractory period (AVNERP) of NPR-$C^{-/-}$ mice (and littermate controls), specific stimulation protocols were used (see Figures 4-6). All stimulation pulses were given at 0.5 mA for 2 ms and the basic cycle length for stimulation was 100 ms or 20 ms shorter than the mouse basic cycle length, whichever value was shorter. SNRT at a basic cycle length of 100 ms ($SNRT_{100}$) was measured following a 12 stimulus drive train with a basic cycle length of 100 ms. SNRT is defined as the time between the last stimulus in

Figure 3.

A representative mouse ECG trace illustrating the standard ECG parameters measured by surface ECG. All intervals are measured in ms.

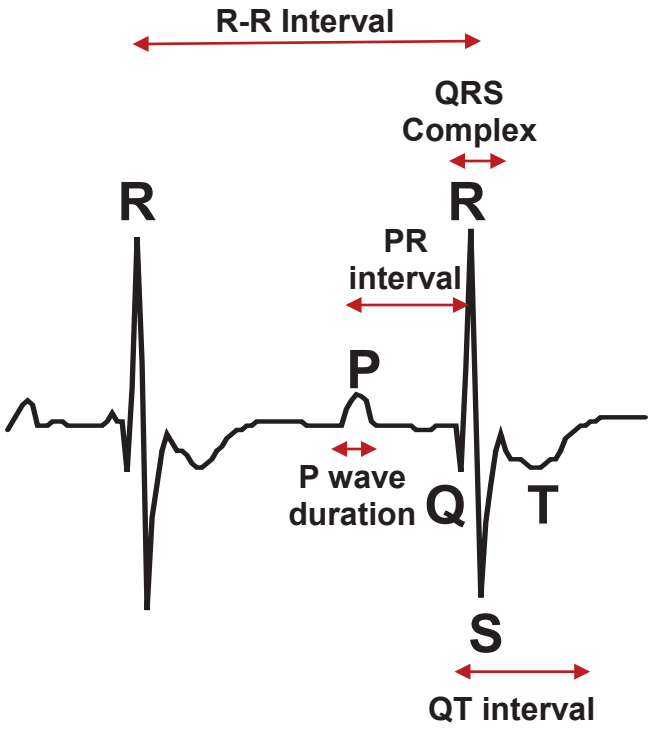

R-R Interval
QRS
Complex
R
R
PR
interval
P
P wave
duration
Q
T
S
QT interval

Figure 3.

the drive train and the onset of the first spontaneous sinus beat (designated by the start of the P wave). To correct for heart rate, corrected SNRT (cSNRT) was calculated by subtracting the prestimulus R-R interval from the measured SNRT (cSNRT= SNRT-RR; Figure 4). An 8 stimulus drive train (S1) at a cycle length of 100 ms was followed by a prestimulus (S2) at progressively shorter cycle lengths to determine the AERP (Figure 5) and AVNERP (Figure 6). To assess the inducibility of atrial fibrillation (AF) in NPR-$C^{-/-}$ mice, a three burst pacing protocol was used as described in Fukui *et al*. (2013). AF was defined by rapid and irregular atrial rhythm in association with irregular R-R intervals that persisted for at least 1 s. The duration of AF was measured from the end of the last stimulus of the burst pacing to the first P wave upon spontaneous reversion to normal sinus rhythm.

2.3.2 Blood Pressure

Blood Pressure was measured using a Mouse Rat Tail Cuff Blood Pressure System (IITC Life Science). Mice were trained in the apparatus for two consecutive days prior to three days of recording. Systolic pressure and mean arterial pressure (MAP) were measured in conscious mice at 33°C. The diastolic pressure was calculated from the equation: Diastolic Pressure= (3 x MAP-systolic pressure)/2.

2.4 Statistical Analysis

All graphs were constructed using GraphPad Prism (version 4.01; GraphPad Software, Inc., La Jolla, CA). Statistical analyses were performed using SigmaPlot (version 11.0; Systat Software, Inc., San Jose, CA). Paired Student`s *t*-tests were used for

comparison of single parameter means. Differences between means were also evaluated by one-way analysis of variance (ANOVA), or one-way repeated measures ANOVA followed by Tukey's post-hoc test when appropriate. In the event of a failed normality test, a one-way ANOVA on ranks was performed followed by a Dunn`s post hoc test when appropriate. For comparing proportions of multiple groups, a Fisher`s exact test was used. All summary data are presented as mean ± SEM. In all cases, $P<0.05$ was considered statistically significant.

Figure 4.

Assessment of sinoatrial node function *in vivo* by determination of sinoatrial node recovery time (SNRT). An octapolar catheter was used to deliver 12 stimulated beats to the atria and the interval between the last stimulated beat and the P wave of the first spontaneous sinus beat was measured. In this example, it took 144 ms for the first P wave to appear after the last stimulated beat. SNRT is corrected by subtracting the intrinsic RR interval during normal sinus rhythm (108 ms in the above example) for a corrected SNRT (cSNRT) of 36 ms.

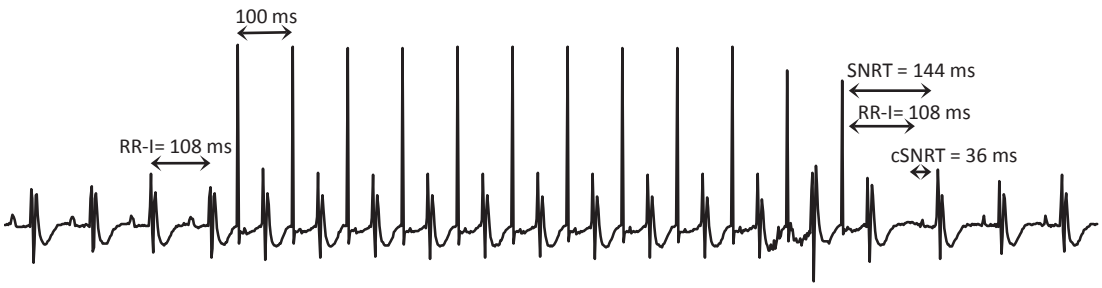


Figure 4.

Figure 5.

Determination of atrial effective refractory period (AERP) *in vivo* by delivering an 8 stimulus drive train (S1; cycle length= 100 ms) followed by a premature stimulus (S2) in progressively reduced increments from electrodes located in the atria above the AVN. In this example, an S1-S2 interval of 38 ms (panel A) results in an atrial activation, however as shown in panel B, a reduction of the S1-S2 interval to 36 ms does not result in an atrial activation. Therefore, AERP= 38 ms.

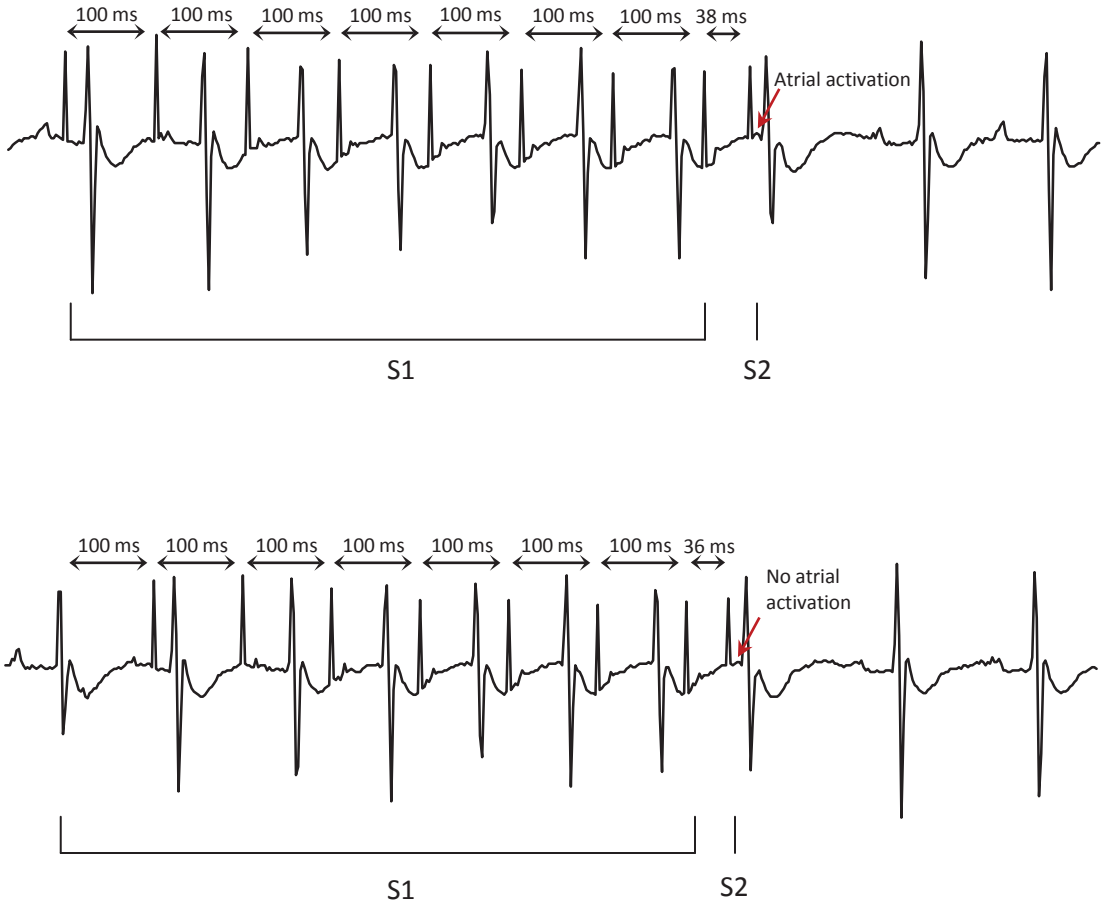

100 ms
100 ms
100 ms
100 ms
100 ms
100 ms
100 ms
38 ms
Atrial activation
S1
S2
100 ms
100 ms
100 ms
100 ms
100 ms
100 ms
100 ms
36 ms
No atrial activation
S1
S2

Figure 5.

Figure 6.

Determination of atrioventricular node effective refractory period (AVNERP) *in vivo* by delivering an 8 stimulus drive train (S1; cycle length= 100 ms) followed by a premature stimulus (S2) in progressively reduced increments from electrodes located in the atria above the AVN. In this example, an S1-S2 interval of 50 ms (panel A) results in a conducted QRS complex, however as shown in panel B, a reduction of the S1-S2 interval to 48 ms results in a loss of conducted QRS complex. Therefore, AVNERP= 50 ms.

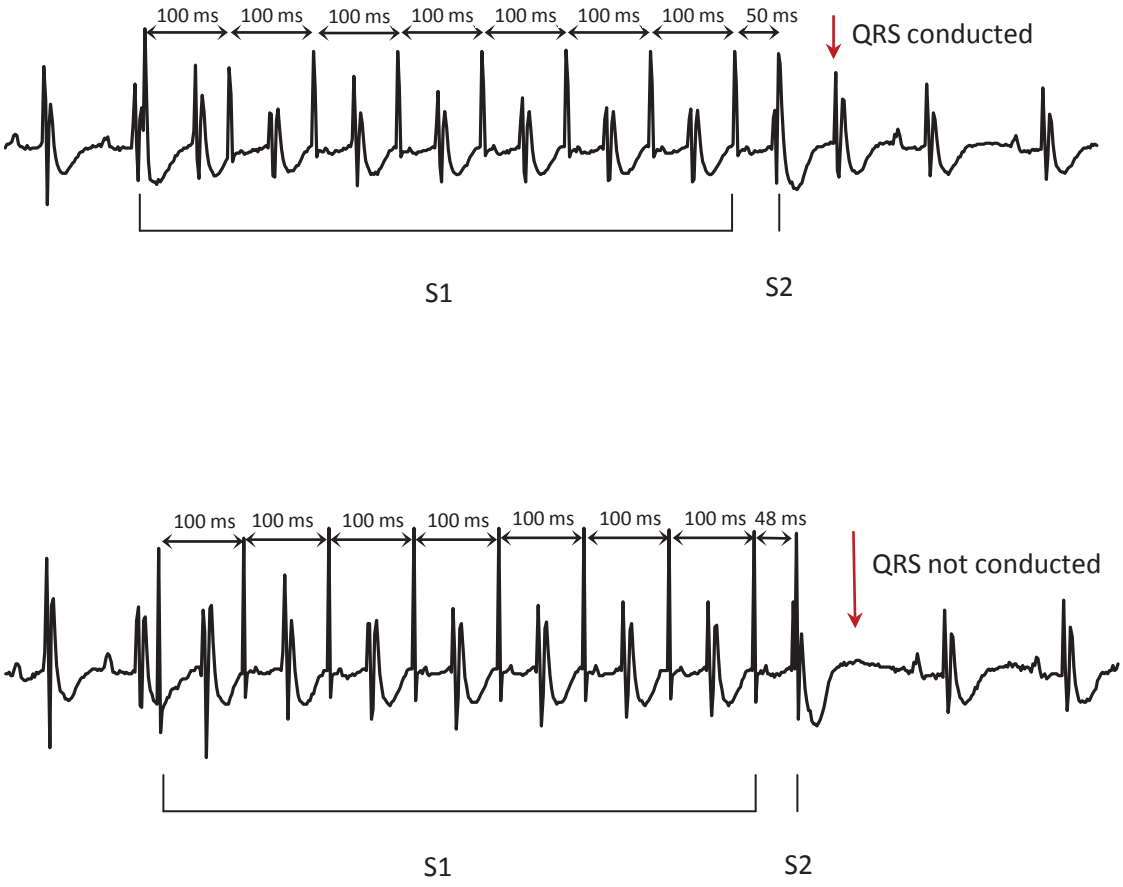

100 ms
100 ms
100 ms
100 ms
100 ms
100 ms
100 ms
50 ms
QRS conducted
S1
S2
100 ms
100 ms
100 ms
100 ms
100 ms
100 ms
100 ms
48 ms
QRS not conducted
S1
S2

Figure 6.

CHAPTER 3: RESULTS

3.1 Effects of CNP on Heart Rate and Electrical Conduction *in vivo*

In the first set of experiments to evaluate the electrophysiological effects of NPs *in vivo,* the effects of CNP on heart rate and electrical conduction were measured in anesthetized mice. Heart rate and ECG parameters were analyzed before and after intravenous injection of CNP (10 and 100 μg/kg doses) or an equal volume of saline as a control. Acute intravenous injection of CNP in basal conditions rapidly and reversibly increased heart rate in a dose-dependent manner (Figures 7 and 8). At a dose of 10 μg/kg, CNP increased heart rate by approximately 40 beats/min (483 ± 27 beats/min at baseline vs. 521 ± 24 beats/min after CNP, $P<0.05$; $n=8$) as shown in Figure 7. This increase in heart rate occurred in conjunction with a shortening of the QT interval (45.70 ± 0.74 ms at baseline vs. 44.40 ± 0.79 ms after CNP, $P<0.05$). A summary of all ECG parameters before and after infusion of CNP is presented in Table 2.

Figure 8 demonstrates that a higher dose of CNP (100μg/kg) increased heart rate by approximately 80 beats/min (500 ± 16 beats/min at baseline vs. 580 ± 18 beats/min after CNP, $P<0.05$; $n=7$). This increase in heart rate was associated with a shortening of the PR interval (38.63 ± 1.03 ms at baseline vs. 37.20 ± 1.05 ms after CNP, $P<0.05$) and QT interval (47.20 ± 1.45 ms at baseline vs. 43.94 ± 0.84 ms after CNP, $P<0.05$) as summarized in Table 3. Control experiments demonstrated that injection of saline had no effect on heart rate (565 ± 20 beats/min at baseline vs. 564 ± 21 beats/min, $P<0.05$; $n=11$; data not shown).

Figure 7.

Effect of CNP (10 μg/kg IV) on heart rate in anesthetized mice. **A.** Representative ECG traces (1 s duration) in baseline conditions and following injection of CNP. R-R intervals indicated in scale bars. **B.** Representative time course of the effect of CNP on heart rate. **C.** Summary data illustrating the effect of CNP on heart rate. Data are means ± SEM; n=8 mice per group. *P<0.05 vs. baseline by paired Student's t-test. See Table 2 for analysis of additional ECG parameters.

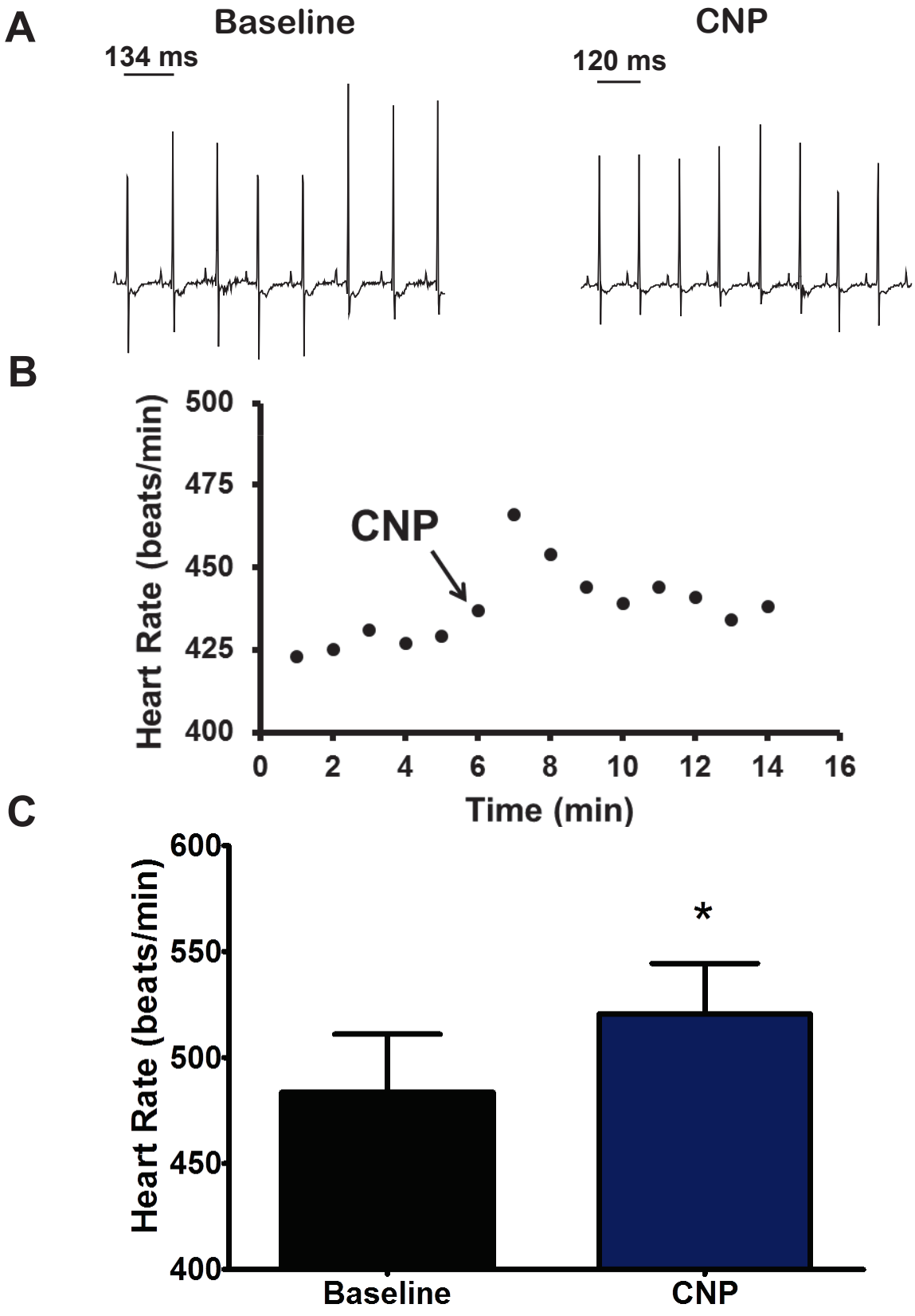

A
Baseline
CNP
134 ms
120 ms
B
Heart Rate (beats/min)
500
475
450
425
400
CNP
0
2
4
6
8
10
12
14
16
Time (min)
C
Heart Rate (beats/min)
600
550
500
450
400
*
Baseline
CNP

Figure 7.

TABLE 2:

Summary of ECG parameters following intravenous injection of CNP (10 μg/kg) in anesthetized mice.

	Baseline	**CNP**
Heart Rate (beats/min)	483 ± 27	521 ± 24*
R-R interval (ms)	127.45 ± 6.89	119.05 ± 5.58*
P wave duration (ms)	18.20 ± 0.47	18.00 ± 0.48
PR interval (ms)	39.35 ± 0.43	39.15 ± 0.60
QRS Complex (ms)	12.20 ± 0.43	12.00 ± 0.52
QT interval (ms)	45.70 ± 0.74	44.40 ± 0.79*
QTc interval (ms)	128.87 ± 3.20	129.26 ± 2.69

PR interval was measured from the start of the P wave to the peak of the QRS complex, QT interval was measured from the start of the QRS complex to the end of the T wave. Data are means ± SEM; n=8 mice per group. *P<0.05 vs. baseline by paired Student's t-test.

Figure 8.

Effect of CNP (100 μg/kg IV) on heart rate in anesthetized mice. **A.** Representative ECG traces (1 s duration) in baseline conditions and following infusion of CNP. R-R intervals indicated in scale bars. **B.** Representative time course of the effect of CNP on heart rate. **C.** Summary data illustrating the effect of CNP on heart rate. Data are means ± SEM; n=7 mice per group. *P<0.05 vs. baseline by paired Student's t-test. See Table 3 for analysis of additional ECG parameters.

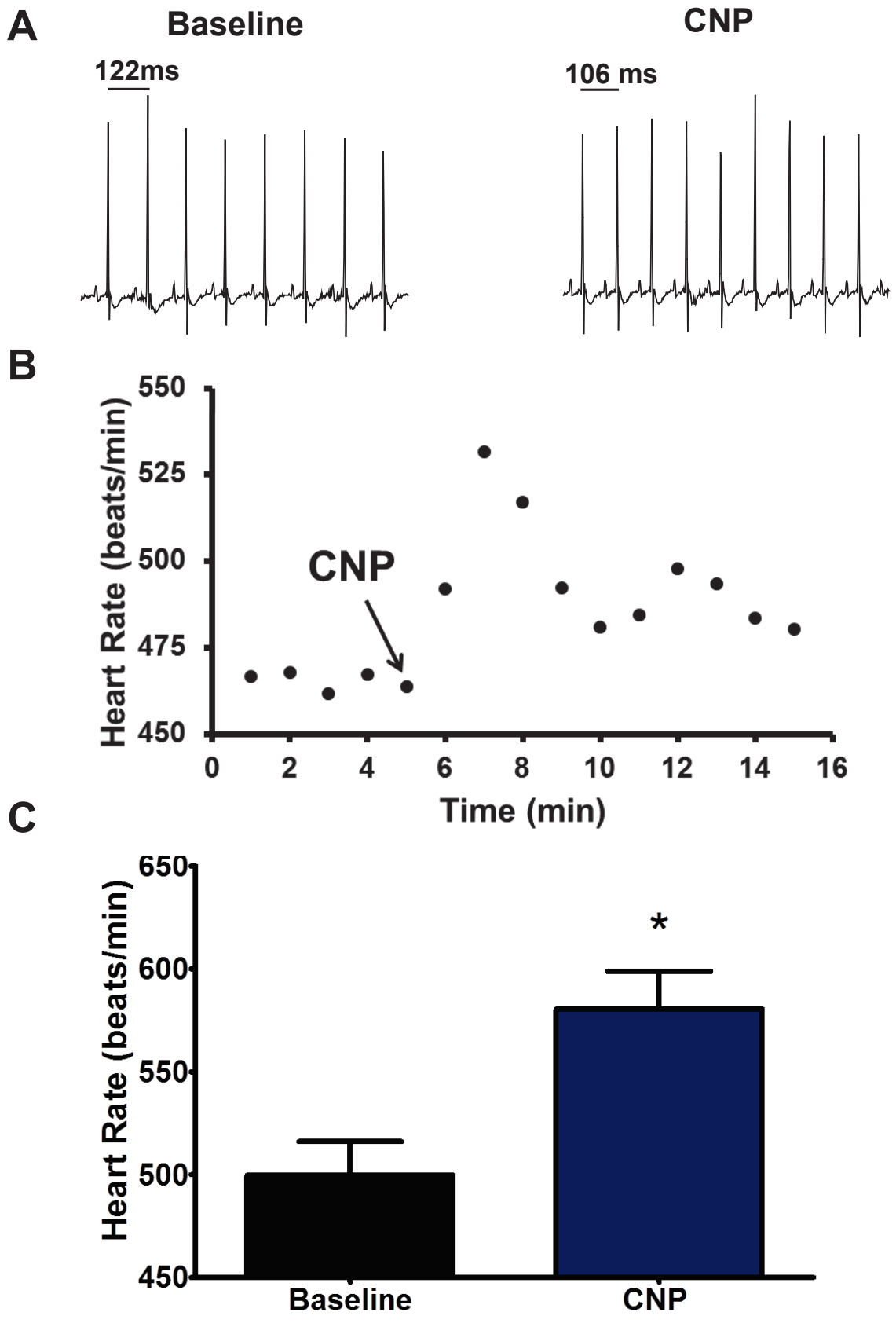

A
Baseline
CNP
122ms
106 ms
B
Heart Rate (beats/min)
550
525
500
475
450
CNP
0
2
4
6
8
10
12
14
16
Time (min)
C
Heart Rate (beats/min)
650
600
550
500
450
*
Baseline
CNP

Figure 8.

TABLE 3:

Summary of ECG parameters following intravenous injection of CNP (100 μg/kg) in anesthetized mice.

	Baseline	**CNP**
Heart Rate (beats/min)	500 ± 16	580 ± 18*
R-R interval (ms)	122.57 ± 3.81	105.14 ± 3.67*
P wave duration (ms)	18.40 ± 0.46	18.29 ± 0.42
PR interval (ms)	38.63 ± 1.03	37.20 ± 1.05*
QRS Complex (ms)	12.63 ± 0.65	12.80 ± 0.74
QT interval (ms)	47.20 ± 1.45	43.94 ± 0.84*
QTc interval (ms)	134.90 ± 3.31	135.73 ± 2.21

PR interval was measured from the start of the P wave to the peak of the QRS complex, QT interval was measured from the start of the QRS complex to the end of the T wave. Data are means ± SEM; n=7 mice per group. *P<0.05 vs. baseline by paired Student's t-test.

3.2 Effects of CNP on Heart Rate and Electrical Conduction Following Autonomic Nervous System Blockade

The above findings demonstrate that CNP increases heart rate *in vivo;* however, regulation of heart rate *in vivo* is complex and can be modulated by the autonomic nervous system in addition to intrinsic changes in sinoatrial node activity. To assess whether the increase in heart rate observed with CNP was due to changes in autonomic nervous system activity or due to direct effects on the sinoatrial node, CNP was infused following autonomic nervous system blockade by atropine and propranolol. As illustrated in Figures 9 and 10, intraperitoneal injection of atropine (10 mg/kg; parasympathetic antagonist) and propranolol (10 mg/kg; sympathetic antagonist) rapidly decreased heart rate which is consistent with a higher level of sympathetic tone than parasympathetic tone in the mouse (Gehrmann *et al*. 2000; Just *et al*. 2000; Janssen and Smits 2002; Rose *et al*. 2007).

Similar to basal conditions, CNP (10 μg/kg and 100 μg/kg) rapidly and reversibly increased heart rate in the absence of sympathetic and parasympathetic input (Figures 9 and 10). At a dose of 10μg/kg, CNP increased heart rate by approximately 15 beats/min (Figure 9); however, this did not reach statistical significance (405 ± 8 beats/min after autonomic nervous system blockade vs. 421 ± 9 beats/min after CNP, P=0.566). Following autonomic nervous system blockade, the PR interval and QT interval were prolonged relative to baseline (P<0.05) but were unaffected by CNP (Table 4).

Figure 10 illustrates that at a dose of 100μg/kg, CNP increased heart rate by approximately 30 beats/min (473 ± 15 beats/min after autonomic nervous system blockade vs. 505 ± 15 beats/min after CNP, P<0.05; n=7). A summary of the ECG

Figure 9.

Effect of CNP (10 μg/kg IV) on heart rate in anesthetized mice following inhibition of the autonomic nervous system by atropine and propranolol (10 mg/kg IP). **A.** Representative ECG traces (1 s duration) at baseline, after autonomic nervous system blockade and following injection of CNP. R-R intervals indicated in scale bars. **B.** Representative time course of the effects of autonomic nervous system blockade and infusion of CNP on heart rate. **C.** Summary data illustrating the effect of autonomic nervous system blockade and CNP on heart rate. Data are means ± SEM; n=6 mice per group. *P<0.05 vs baseline by repeated measures One-way ANOVA and a Tukey's post-hoc test. See Table 4 for analysis of additional ECG parameters.

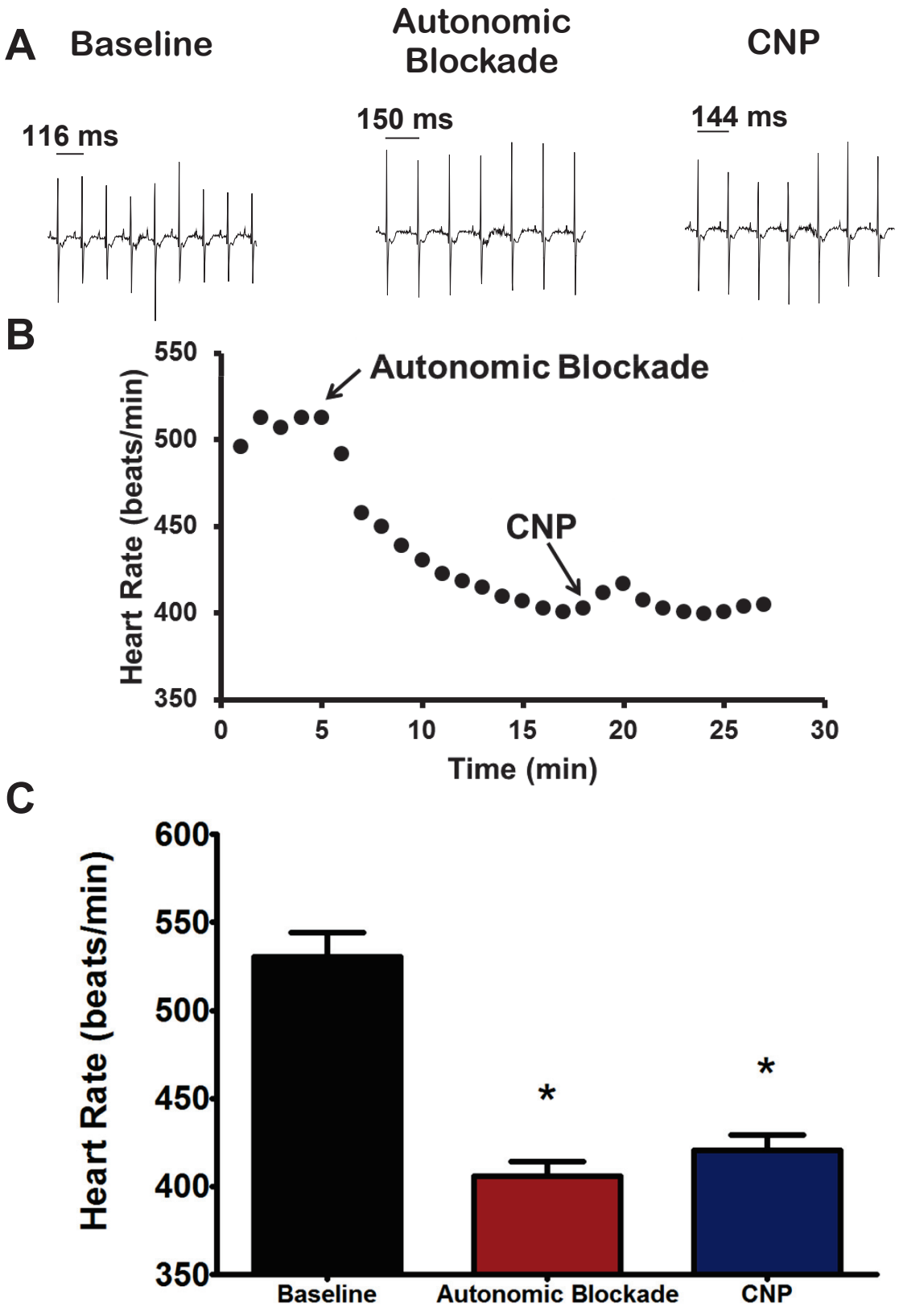

A
Baseline
Autonomic Blockade
CNP
116 ms
150 ms
144 ms
B
Autonomic Blockade
CNP
Heart Rate (beats/min)
550
500
450
400
350
0
5
10
15
20
25
30
Time (min)
C
Heart Rate (beats/min)
600
550
500
450
400
350
*
*
Baseline
Autonomic Blockade
CNP

Figure 9.

TABLE 4.

Summary of ECG parameters following autonomic nervous system blockade and intravenous injection of CNP (10 μg/kg) in anesthetized mice.

	Baseline	**Autonomic Blockade**	**CNP**
Heart Rate (beats/min)	530 ± 14	406 ± 8*	421 ± 9*
R-R Interval (ms)	113.67 ± 2.80	149.13 ± 2.92*	143.27 ± 2.82*
P wave Duration (ms)	16.40 ± 0.44	17.00 ± 0.48	17.13 ± 0.41
PR Interval (ms)	37.33 ± 1.24	44.93 ± 0.93*	44.53 ± 1.13*
QRS Complex (ms)	15.27 ± 0.50	15.40 ± 0.37	15.73 ± 0.41
QT Interval (ms)	44.67 ± 0.88	49.80 ± 0.97*	49.13 ± 1.16*
QTc Interval (ms)	132.64 ± 2.99	129.03 ± 2.65	129.85 ± 2.89

PR interval was measured from the start of the P wave to the peak of the QRS complex, QT interval was measured from the start of the QRS complex to the end of the T wave. Data are means ± SEM; n=6 mice per group. *P<0.05 vs. baseline by repeated measures One-way ANOVA and a Tukey's post-hoc test.

Figure 10.

Effect of CNP (100 μg/kg IV) on heart rate in anesthetized mice following inhibition of the autonomic nervous system by atropine and propranolol (10 mg/kg IP). **A.** Representative ECG traces (1 s duration) in baseline, autonomic nervous system blockade and following injection of CNP. R-R intervals indicated in scale bars. **B.** Representative time course of the effects of autonomic nervous system blockade and infusion of CNP on heart rate. **C.** Summary data illustrating the effect of autonomic nervous system blockade and CNP on heart rate. Data are means ± SEM; n=7 mice per group. *P<0.05 vs. baseline, †P<0.05 vs. autonomic blockade by repeated measures One-way ANOVA and a Tukey's post-hoc test. See Table 5 for analysis of additional ECG parameters.

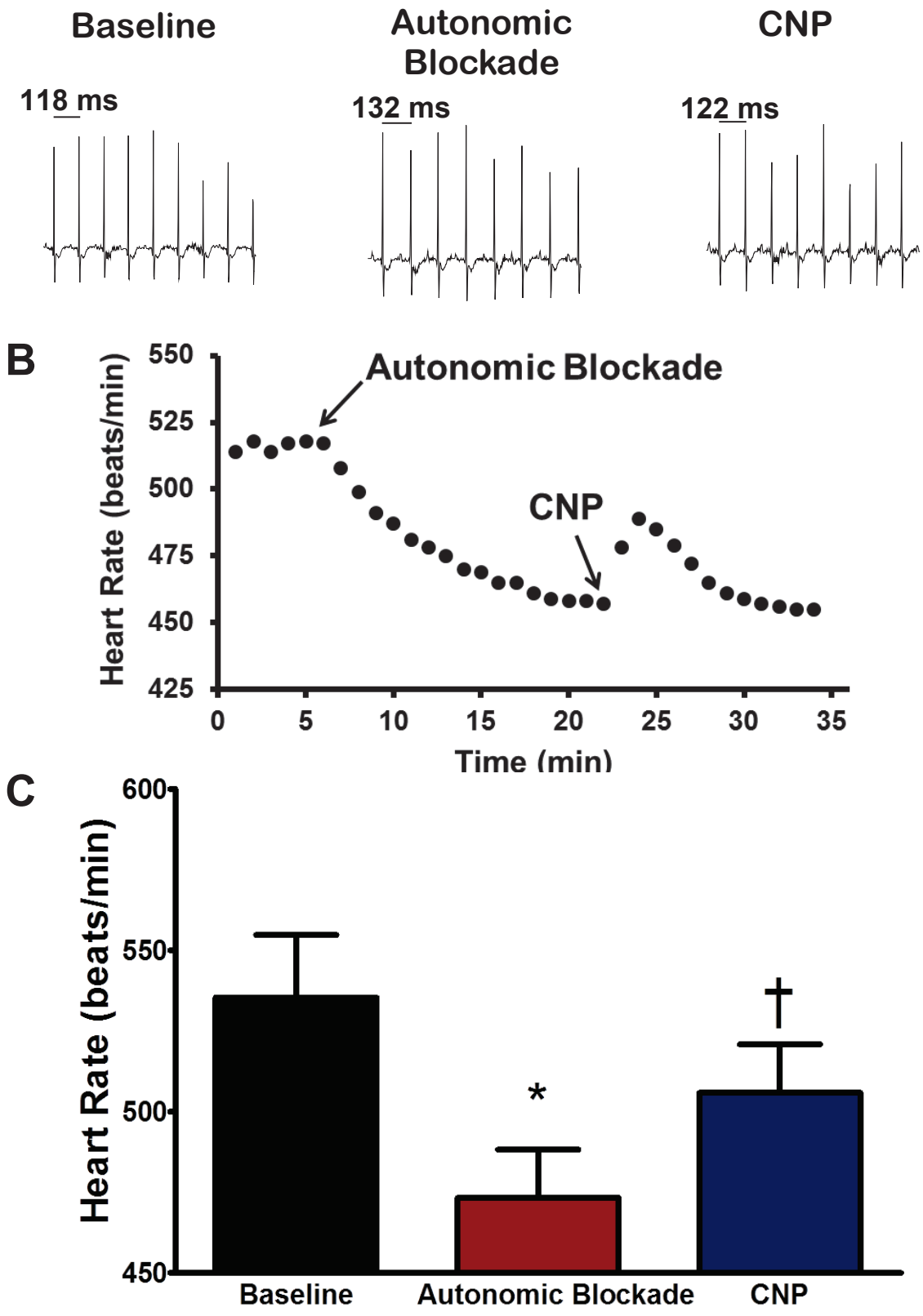

Baseline
Autonomic Blockade
CNP
118 ms
132 ms
122 ms
B
Heart Rate (beats/min)
550
525
500
475
450
425
Autonomic Blockade
CNP
0
5
10
15
20
25
30
35
Time (min)
C
Heart Rate (beats/min)
600
550
500
450
*
†
Baseline
Autonomic Blockade
CNP

Figure 10.

TABLE 5.

Summary of ECG parameters following autonomic nervous system blockade and intravenous infusion of CNP (100 μg/kg) in anesthetized mice.

	Baseline	**Autonomic Blockade**	**CNP**
Heart Rate (beats/min)	535 ± 19	473 ± 15*	506 ± 15†
R-R Interval (ms)	113.20±4.63	127.77±4.13*	119.83±3.66†
P wave Duration (ms)	16.51 ± 0.82	16.69 ± 0.56	16.40 ± 0.59
PR Interval (ms)	40.69 ± 0.80	46.06 ± 1.67*	45.65 ± 1.09*
QRS Complex (ms)	13.14 ± 0.68	14.00 ± 0.62	13.94 ± 0.49
QT Interval (ms)	41.26 ± 0.94	45.94 ± 0.55*	45.09 ± 0.68*
QTc Interval (ms)	123.21 ± 3.32	128.81 ± 2.41*	130.57 ± 2.64*

PR interval was measured from the start of the P wave to the peak of the QRS complex, QT interval was measured from the start of the QRS complex to the end of the T wave. Data are means ± SEM; n=7 mice per group. *P<0.05 vs. baseline, †P<0.05 vs. autonomic blockade by repeated measures One-way ANOVA and a Tukey's post-hoc test.

parameters is shown in Table 5. Following autonomic nervous system blockade the PR interval, QT interval were prolonged ($P<0.05$) compared to baseline conditions. These values were not altered after injection of CNP.

3.3 Effects of BNP Infusion on Heart Rate and Electrical Conduction *in vivo*

We have previously shown that BNP has comparable effects on heart rate to CNP in isolated hearts and isolated sinoatrial node myocytes (Springer et al. 2012). Accordingly, we next investigated the effects of BNP injection on heart rate and electrical conduction *in vivo*. As illustrated in Figure 11, acute intravenous injection of BNP (100 μg/kg) rapidly and reversibly increased heart rate by approximately 35 beats/min (539 ± 8 beats/min at baseline vs. 576 ± 7 beats/min after BNP, $P<0.05$; n=8). Infusion of BNP also shortened the PR interval (39.20 ±0.70 ms at baseline vs. 38.15 ±0.68 ms after BNP injection, $P<0.05$) and QT interval (43.00 ± 0.64 to 41.05 ± 0.64 ms, $P<0.05$). See Table 6 for analysis of all ECG parameters.

3.4 Effect of the NPR-C Agonist cANF on Heart Rate and Electrical Conduction *in vivo*

The next set of experiments was designed to specifically investigate the role of NPR-C in the heart. Originally, NPR-C was classified as a clearance receptor that had no signaling function and served only to control the circulating levels of NPs (Maack *et al*. 1987). It is now clear; however, that NPR-C is coupled to the activation of inhibitory G proteins (G_i) and activation of this receptor leads to a reduction in cAMP levels (Anand-

Figure 11.

Effect of BNP (100 μg/kg IV) on heart rate in anesthetized mice. **A.** Representative ECG traces (1 s duration) in baseline conditions and following infusion of BNP. R-R intervals indicated in scale bars. **B.** Representative time course of the effect of BNP on heart rate. **C.** Summary data illustrating the effect of BNP on heart rate. Data are means ± SEM; n=7 mice per group. *P<0.05 vs. baseline by paired Student's t-test. See Table 6 for analysis of additional ECG parameters.

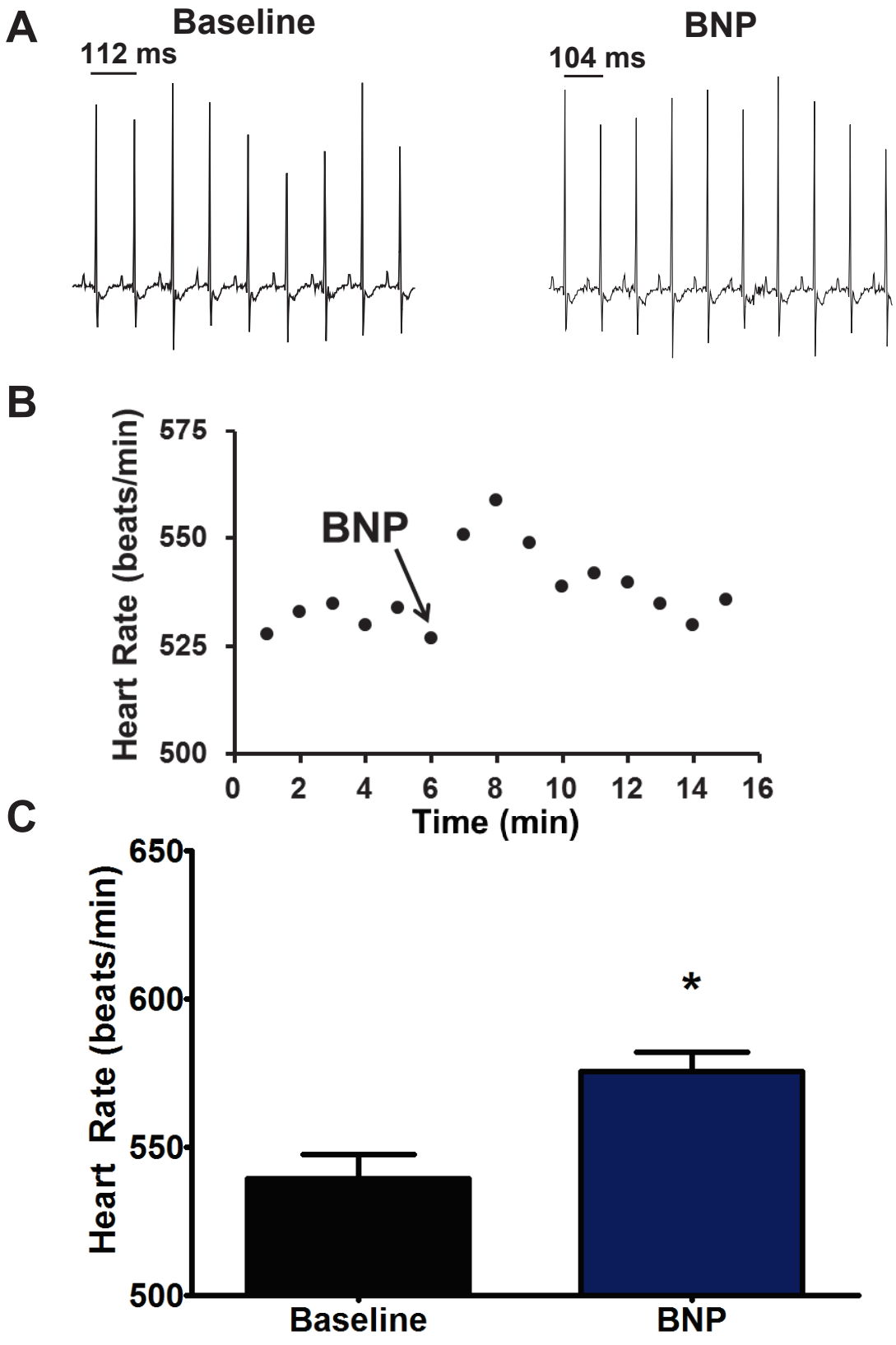

A
Baseline
112 ms
BNP
104 ms
B
Heart Rate (beats/min)
575
550
525
500
BNP
0
2
4
6
8
10
12
14
16
Time (min)
C
Heart Rate (beats/min)
650
600
550
500
*
Baseline
BNP

Figure 11.

TABLE 6.

Summary of ECG parameters following intravenous injection of BNP (100 μg/kg) in anesthetized mice.

	Baseline	**BNP**
Heart Rate (beats/min)	539 ± 8	576 ± 7*
R-R interval (ms)	111.90 ± 1.67	104.80 ± 1.18*
P wave duration (ms)	19.05 ± 0.39	18.9 ± 0.34
PR interval (ms)	39.20 ± 0.70	38.15 ± 0.68*
QRS Complex (ms)	14.30 ± 1.12	14.10 ± 1.09
QT interval (ms)	43.00 ± 0.64	41.05 ± 0.64*
QTc interval (ms)	128.64 ± 2.29	126.86 ± 2.17

PR interval was measured from the start of the P wave to the peak of the QRS complex, QT interval was measured from the start of the QRS complex to the end of the T wave. Data are means ± SEM; n=8 mice per group. *$p<0.05$ vs. baseline by paired Student's *t*-test.

Srivastava *et al*. 1996; Pagano and Anand-Srivastava, 2001; Zhou and Murthy, 2003). In order to specifically study the contribution of NPR-C, a selective NPR-C agonist, cANF is utilized. We have demonstrated that cANF causes a decrease in heart rate and spontaneous AP frequency in isolated heart preparations and isolated SAN cells respectively in the presence of the β-adrenergic receptor agonist isoproterenol (ISO) (Rose *et al.* 2004; Azer *et al.* 2012). The effects of NPR-C activation on heart rate and electrical conduction *in vivo* are not known, thus we next studied the effects of intravenous cANF injection on heart rate and ECG parameters (Figure 12).

Initially we tried injection of cANF through the tail vein as we had done with BNP and CNP but could not obtain clear results therefore we altered the route of delivery of the peptide. Acute injection of cANF (100 μg/kg) through the jugular vein had the opposite effect to the native peptides (BNP and CNP) whereby it rapidly and reversibly decreased heart rate by approximately 40 beats/min (533 ± 16 beats/min at baseline vs. 493 ± 18 beats/min after cANF, $P<0.05$; n=7). This decrease in heart rate was associated with a prolongation of the QT interval (42.17 ± 2.06 ms at baseline vs. 44.46 ± 1.85 ms after cANF). A summary of additional ECG parameters is shown in Table 7.

3.5 Heart Rate and Electrical Conduction in Mice Lacking Functional NPR-C Receptors (NPR-C$^{-/-}$)

As mentioned above, the role of NPR-C is quite controversial and its role in the heart is not well understood. The above experiments using cANF, in addition to our previous work in isolated cells (Azer *et al.* 2012)*;* demonstrate that NPR-C can be activated

Figure 12.

Effect of the natriuretic peptide receptor C agonist, cANF (100 μg/kg iv) on heart rate in anesthetized mice. **A.** Representative ECG traces (1 s duration) in baseline conditions and following injection of cANF. R-R interval indicated in scale bars. **B.** Representative time course of the effect of cANF on heart rate. **C.** Summary data illustrating the effect of cANF on heart rate. Data are means ± SEM; n=7 mice per group. *P<0.05 vs. baseline by paired Student's t-test. See Table 7 for analysis of additional ECG parameters.

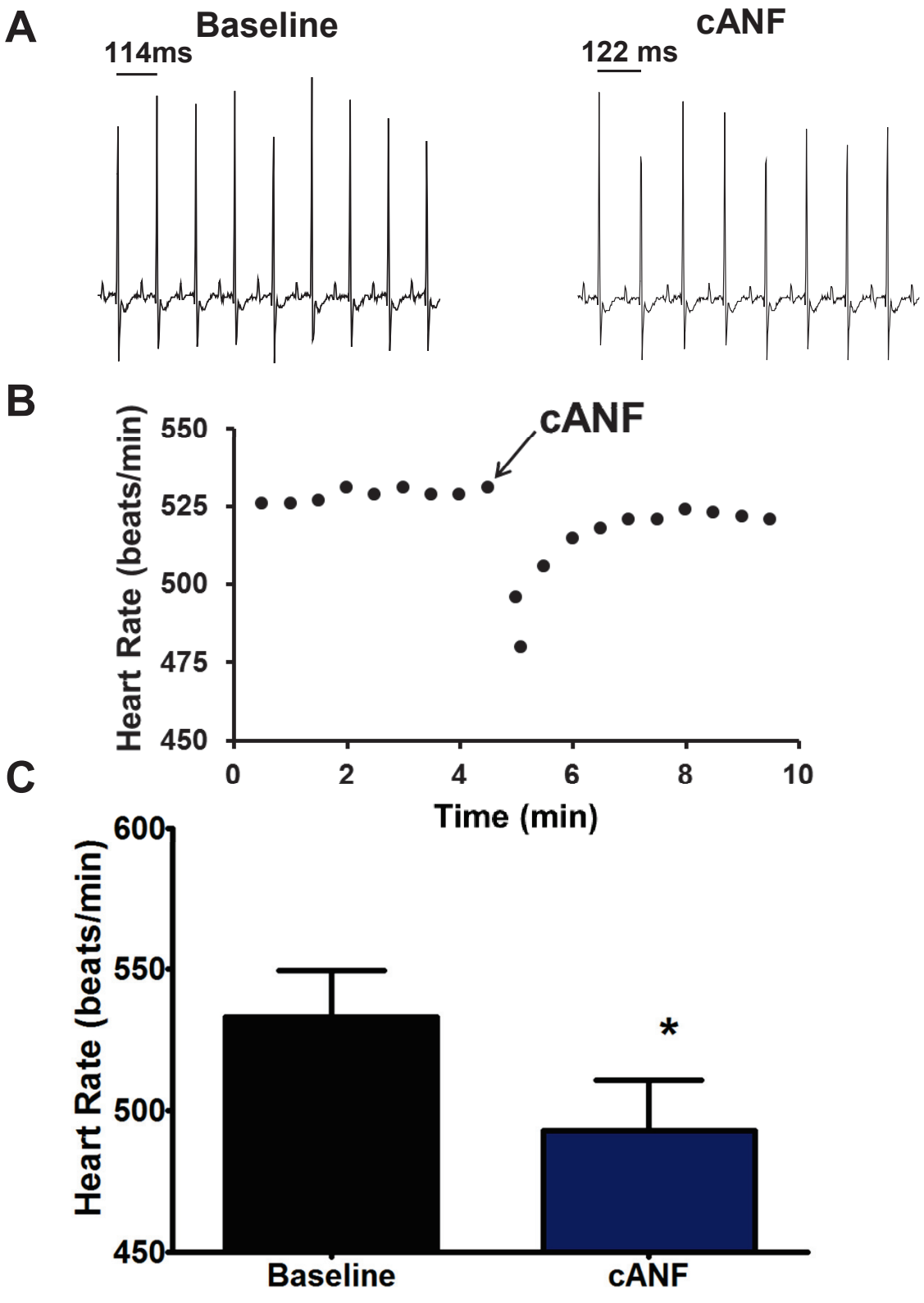

A
Baseline
114ms
cANF
122 ms
B
cANF
Heart Rate (beats/min)
550
525
500
475
450
0
2
4
6
8
10
Time (min)
C
Heart Rate (beats/min)
600
550
500
450
*
Baseline
cANF

Figure 12.

TABLE 7.

Summary of ECG parameters following infusion of the NPR-C agonist, cANF (100μg/kg) in anesthetized mice.

	Baseline	**cANF**
Heart Rate (beats/min)	533 ± 16	493 ± 18*
R-R interval (ms)	113.14 ± 3.38	120.40 ± 3.98*
P wave duration (ms)	17.43 ± 0.77	17.20 ± 0.72
PR interval (ms)	45.89 ± 2.53	46.00 ± 2.60
QRS Complex (ms)	15.71 ± 0.53	15.89 ± 0.56
QT interval (ms)	42.17 ± 2.06	44.46 ± 1.85*
QTc interval (ms)	125.54 ± 6.04	128.46 ± 5.73

PR interval was measured from the start of the P wave to the peak of the QRS complex, QT interval was measured from the start of the QRS complex to the end of the T wave. Data are means ± SEM; n=7 mice per group. *P<0.05 vs. baseline by paired Student's t-test.

acutely and that this results in a slowing of heart rate. These data suggest that NPR-C contributes to the effects of NPs on heart rate and electrical conduction. To further study the role of NPR-C we have characterized the cardiovascular phenotype of mice lacking NPR-C receptors (NPR-$C^{-/-}$) using both surface and intracardiac ECG recordings with programmed stimulation protocols to assess electrical conduction and susceptibility to atrial arrhythmias.

Heart rates were measured in NPR-$C^{-/-}$ mice and compared to those measured in age matched wildtype (NPR-$C^{+/+}$) and heterozygote (NPR-$C^{+/-}$) littermates. As illustrated in Figure 13, heart rate did not differ between the three genotypes (568 ± 9 beats/min in NPR-$C^{+/+}$ mice, 582 ± 9 beats/min in NPR-$C^{+/-}$ mice, 587 ± 12 beats/min in NPR-$C^{-/-}$ mice, P=0.409; n= 13 for NPR-$C^{+/+}$ and NPR-$C^{+/-}$ mice, n=15 for NPR-$C^{-/-}$). Additional ECG parameters are summarized in Table 8.

3.6 Electrophysiological Properties and Susceptibility to Atrial Fibrillation in Mice Lacking Functional NPR-C Receptors (NPR-$C^{-/-}$)

To further characterize sinus node function and atrial electrophysiological properties of mice lacking the NPR-C receptor, intracardiac ECGs were performed in NPR-$C^{-/-}$ mice and compared to littermate controls. As shown in Table 9, there was no difference in sinus node function between the three genotypes as indicated by measures of $SNRT_{100}$ (147.09 ± 4.17 ms in NPR-$C^{+/+}$ mice, 157.23 ± 6.03 ms in NPR-$C^{+/-}$ mice and 163.61 ± 10.34 ms in NPR-$C^{-/-}$ mice; P=0.450), $cSNRT_{100}$ (37.74 ± 2.85 ms in NPR-$C^{+/+}$, 39.73 ± 4.69 ms in NPR-$C^{+/-}$ mice and 49.39 ± 7.51 ms in NPR-$C^{-/-}$ mice, P=0.472) and $SNRT_{100}$/SCL (134.33 ± 2.45% in NPR-$C^{+/+}$ mice, 133.62 ± 3.68% in NPR-$C^{+/-}$ mice

Figure 13.

Basal heart rate of mutant mice lacking functional NPR-C receptors (NPR-$C^{-/-}$) in comparison to wildtype (NPR-$C^{+/+}$) and heterozygous (NPR-$C^{+/-}$) littermates. **A.** Representative ECG traces (1 s duration) illustrating the R-R intervals of NPR-$C^{+/+}$, NPR-$C^{+/-}$ and NPR-$C^{-/-}$ mice. **B.** Summary of heart rate recorded by surface ECGs in anesthetized mice of each genotype. Data are means ± SEM; n=13 for NPR-$C^{+/+}$ and NPR-$C^{+/-}$, n=15 for NPR-$C^{-/-}$ mice. There was no difference in heart rate (P=0.409; One-way ANOVA with Tukey's post-hoc test) between genotypes. See Table 8 for analysis of ECG parameters.

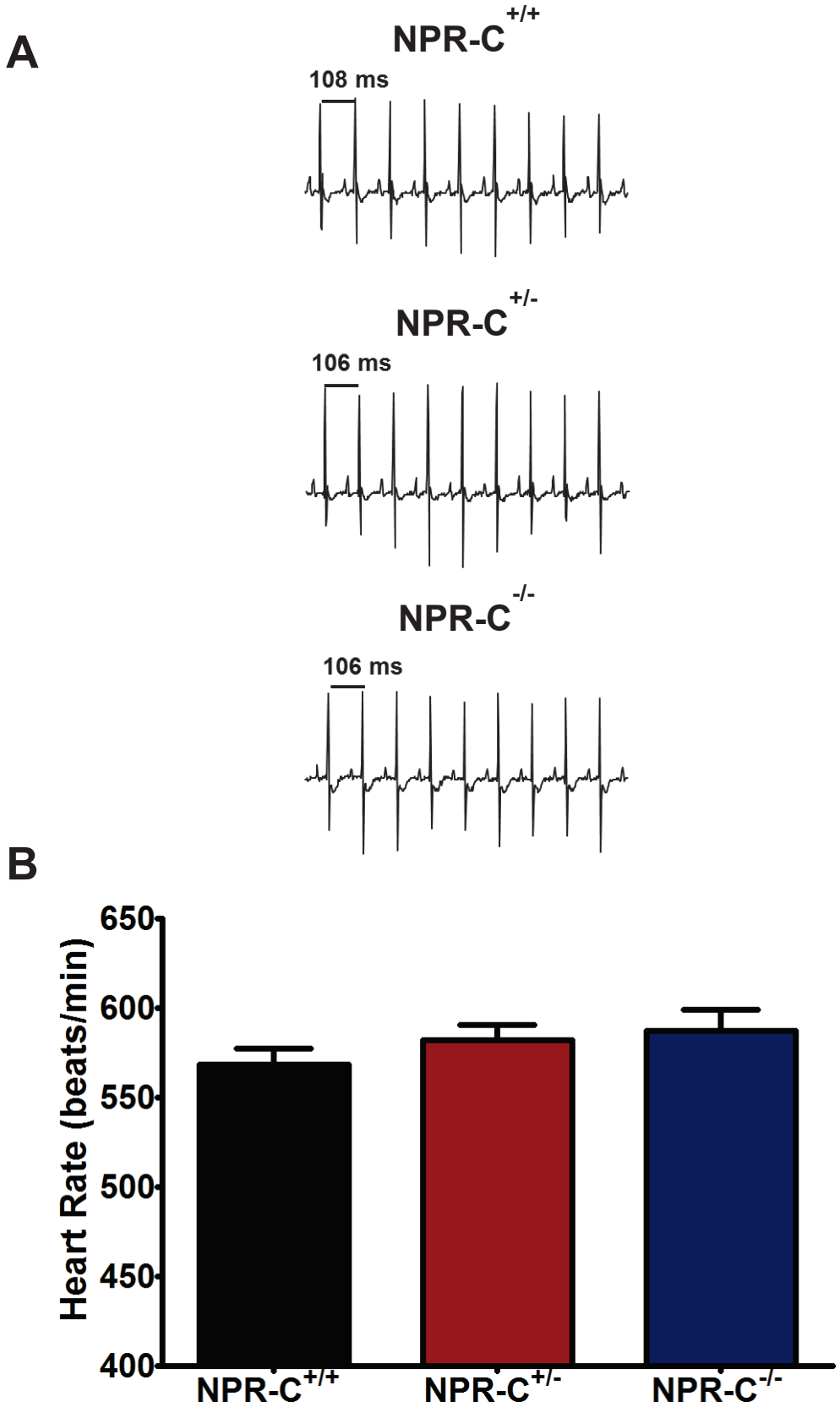

A
NPR-C+/+
108 ms
NPR-C+/-
106 ms
NPR-C-/-
106 ms
B
Heart Rate (beats/min)
650
600
550
500
450
400
NPR-C+/+
NPR-C+/-
NPR-C-/-

Figure 13.

TABLE 8.

Summary of ECG parameters measured by surface ECG in anesthetized NPR-$C^{-/-}$ compared to NPR-$C^{+/+}$ and NPR-$C^{+/-}$ littermates.

	NPR-$C^{+/+}$	**NPR-$C^{+/-}$**	**NPR-$C^{-/-}$**
Heart Rate (beats/min)	568 ± 9	582 ± 9	587 ± 12
R-R interval (ms)	106.09 ± 1.58	103.32 ± 1.58	103.09 ± 2.07
P wave duration (ms)	14.06 ± 0.38	13.44 ± 0.38	13.04 ± 0.32
PR interval (ms)	37.60 ± 0.73	37.05 ± 0.75	38.64 ± 0.64
QRS Complex (ms)	11.69 ± 0.35	12.03 ± 0.41	12.67 ± 0.39
QT interval (ms)	40.49 ± 0.87	39.20 ± 0.62	38.69 ± 0.89
QTc interval (ms)	124.36 ± 2.54	121.98 ± 1.67	120.51 ± 2.19

PR interval was measured from the start of the P wave to the peak of the QRS complex, QT interval was measured from the start of the QRS complex to the end of the T wave. Data are means ± SEM; *n*=13 for NPR-$C^{+/+}$ and NPR-$C^{+/-}$, *n*=15 for NPR-$C^{-/-}$ mice.

and 142.09 ± 5.24% in NPR-$C^{-/-}$ mice; P=0.428). While these measurements of SNRT were not statistically different between genotypes, there was a trend towards prolongation, including after correction for heart rate, in NPR-$C^{-/-}$ mice compared to wildtype animals. Notably, the NPR-$C^{-/-}$ mice displayed more variability than NPR-$C^{+/+}$ and $NPR^{+/-}$ mice.

Additional analysis demonstrates that the AERP was prolonged in NPR-$C^{+/-}$ mice as compared to the wildtype mice (31.00 ± 1.79 ms in NPR-$C^{+/+}$ mice vs. 39.30 ± 1.92 ms in NPR-$C^{+/-}$ mice, $P<0.05$). The AERP also tended to be longer in NPR-$C^{-/-}$ mice compared to wildtype animals (31.00 ± 1.79 ms in NPR-$C^{+/+}$ mice vs. 40.087 ± 4.08 ms in NPR-$C^{-/-}$ mice); however this difference did not reach statistical significance likely due to the increased variability in NPR-$C^{-/-}$ animals (Table 9). There was no difference in the AVNERP between the three genotypes (56.71 ± 3.67 ms in NPR-$C^{+/+}$ mice; 48.87 ± 2.09 ms in NPR-$C^{+/-}$ mice; 52.37 ± 1.28 ms in NPR-$C^{-/-}$ mice, P=0.104*)*.

Finally, we evaluated the susceptibility to AF in NPR-$C^{-/-}$ mice using a burst pacing protocol (Figure 14). Our results demonstrate an increased susceptibility to AF in NPR-$C^{-/-}$ mice (AF in 1 of 17 NPR-$C^{+/+}$ mice vs. AF in 7 of 15 NPR-$C^{-/-}$ mice, P<0.05). AF was not induced in any NPR-$C^{+/-}$ mice (n=7). Interestingly, the duration of AF observed in the one NPR-$C^{+/+}$ mouse was 4.6 s before spontaneous conversion back to normal sinus rhythm. In contrast, the average duration of AF in NPR-$C^{-/-}$ mice was 19.7 s. A representative trace of AF induction in an NPR-$C^{-/-}$ mouse is shown in Figure 14.

TABLE 9.

Summary of the electrophysiological parameters measured by intracardiac ECG in NPR-$C^{-/-}$ compared to NPR-$C^{+/+}$ and NPR-$C^{+/-}$ littermates.

	NPR-$C^{+/+}$	**NPR-$C^{+/-}$**	**NPR-$C^{-/-}$**
$SNRT_{100}$ (ms)	147.09 ± 4.17 (11)	157.23 ± 6.03 (10)	163.61 ± 10.34 (10)
$cSNRT_{100}$ (ms)	37.74 ± 2.85 (11)	39.73 ± 4.69 (10)	49.39 ± 7.51 (10)
$SNRT_{100}$/SCL (%)	134.33±2.45 (11)	133.62±3.68 (10)	142.09 ± 5.24 (10)
AERP (ms)	31.00 ± 1.79 (9)	39.30 ± 1.92* (10)	40.87 ± 4.08 (8)
AVNERP (ms)	56.71 ± 3.67 (7)	48.87±2.09 (8)	52.37 ± 1.28 (8)

Data are means ± SEM; values in brackets represent number of animals. *P<0.05 vs. NPR-$C^{+/+,}$ by One-way ANOVA and Tukey's post-hoc test. Experiments were performed with assistance from Dr. Egom.

Figure 14.

Inducibility of atrial fibrillation in mice lacking functional NPR-C receptors (NPR-$C^{-/-}$) in comparison to wildtype (NPR-$C^{+/+}$) and heterozygous (NPR-$C^{+/-}$) littermates. **A.** Representative image demonstrating induction of AF following burst pacing and spontaneous reversion to sinus rhythm. Inset demonstrates abnormal atrial rhythm and irregular RR intervals associated with AF. **B.** Summary data illustrating the inducibility of AF in wildtype and NPR-C mutant mice. AF was induced in 1 of 17 NPR-$^{+/+}$ mice (5.9%) and in 7 of 15 NPR-$C^{-/-}$ mice (46.7%). *P<0.05 vs. NPR-$C^{+/+}$ by Fisher`s exact test. AF was not induced in any NPR-$C^{+/-}$ mice (n=7).

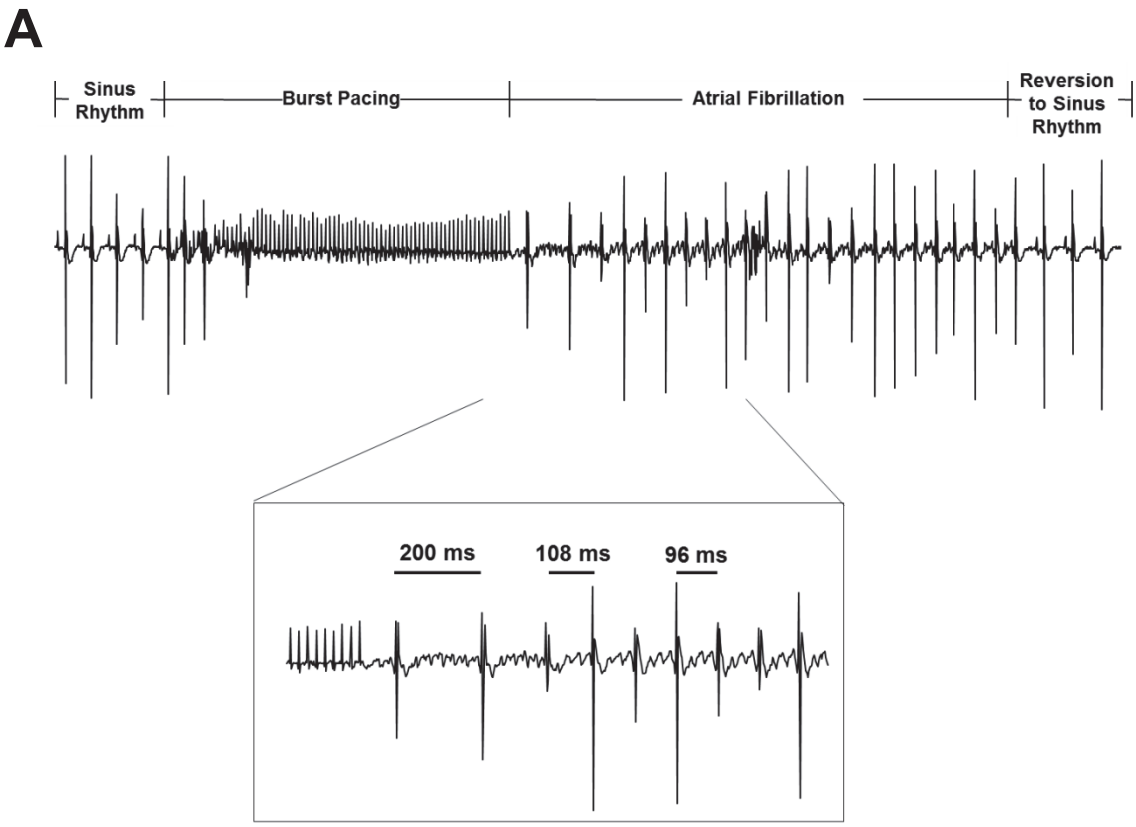

A
Sinus Rhythm
Burst Pacing
Atrial Fibrillation
Reversion to Sinus Rhythm
200 ms
108 ms
96 ms
B
Inducibility of Atrial Fibrillation (%)
60
50
40
30
20
10
0
*
NPR-C+/+
NPR-C+/-
NPR-C-/-

Figure 14.

3.7 Blood Pressure of Conscious Wildtype Mice and NPR-C Mutant Mice Lacking Functional NPR-C (NPR-$C^{-/-}$)

In addition to heart rate, blood pressure is another critical determinant of cardiac performance. Furthermore, NPs are known to modulate blood pressure (Potter et al. 2006), which could impact heart rate through activation of baroreceptors. Accordingly, blood pressure was measured in conscious NPR-$C^{-/-}$ mice and littermate controls via the tail cuff method (Figure 15). No differences in systolic blood pressure, diastolic blood pressure or mean arterial pressure (MAP) were observed in NPR-$C^{-/-}$ when compared to wildtype and heterozygous mice (n= 7 mice for NPR-$C^{+/+}$, n=9 mice for $NPR^{+/-}$ and NPR-$C^{-/-}$). Specifically, systolic blood pressure was 126.22 ± 1.83 mmHg in NPR-$C^{+/+}$ mice, 126.42 ± 2.23 mmHg in NPR-$C^{+/-}$ mice and 129.72 ± 3.11 mmHg in NPR-$C^{-/-}$ mice (P= 0.552). Diastolic blood pressure was calculated as 90.25 ± 4.63 mmHg in NPR-$C^{+/+}$ mice, 84.03 ± 1.53 mmHg in NPR-$C^{+/-}$ mice and 93.73 ± 4.13 mmHg in NPR-$C^{-/-}$ mice (P=0.150). Finally, MAP was 102.01 ± 3.59 mmHg in NPR-$C^{+/+}$ mice, 97.49 ± 1.40 mmHg in NPR-$C^{+/-}$ mice and 105.55 ± 3.65 mmHg in NPR-$C^{-/-}$ mice (P=0.165). Interestingly, these findings differ from a previous study that reported a modest reduction in blood pressure in a different NPR-$C^{-/-}$ mouse model (see Discussion).

Figure 15.

Blood pressure of mutant mice lacking functional NPR-C receptors (NPR-$C^{-/-}$) in comparison to wildtype (NPR-$C^{+/+}$) and heterozygous (NPR-$C^{+/-}$) littermates. Blood pressure was recorded in conscious mice using a non-invasive computerized tail-cuff method. **A.** Summary data showing systolic blood pressure recorded in wildtype and NPR-C mutant mice. **B.** Summary data demonstrating diastolic blood pressure calculated from the systolic blood pressure and mean arterial pressure in wildtype and NPR-C mutant mice. **C.** Summary data showing mean arterial pressure recorded in wildtype and NPR-C mutant mice. Data are means ± SEM; n=7 for NPR-$C^{+/+}$ mice, n= 9 for NPR-$C^{+/-}$ and NPR-$C^{-/-}$ mice. There was no difference in blood pressure (P>0.05; One-way ANOVA with Tukey's post hoc test) between genotypes.

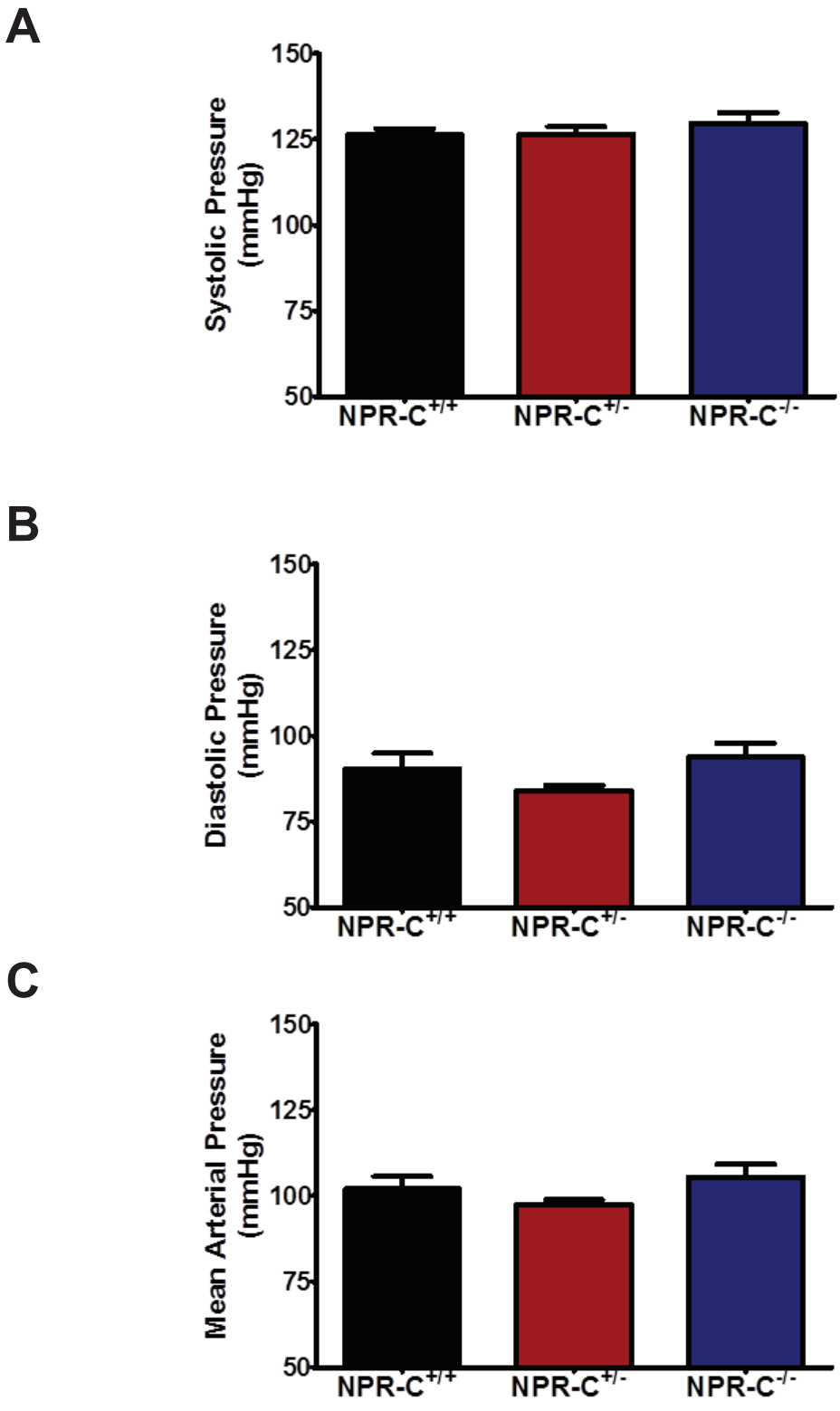

A
Systolic Pressure (mmHg)
150
125
100
75
50
NPR-C+/+
NPR-C+/-
NPR-C-/-
B
Diastolic Pressure (mmHg)
150
125
100
75
50
NPR-C+/+
NPR-C+/-
NPR-C-/-
C
Mean Arterial Pressure (mmHg)
150
125
100
75
50
NPR-C+/+
NPR-C+/-
NPR-C-/-

Figure 15.

CHAPTER 4: DISCUSSION

4.1 Overview of Key Findings

Our laboratory has recently characterized the effects of natriuretic peptides (BNP and CNP) on cardiac electrophysiology in the SAN and atrial myocardium *in vitro* using single cell and isolated heart preparations (Azer *et al.* 2012; Springer *et al.* 2012); however the effects of these peptides on heart rate and electrical conduction *in vivo* are not well understood. This is due, at least in part, to the complexity of NP signaling in the heart and the possibility of multiple NPRs contributing to the physiological effects of NPs (see below). Accordingly, the purpose of this study was to investigate the effects of BNP and CNP and their NPRs on heart rate and electrical conduction in anesthetized mice. The use of both BNP and CNP as agonists allowed us to assess the role of all three NPRs in the regulation of heart rate. Our data demonstrate that acute injection of BNP or CNP increases heart rate and electrical conduction *in vivo.* Similar changes in heart rate were observed in the presence of autonomic nervous system blockade. Together, these results suggest that NPs have direct effects on SAN function to modulate heart rate and electrical conduction *in vivo*. To further understand the role of NPR-C in mediating the electrophysiological effects of NPs we utilized the selective NPR-C agonist cANF. Acute injection of cANF decreased heart rate and electrical conduction. This result demonstrates that NP signaling in the heart is complex and is mediated by the activation of multiple NPRs.

A second objective of this study was to investigate the electrophysiological role of NPR-C in the SAN and atrial myocardium using NPR-$C^{-/-}$ mice. These results demonstrated that heart rate, sinoatrial node function and blood pressure do not differ

between NPR-$C^{-/-}$ mice and wildtype controls. However, the atrial effective refractory period tended to be prolonged in NPR-$C^{+/-}$ and $NPR^{-/-}$ mice compared to wildtype controls. Additionally, NPR-$C^{-/-}$ mice are more susceptible to AF induced by burst pacing than wildtype controls. These data suggest that NPR-C plays a protective role in the regulation of cardiac electrophysiology and generation of atrial arrhythmias.

4.2 BNP and CNP Increase Heart Rate and Electrical Conduction *in vivo*

The effects of natriuretic peptides on heart rate are both interesting and complex. Previously, BNP and CNP have been shown to elicit an increase (Beaulieu *et al.* 1996; Beaulieu *et al.* 1997; Herring *et al.* 2001; Springer *et al.* 2012) or decrease (Azer *et al.* 2012) in heart rate depending on the experimental conditions and which NPR was activated. Thus, it is essential to determine the effects of these peptides *in vivo* in the presence of autonomic nervous system regulation and to ascribe these effects to the different NPRs. The present investigation demonstrates that acute injection of BNP and CNP in anesthetized mice increases heart rate in association with a shortening of the PR interval and QT interval demonstrating increased conduction through the AVN and the ventricular myocardium. This positive chronotropic effect of CNP was dose-dependent and was maintained even in the presence of sympathetic and parasympathetic nervous system blockade, a well-established technique used to assess intrinsic SAN function *in vivo* (Just *et al.* 2000; Rose *et al.* 2007; Mangoni and Nargeot, 2008). This finding, in addition to our previous findings *in vitro* provides further evidence for the direct effects of NPs on the SAN.

Interestingly, the increase in heart rate in response to CNP tended to be larger than that observed in response to BNP. This is consistent with what has been previously reported by Herring *et al.* (2001) who showed that all NPs increased baseline heart rate in isolated guinea pig sinoatrial node preparations and that CNP was more potent than both ANP and BNP (CNP>> BNP=ANP). Hirose *et al.* (1998) also demonstrated that CNP was more potent in increasing sinus rate in right atrial canine preparations compared to ANP and BNP. In contrast to these findings, we have previously reported that BNP and CNP increased spontaneous AP frequency in SAN myocytes and heart rate in Langendorff-perfused mouse hearts to the same extent. The basis for these differential findings is currently unknown; nevertheless all studies clearly demonstrate that BNP and CNP consistently increase heart rate.

BNP binds to NPR-A, CNP binds to NPR-B and both peptides bind to NPR-C with a similar affinity (Bennett *et al*. 1991; Koller *et al.* 1991; Suga *et al.* 1992a). All NPRs are known to be present in the SAN and right atrium (Nunez *et al.* 1992; Lin *et al.* 1995; Beaulieu *et al.* 1997; Springer *et al.* 2012). However, previous work *in vitro* has shown that the positive chronotropic effects of BNP and CNP are blocked by NPR-A and NPR-B antagonists respectively (Herring *et al*. 2001; Springer *et al*. 2012). We have also shown that BNP and CNP increased AP frequency in SAN myocytes from NPR-$C^{-/-}$ mice to the same extent as in wildtype myocytes (Springer *et al.* 2012). Taken together, these findings suggest that the positive chronotropic effects of BNP and CNP are mediated by the GC-linked receptors NPR-A and NPR-B respectively. Activation of the GC-linked NPRs leads to an increase in the intracellular levels of the cGMP that regulates the activity of several downstream targets including PKG, PDE2 and PDE3 (Potter *et al.*

2006). Previously it was have shown that the stimulatory effects of BNP and CNP on SAN myocytes are completely abolished in the presence of the PDE3 inhibitor milrinone, suggesting that BNP and CNP modulate heart rate in a NPR-A/B-PDE3- dependent manner (Herring *et al.* 2001; Springer *et al.* 2012). Both cAMP and cGMP are substrates for PDE3 and it has recently been demonstrated that PDE3 is expressed in the SAN (Hua *et al.* 2012) and is constitutively active in basal conditions (Vinogradova *et al.* 2008; Hua *et al*. 2012). Therefore, inhibition of PDE3 would likely increase intracellular cAMP which can mediate an increase in heart rate through modulation of ionic currents. $I_{Ca,L}$ and I_f are both modulated by cAMP (Mangoni and Nargeot, 2008) either through PKA mediated phosphorylation of ion channels (both I_{Ca} and I_f) or through direct binding of cAMP to HCN channels that carry I_f. Herring *et al.* (2001) showed that the increase in heart rate in response to CNP was significantly reduced in the presence of specific I_f blockers (cesium chloride and ZD-7288) suggesting that the chronotropic effect of CNP was at least partially mediated by changes in I_f. We have also shown that BNP and CNP increased the peak current densities of $I_{Ca,L}$ and I_f in SAN myocytes (Springer *et al*. 2012). Based on these previous findings, we propose that BNP and CNP increase heart rate *in vivo* by activating their GC-linked receptors leading to a cGMP-dependent inhibition of PDE3 and increase in cAMP (as summarized in Figure 16). This increase in cAMP modulates $I_{Ca,L}$ and I_f leading to an increase in the slope of diastolic depolarization and ultimately increasing the rate of spontaneous AP frequency in SAN cells. There are several other ion channels that contribute to the diastolic depolarization in the SAN that may be modulated by NPs. SR Ca^{2+} release is modulated by PKA and can lead to an increase in I_{NCX} which may contribute to the increase in heart rate in response to NPs.

Figure 16.

Potential mechanisms of heart rate regulation by natriuretic peptides and condition dependent activation of NPRs *in vivo*. NPR-A/B: natriuretic peptide receptors A and B; NPR-C: natriuretic peptide receptor C. GC: guanylyl cyclase; cGMP: cyclic guanosine monophosphate; PDE3: phosphodiesterase 3; G_i; inhibitory G-protein, α, β, γ; accessory G-protein subunits- α, β, γ; cAMP: cyclic adenosine monophosphate.

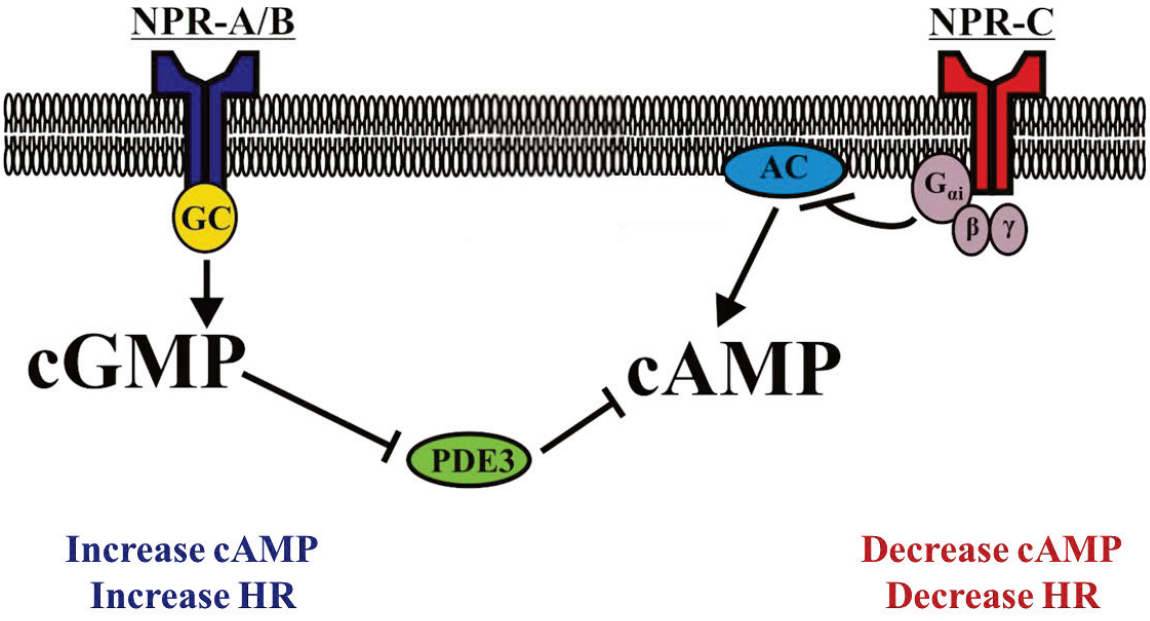

NPR-A/B
NPR-C
AC
GC
$G_{\alpha i}$
β
γ
cGMP
cAMP
PDE3
Increase cAMP
Increase HR
Decrease cAMP
Decrease HR

Figure 16.

Therefore, further studies to evaluate a contribution of altered Ca^{2+} handling to the chronotropic effects of NPs should be conducted.

In addition to PDE3, cGMP also regulates the activity of PDE2 and PKG. Activation of PDE2 by cGMP has been shown to regulate the cAMP response to β-adrenergic stimulation in rabbit atrioventricular nodal cells (Han *et al.* 1996) and rat ventricular myocytes (Mongillo *et al.* 2006). Additionally, the cAMP-enhanced I_{Ca} is inhibited by activation of a cGMP-stimulated PDE in amphibian ventricular myocytes (Mery *et al.* 1993) and human atrial myocytes (Rivet-Bastide *et al.* 1997). Interestingly, an increase in cGMP has been associated with negative inotropic and chronotropic effects through a PKG-mediated inhibition of calcium channels (Imai, 1995; Rodriguez-Pascual *et al.* 1996). Whether PDE2 and PKG are involved in the effects of NPs observed here is still unknown and requires further investigation.

The present study as well as our previous studies, clearly demonstrates direct effects of NPs on heart rate and electrical conduction. Testing the effects of the peptides *in vivo* is critical because NPs have also been shown to have effects on non-cardiac tissues including blood vessels and nerves that innervate the heart (Pandey, 2005; Potter *et al.* 2006). An advantage of using an *in vivo* approach is the ability to study the effects of NPs on heart rate regulation in the presence of an intact autonomic nervous system. Here we have shown that CNP increases heart rate in a dose-dependent manner even in the presence of autonomic nervous system blockade with atropine and propranolol. Interestingly, the magnitude of the increase in heart rate with autonomic nervous system blockade was approximately half of that observed with an intact autonomic nervous system. This suggests that NPs have direct effects on intrinsic SAN activity; however

NPs also interact with the autonomic nervous system to mediate their chronotropic effects. This concept has been suggested previously by Whalen *et al.* (2006) who showed that bolus injection of a membrane permeable cGMP analogue, 8-(4-chlorophenylthio)-cGMP (8-CPT-cGMP) increased heart rate through the release of Ca^{2+}-sensitive and Ca^{2+}-insensitive stores of catecholamines from sympathetic nerve terminals in conscious rats. Recently Chan *et al.* (2012) reported that BNP increased norepinephrine release in isolated guinea pig hearts and both ANP and BNP increased norepinephrine release from sympathetic nerves isolated from the guinea pig heart. This increase in catecholamine release was inhibited by both PKG inhibition and PDE3 stimulation and was associated with an increase in intracellular Ca^{2+}, cAMP and PKA activity. This suggests that activation of cGMP by BNP leads to a PKG mediated inhibition of PDE3 and an increase in Ca^{2+} mediated exocytosis of catecholamines. Chronic infusion of BNP is also associated with a decrease in heart rate variability suggesting an elevation of sympathetic tone (Thireau *et al.* 2012). Tsutsui *et al.* (1994) also demonstrated that CNP stimulates catecholamine synthesis however this was shown in cultured bovine adrenal medullary cells. Whether CNP increases catecholamine release from sympathetic nerves in the heart, and to what extent, requires further investigation. Contrary to these studies, Herring *et al*. (2001) demonstrated that BNP and CNP significantly enhanced the heart rate response to vagal nerve stimulation and increased ACh release through a cGMP-PDE3 dependent increase in PKA phosphorylation of presynaptic N-type calcium channels. Taken together, these results demonstrate an interaction between NPs and the autonomic nervous system and suggest that this interaction may contribute to the effects of NPs on heart rate regulation *in vivo*.

4.3 Activation of NPR-C Decreases Heart Rate and Electrical Conduction *in vivo*

Regulation of heart rate by NPs involves multiple receptors that can regulate heart rate in different conditions. In addition to the stimulatory effects of BNP and CNP on heart rate (which we have previously shown to be mediated by NPR-A and NPR-B) we have also shown that NPs can decrease heart rate in some conditions via activation of NPR-C. Specifically, in isolated hearts, we demonstrated that selective activation of NPR-C with cANF resulted in a decrease in heart rate; however, this effect was only seen in the presence of acute β-AR activation with ISO. This decrease in heart rate was absent in NPR-$C^{-/-}$ mice confirming the role of this receptor in mediating the negative chronotropic effect of cANF (Azer et al. 2012). Since mice, as well as other mammals (including humans) display sympathetic tone that will ensure activation of adenylyl cyclase in the heart (Mangoni and Nargeot, 2008), we tested whether acute selective NPR-C activation with cANF would modulate heart rate *in vivo* without requiring acute activation of β-ARs. Our data show that acute activation of NPR-C by cANF *in vivo* decreases heart rate and electrical conduction. This result is consistent with our previous findings *in vitro* and suggests that the level of adenylyl cyclase activity and intracellular cAMP *in vivo* exceeds the threshold level for activation of NPR-C. It is well documented that the level of sympathetic tone in the mouse is greater than the level of parasympathetic tone (Gehrmann *et al*. 2000; Just *et al*. 2000; Janssen and Smits, 2002; Rose *et al*. 2007) which may contribute to this result.

As mentioned previously, all endogenous NPs bind to NPR-C (Bennett *et al*. 1991; Suga *et al.* 1992a). Here we showed that BNP and CNP increase heart rate and electrical conduction *in vivo*, an effect that is likely due to activation of NPR-A and NPR-

B respectively. However, the above result with cANF suggests that NPR-C can be acutely activated *in vivo* without prior stimulation of β-AR. Based on these results; one can infer that the effects of BNP and CNP are due to activation of multiple NPRs and that the increase in heart rate mediated by the GC-linked NPRs may be partially counteracted by activation of NPR-C. To test this hypothesis and evaluate the specific contribution of NPR-C in mediating the effects of NPs, acute injection of NPs into NPR-$C^{-/-}$ mice should be performed.

Activation of NPR-C by cANF has been shown to activate G_i proteins which lead to inhibition of adenylyl cyclase activity (Anand-Srivastava *et al.* 1996; Pagano and Anand-Srivastava, 2001; Zhou and Murthy, 2003). Inhibition of adenylyl cyclase would be expected to decrease intracellular cAMP levels (see Figure 16) which can modulate several ionic currents that contribute to the slope of diastolic depolarization. Accordingly, we have previously shown that activation of NPR-C by cANF and CNP inhibited $I_{Ca,\,L}$ and decreased spontaneous firing rate in isolated mouse SAN cells in the presence of ISO (Rose *et al.* 2004; Springer *et al.* 2012). However, as discussed earlier, there are several cAMP dependent currents that contribute to diastolic depolarization in the SAN that may be affected. Therefore, effects of cANF on each of these currents require further study.

Interestingly, the route of delivery of cANF was shown to play a critical role in mediating its effects *in vivo*. Similar to the native peptides BNP and CNP, cANF was initially injected intravenously through the tail vein however no clear response was observed. Similar findings were observed following cANF injection in the presence of ISO. This was a surprising result and led us to question the delivery method of this peptide. When cANF was injected directly into the jugular vein, a rapid and reversible

decrease in heart rate was observed. Several explanations for this observation can be proposed. The metabolism and degradation of cANF is not known and it is possible that it was degraded in the circulation by proteases before reaching the heart. cANF is a ring deleted synthetic analog of ANP (Maack *et al.* 1987; Anand-Srivastava *et al.* 1990) and may be more susceptible to degradation than the native peptides. cANF is a selective agonist for NPR-C which is widely expressed in several cell types throughout the body including platelets and vascular smooth muscle cells (Anand-Srivastava 2005; Rose and Giles, 2008). Therefore, infusion of cANF in the tail vein may not reach the heart if it is bound to NPR-C within the circulation. Further investigation into the degradation of cANF is required in order to provide a more conclusive explanation for this observation.

4.4. Cardiovascular Phenotype of Mice Lacking Functional NPR-C Receptors (NPR-C$^{-/-}$)

To further study the role of NPR-C in the heart we have performed both surface and intracardiac ECGs in NPR-C$^{-/-}$ mice to determine how the absence of NPR-C affects SAN and atrial electrophysiology. Here we have shown that there is no difference in heart rate, sinoatrial node function and blood pressure in NPR-C$^{-/-}$ mice compared to wildtype littermates. Initially this result was surprising as we have previously shown that NPR-C can be acutely activated in the heart *in vivo*. All endogenous peptides bind to NPR-C and the expression of NPR-A and NPR-B in the heart are unaltered in NPR-C$^{-/-}$ mice (Springer *et al.* 2012). Given this information, one might anticipate that in the absence of NPR-C, more endogenous peptide would be free to bind the guanylyl cyclase linked receptors to elicit an increase in heart rate. However, Matsukawa *et al*. (1999)

demonstrated that while the half-life of ANP is significantly prolonged, the circulating plasma concentrations of ANP and BNP in NPR-$C^{+/-}$ and NPR-$C^{-/-}$ mice were not significantly different compared to wildtype littermates. No data was reported about the circulating levels of CNP. Circulating levels of BNP and CNP are very low in normal conditions (in the pmol/L range) and if these levels remain unchanged in the absence of NPR-C then this may account for the comparable heart rates in the NPR-$C^{-/-}$ mice. Whether we observe similar findings in conditions where there are elevated endogenous peptide concentrations, such as in heart failure, is currently under investigation by our laboratory. While it has been shown that there is no compensation in the levels of NPR-A and NPR-B in NPR-$C^{-/-}$ mice (Springer *et al.* 2012), we cannot exclude the possibility of compensation by the autonomic nervous system to maintain normal SAN function in the chronic absence of NPR-C. Further experiments using atroprine and propranolol to block autonomic nervous system activity should be conducted to further investigate intrinsic regulation of heart rate in NPR-$C^{-/-}$ mice.

In addition to heart rate, blood pressure is also an important determinant of cardiac output. Here we reported no difference in systolic blood pressure, diastolic blood pressure and mean arterial pressure across the three genotypes. Interestingly, in a separately generated NPR-$C^{-/-}$ mouse model, a slight reduction (8 mmHg) in blood pressure in $NPR^{-/-}$ mice was reported when compared to wild type and heterozygote animals (Matsukawa *et al.* 1999). The basis for these conflicting results is unknown; however it may be attributed to the small sample size used in the previous study or due to differences in experimental protocol.

While we did not observe any significant difference in heart rate and sinus node function in NPR-$C^{-/-}$ mice compared to wildtype controls, we tended to observe a greater variability between the NPR-$C^{-/-}$ population compared to wildtype and heterozygote animals. Notably, three out of ten NPR-$C^{-/-}$ mice had an indication of a prolonged sinus node recovery time suggesting sinus node dysfunction. The cause of this variability is unknown and requires further investigation. It may be attributed to differences in penetrance or may be part of the cardiac phenotype that has developed earlier in some animals compared to others.

To further evaluate the electrophysiological properties of NPR-$C^{-/-}$ mice, we measured atrial and AVN effective refractory periods in addition to susceptibility to AF. There was no difference in the AVNERP between genotypes; however the AERP tended to be prolonged in NPR-$C^{+/-}$ and $NPR^{-/-}$ mice. Initially this finding was intriguing as we have also shown that NPR-$C^{-/-}$ mice are more susceptible to AF which can be associated with shortening of the AERP due to electrical remodeling (Allessie *et al.* 2002). It seems unlikely that the prolonged AERP is related to the increased susceptibility to AF in NPR-$C^{-/-}$ mice as there was a comparable increase in AERP in NPR-$C^{+/-}$ mice and AF could not be induced in NPR-$C^{+/-}$ mice. AF is the most common sustained cardiac arrhythmia in humans (Chen and Shen, 2007) and is associated with not only electrical remodeling but also structural remodeling (Nattel, 2002). A prolongation of the AERP rather than shortening suggests that the increased susceptibility to AF in NPR-$C^{-/-}$ mice is not due to shortening of the atrial action potential duration. Additionally, there was no direct correlation between the NPR-$C^{-/-}$ mice that exhibited a prolonged SNRT and the animals in which AF was induced as only one out of the seven mice in which AF was induced had

an indication of a prolonged SNRT. Structural remodeling of the atria and SAN due to cardiac hypertrophy or fibrosis may contribute to the prolonged SNRT and increased susceptibility to AF. Interestingly, echocardiograms performed on NPR-$C^{-/-}$ mice revealed enlargement of the right atria in NPR-$C^{-/-}$ mice compared to wildtype controls (unpublished data). The potential involvement of this right atrial enlargement in the increased susceptibility to AF in NPR-$C^{-/-}$ mice is currently under investigation. There is also substantial evidence to suggest that fibrosis is a key contributor to atrial re-entry and development of AF (Burstein and Nattel, 2008). Interestingly, Huntley *et al.* (2006) suggested that NPR-C mediated the antiproliferative effects of BNP on cardiac fibroblasts while others have shown that NPR-C is involved in the antiproliferative process of smooth muscle cells (Cahill and Hassid, 1994). While we did not observe any abnormalities in the baseline ECG, we cannot exclude the possibility of an alteration in fibrosis causing conduction slowing in localized regions of the heart. Further examination into the tissue ultrastructure and inter-myocyte coupling of NPR-$C^{-/-}$ mice is required to develop a more conclusive explanation for this increase in susceptibility to atrial tachyarrhythmias in NPR-$C^{-/-}$ mice. Nevertheless, these findings demonstrate how NPR-C contributes to the regulation of cardiac electrophysiology and arrhythmogenesis *in vivo*.

4.5 Study Limitations

The present study investigated the electrophysiological effects of natriuretic peptides and their receptors *in vivo*. While *in vivo* studies are of obvious importance, they do not allow for direct identification of the underlying signaling pathways that mediate

the physiological effects. Fortunately our previous work in isolated SAN myocytes and isolated heart preparations allows us to extrapolate potential pathways that may underlie our observations *in vivo*. Additionally, heart rates were recorded in mice anesthetized with isoflurane and blood pressures were measured in restrained mice. While stable heart rate and MAP have previously been observed in mice anesthetized with isoflurane (Szczęsny *et al.* 2004; Constantinides *et al.* 2011) we cannot exclude the possibility that isoflurane inhalation caused alterations in cardiac and metabolic function that may influence our results. Furthermore, in this study we only studied the effects of acute infusion of NPs. The use of radiotelemetry would allow for chronic measurements of heart rate, blood pressure and core body temperature in conscious, freely moving animals. This would enable us to study the chronic effects of NPs in more physiological conditions and in the absence of anesthesia. Finally, the doses that were used in this study were based on our previous data *in vitro* and are significantly higher than those typically found in the circulation in normal physiological conditions. However, NPs are produced in the heart and released in the circulation so local concentrations in the myocardium and coronary circulation are thought to be much higher than concentrations of NPs in the general circulation (Barr *et al.* 1996; Ellmers *et al.* 2002; Kuhn, 2004; Del Ry *et al*. 2006). Therefore the doses of NPs used in this study may be closer to the local concentration of NPs *in vivo.*

4.6 Future Directions

As previously mentioned, the levels of NPs are significantly increased in proportion to the severity of several cardiac diseases including cardiac hypertrophy,

hypertension and heart failure. Due to their cardioprotective effects, NPs are an attractive treatment option for heart failure patients. Currently, recombinant human BNP, nesiritide (Natrecor) is approved for treatment of acute decompensated heart failure, a condition commonly associated with SAN dysfunction and arrhythmias including atrial fibrillation (Lee and Burnett, 2007). However questions about its efficacy have recently been raised (O'Connor *et al.* 2011). Therefore further studies evaluating the role of BNP infusion in a heart failure model should be conducted. Additionally, due to the antihypertrophic and antifibrotic effects associated with CNP, investigation into its role as a potential therapeutic target in comparison to BNP should be conducted. Currently, we are evaluating the effects of chronic BNP and CNP infusion in a model of cardiac hypertrophy induced by angiotensin II to further study the effects of NPs on cardiac function and cardiac electrophysiology. The role of NPR-C in conditions of elevated NPs such as heart failure is also under investigation using NPR-$C^{-/-}$ mice. Furthermore, based on our present findings demonstrating an increased susceptibility to atrial arrhythmias in NPR-$C^{-/-}$ mice, further histological and functional analysis should be performed in order to discern a role of NPR-C in arrhythmogenesis.

4.7 Conclusions

In summary, the present findings demonstrate that natriuretic peptides regulate heart rate and electrical conduction *in vivo*. BNP and CNP increase heart rate and enhance electrical conduction presumably through the guanylyl cyclase linked NPRs as we have shown previously (Springer *et al.* 2012). This increase in heart rate was maintained in the absence of the autonomic nervous system providing further evidence

for direct effects of NPs on the SAN. In contrast, activation of NPR-C by the selective agonist cANF decreased heart rate and electrical conduction *in vivo* suggesting that this receptor also contributes to the acute effects of NPs. Additionally, we observed no change in heart rate, sinus node function and blood pressure in NPR-$C^{-/-}$ mice. However, NPR-$C^{-/-}$ mice are more susceptible to AF compared to wild type animals.

Based on these findings, as well as our previous findings in isolated cell and whole heart preparations, it is clear that NPs play a role in regulating heart rate and electrical conduction. It is also clear that signaling by NPs in the heart is complex and is dependent on differential receptor activation. The role of NPs and NPRs in pathological conditions is even less understood yet our data suggests NPR-C plays an important protective role against the development of atrial tachyarrhythmias. The results presented in this thesis provide novel insight into the direct electrophysiological effects of NPs in the atria and sinus node and will contribute to a further understanding of how NPs act in both physiological and pathological conditions such as heart failure and AF.

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