Characterization of Murine Cardiac Cholinergic Innervation and its Remodeling in Type 1 Diabetes

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ABSTRACT

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Murine models have become increasingly popular to study various aspects of cardiovascular diseases due to their ease of genetic manipulation. Unfortunately, there has been little effort put into describing the distribution of autonomic nerves in the mouse heart, making it difficult to compare current findings from clinical and experimental models related to cardiovascular diseases. Furthermore, determination of the requirements for the development of this system and its maintenance in adult mice remains largely unexplored. This study represents the first detailed mapping of cholinergic neuroanatomy of the mouse heart based on immunohistochemical staining using true cholinergic markers. We found cholinergic innervation of the mouse heart to be largely focused in the atrium and conducting system. We investigated the involvement of the neurotrophic factor neurturin (NRTN) in the development of cholinergic innervation, because there was indirect evidence that implicated it as a crucial factor. Results from our work definitively demonstrate that NRTN plays a major role in the development of cardiac parasympathetic ganglia and cholinergic innervation of the mouse heart. Adult NRTN knockout mice exhibited a drastic reduction in the number of intracardiac neurons with decreased atrial acetylcholine, cholinergic nerve density at the sinoatrial node and negative chronotropic responses to vagal stimulation. The presence of NRTN and its receptors in hearts from adult wild-type mice suggests
that this neurotrophic factor might also be required for maintenance of cardiac cholinergic innervation. Finally, we wanted to determine how intracardiac neurons and their processes change during diseased states, specifically type 1 diabetes. This work has shown that the cardiac cholinergic nervous system in the mouse undergoes structural and functional remodeling when challenged with streptozotocin-induced diabetes. Cholinergic nerves in diabetic hearts undergo extensive sprouting at the sinoatrial node with no change in the number of intracardiac neurons. Cholinergic function appears to be enhanced in diabetic mice, based on pharmacological testing, despite decreased response to direct vagal nerve stimulation. Evidence also suggests that diabetic mice have an imbalance in autonomic control of heart rate. The latter findings suggest that disruption of central input into intrinsic cardiac ganglia also contributes to the neuropathology of type 1 diabetes.
DEDICATION

This dissertation is dedicated to the loving memory of my grandmother, Edith Mabe.
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CHAPTER 1

INTRODUCTION

Autonomic Nervous System and Control of the Heart

The autonomic nervous system (ANS) is a subdivision of the peripheral nervous system that acts to maintain homeostasis in the body. In general, these activities are performed without conscious control. The ANS assists in regulation of numerous bodily functions including heart rate (HR), blood pressure, respiration rate, perspiration and many others. Autonomic control of the heart is accomplished via two antagonistic branches of the ANS, the sympathetic and the parasympathetic, that modulate the intrinsic pacemaker activity of the heart.

Parasympathetic control of the heart includes regulation of HR and the conduction system. Parasympathetic innervation of the heart is accomplished by the vagus nerve. Central stimulation of the vagus results in the release of acetylcholine (ACh) from post-ganglionic cholinergic nerve endings at the sinoatrial (SA) node. Once released, the ACh binds to post-junctional muscarinic receptors (M2) on SA node myocytes to decrease HR. Activation of muscarinic receptors is important for returning cardiac function to baseline after sympathetic activation by reducing HR, contractile force of atrial cardiac muscle and conduction velocity through the atrioventricular (AV) node, bundle of His and bundle branches. In large animals and humans with a lower resting HR, the HR is controlled by the parasympathetic nervous system under normal conditions, when the intrinsic firing rate of the SA node is decreased by vagal input.
Sympathetic control of the heart is accomplished through pre- and post-ganglionic synapses within the sympathetic nervous system. Preganglionic sympathetic neurons release ACh, which then binds to nicotinic ACh receptors on postganglionic neurons. In response to a stimulus, postganglionic neurons release norepinephrine (NE). The NE then binds to adrenergic receptors, termed beta 1 (β1), in the SA node to increase HR. Activation of β1-adrenergic receptors also mediates increased contractility of atrial and ventricular cardiac muscle, increased cardiac output and automaticity.

Impact of Cardiovascular Diseases on Autonomic Control of the Heart

In order to achieve normal cardiovascular function, there must be a balance between sympathetic and parasympathetic inputs to the heart (Moravec 2008). In certain cardiovascular diseases an imbalance between the two branches causes manifestation of the disease and often contributes to its progression. Imbalance within the ANS has been observed in several cardiovascular diseases including hypertension, heart failure, and diabetes (Curtis and O'Keefe, Jr. 2002; Brook and Julius 2000; Machado et al. 2000; Meredith et al. 1993; Esler 2000; Jermendy 2003; Oberhauser et al. 2001). The presence of autonomic imbalance implies that one branch has inappropriate domination over the other, which has been viewed as a lack of “dynamic flexibility and health” (Thayer and Lane 2007). Typically, sympathetic tone becomes increased and/or parasympathetic (vagal) tone decreased (Thayer and Lane 2007). A central theme within the autonomic imbalances observed in cardiovascular disease is decreased vagal function. A reduction in vagal activity shifts the balance of
autonomic control in favor of sympathetic nerves. Remodeling of this nature is very unfavorable because dominant sympathetic activity could increase the automaticity of the ventricles, thereby increasing risk for development of arrhythmias (Hjalmarson 1980; Kent and Epstein 1976). Decreased vagal inhibitory influence can be identified in several ways including increased HR, decreased heart rate variability (HRV), decreased HR recovery following exercise or decreased baroreflex sensitivity, as well as many others, all of which are associated with increased risk for cardiovascular disease and mortality (Thayer and Lane 2007). In patients with acute myocardial infarction (MI), an autonomic imbalance has been found to result in excessive elevation in blood pressure and HR during exercise training (Matsunaga et al. 2004).

Changes to the sympathetic nervous system with respect to autonomic imbalance are especially evident in cases of heart failure. Patients with congestive heart failure (CHF) have decreased cardiac tissue levels of the sympathetic neurotransmitter NE with a loss of cardiac sympathetic nerve fibers despite increased sympathetic nerve firing and circulating NE levels (Machado et al. 2000; Meredith et al. 1993). These changes have also been observed in patients with essential hypertension (Esler 2000). Increased sympathetic activation in CHF has been shown to contribute to adverse cardiac remodeling, including apoptosis of myocytes, cause down-regulation of β1 adrenergic receptors, and decrease or abolish inotropic and chronotropic responses to NE and other catecholamines (Kaye and Esler 2005; Lohse et al. 2003). The increased activation of sympathetic nerves in patients with heart failure has been shown to contribute to poor prognosis and is associated with even more severe heart failure (Kaye et al. 1995).
Several studies have suggested that autonomic imbalance, such as that observed in cardiovascular diseases, may be due to a disruption of central input into the cardiac ANS (Thayer and Lane 2007; Benarroch 1993; Spyer 1989). These studies found that when central inhibitory pathways involving the neurotransmitter γ-aminobutyric acid (GABA) were interrupted, hypertension and sinus tachycardia resulted.

**Neurotrophic Influences on Cardiac Innervation**

Impaired vagal control in cardiovascular diseases is often assumed to be a consequence of cholinergic nerve damage and denervation caused by the disease (Maser and Lenhard 2005). However, there has been little effort made to investigate this hypothesis. Neurotrophic factors have been implicated in the nerve changes associated with cardiovascular diseases since they are responsible for growth and survival of neurons and their processes during development, and some are required for maintenance of adult sympathetic and sensory neurons (Habecker et al. 2008; Kimura et al. 2007; Mattson and Wan 2008; Mendell 1999; Davies 1996). Noradrenergic activation has been linked to a decrease in nerve growth factor (NGF) expression in cardiac myocytes (Kaye et al. 2000; Qin et al. 2002). Deficiency of this neurotrophic factor causes a loss of NE transporters from sympathetic nerves and potential regression of sympathetic fibers (Kaye and Esler 2005). Conversely, elevated NGF levels around infarcted myocardium were associated with regional sympathetic nerve sprouting (Chen et al. 2001; Oh et al. 2006; Cao et al. 2000b; Cao et al. 2000a). These studies suggest a strong link between sympathetic nerve changes in cardiac disease
and neurotrophic factors; however, the trophic factor requirements for survival and maintenance of cardiac parasympathetic neurons are largely unknown.

**Diabetes and Cardiac Autonomic Control**

Impaired autonomic regulation of the heart occurs in patients with diabetes and is found to be associated with poor prognosis. The presence of autonomic imbalance increases the risk for developing life threatening ventricular arrhythmias and sudden death (Vinik et al. 2003). Several epidemiological studies have provided evidence that diabetes also increases the risk for atrial fibrillation, which often impairs quality of life and can lead to stroke (Movahed et al. 2005; Vinik et al. 2003). The mechanisms underlying these associations are undoubtedly complex and most likely involve pathophysiological remodeling of the ANS and cardiac tissue.

Results from several studies of clinical diabetes indicate dysfunction of the cardiac ANS and remodeling of its structure and function. Cholinergic nerve damage and denervation have been thought to be the cause of the impaired vagal control of heart rate in diabetics (Maser et al. 2003; Vinik et al. 2003; Vinik and Ziegler 2007), but this theory has not been explored experimentally. Several clinical studies have used HRV measurements to assess parasympathetic and sympathetic control of the sinus node in patients with and without diabetes (Burger et al. 1999; Molon et al. 2006; Singh et al. 2000). In human diabetics, decreased HRV is considered to be the earliest sign of cardiac dysfunction (Kardelen et al. 2006; Javorka et al. 2005). This, in combination with decreased cardiac baroreflex observed in type 1 diabetics, has been attributed to cardiac parasympathetic denervation (Vinik and Ziegler 2007). ACh release and choline
uptake were decreased in atrial appendage taken from diabetic patients with poor glycemic control and late complications (neuropathy, nephropathy), which is suggested as being consistent with a loss of nerves (Vinik and Ziegler 2007; Oberhauser et al. 2001; Maser and Lenhard 2005). However, there has been little attempt to look at the nerves themselves to support this idea of parasympathetic denervation. Functional impairment of cardiac sympathetic nerve fibers has been observed in patients with type 1 diabetes and attributed as a major cause of inadequate cardiac contraction response during exercise (Scognamiglio et al. 1998). Examination of post-mortem cardiovascular tissue of diabetics found decreased levels of NE as well (Neubauer and Christensen 1976). Additionally, impairment of noradrenergic uptake has been observed in diabetic patients based on 123I-metaiodobenzylguanidine uptake studies (Langer et al. 1995; Scognamiglio et al. 2000).

Autonomic dysfunction of the cardiovascular system has also been found in experimental animal models of type 1 diabetes. The majority of these studies were performed on animals (usually rats or mice) treated with streptozotocin (STZ). This drug is accumulated selectively by pancreatic beta cells and causes cell death in a substantial portion of this population (Szkudelski 2001). Diabetic rats and pigs treated with STZ have decreased HRV and impaired baroreflex sensitivity (Howarth et al. 2005b; Howarth et al. 2006; Mesangeau et al. 2000; Dall'Ago et al. 2007). These changes mimic effects seen in human diabetics and suggest that diabetic animals also have impaired parasympathetic control of HR. Unlike patients with type 1 diabetes, resting HR is depressed in STZ-treated rats, which has been attributed in part to an effect on the intrinsic pacemaker (Howarth et al. 2005b; Howarth et al. 2005a).
Cardiac sympathetic remodeling in diabetes has been studied extensively using the STZ rat model. Within the first 2 months of disease, cardiac NE levels were increased (Akiyama et al. 1989; Felten et al. 1982), which could be due to increased NGF expression followed by sprouting of noradrenergic nerve fibers (Hellweg and Hartung 1990; Rohrer et al. 1996). NE turnover and stimulation-evoked release in isolated atria tissue are also increased at this time (Ganguly et al. 1986). Early increases in noradrenergic innervation are followed by significant regional decreases in NE levels, which are present between 6 and 12 months after STZ treatment (Rohrer et al. 1996). Reduction of NE levels is most likely a consequence of decreased NGF expression, which has been demonstrated in the distal left ventricle after 9 months of diabetes and was associated with a loss of sympathetic nerve fibers (Rohrer et al. 1996). Nerve degeneration, likely preganglionic, has also been observed in intrinsic cardiac ganglia from STZ-treated rats after 1 year of diabetes, yet the postganglionic cell bodies themselves appeared normal (Kamal et al. 1991). Furthermore, electron microscopy studies on alloxan-treated rats showed a loss of atrial post-ganglionic cholinergic nerves which is consistent with their loss in function after 7-8 months of diabetes (Tomlinson and Yusof 1983).

**Specific Aims**

Murine models have become increasingly more popular in the study of various aspects of cardiovascular diseases due to their ease of genetic manipulation. Unfortunately, there has been little effort put into describing the distribution of autonomic nerves in the mouse heart, making it difficult to compare with findings from previous
clinical and experimental models related to cardiovascular diseases. Of the few studies involving cholinergic innervation of the heart in rodents, none have been carried out using true cholinergic markers such as choline acetyltransferase (ChAT), choline transporter (CHT), or vesicular ACh transporter (VAChT). Furthermore, determination of the requirements for the development of this system and its maintenance in adult mice remains largely unexplored. In order to address these deficits, the first aim of this study was to truly define the anatomy of the cardiac cholinergic nervous system in the mouse. These findings have been published in Cell & Tissue Research and are discussed in Chapter 2. To further address the deficits in knowledge about cholinergic innervation of the mouse, the second aim of this study was to investigate the involvement of the neurotrophic factor neurturin (NRTN) because there was indirect evidence that implicated it in cardiac cholinergic development of neonatal mice. Specifically, we wanted to define expression of NRTN and its receptors in adult hearts and determine the impact of developmental NRTN deficiency on cholinergic structure and function in adult hearts. These experiments are contained within Chapters 2 and 3 and show potential ability for plasticity and remodeling of cardiac cholinergic innervation. The final aim of this study was to determine how intracardiac neurons and their processes change during diseased states, specifically type 1 diabetes. There is evidence for sympathetic trophic factor influences on cardiac innervation during cardiovascular disease, especially in heart failure. Additional evidence suggests decreased vagal tone in cardiovascular diseases such as hypertension and diabetes. Indirect evidence through HR and HR dynamics suggests decreased vagal tone in type 1 diabetes, with no direct investigation into the neuroanatomy and function of the system itself.
Accordingly, the main focus of the final aim, addressed in Chapter 4, was to determine the impact of experimental type 1 diabetes on cholinergic structure and function of the mouse heart.
CHAPTER 2

LOCALIZATION OF CHOLINERGIC INNERVATION AND NEURTURIN RECEPTORS IN ADULT MOUSE HEART AND EXPRESSION OF THE NEURTURIN GENE

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Keywords: Choline acetyltransferase, High affinity choline transporter, Cardiac parasympathetic ganglia, GFRα2, Ret

Abstract

Neurturin (NRTN) is a neurotrophic factor required during development for normal cholinergic innervation of the heart, but whether NRTN continues to function in the adult heart is unknown. We have therefore evaluated NRTN expression in adult mouse heart and the association of NRTN receptors with intracardiac cholinergic neurons and nerve fibers. Mapping the regional distribution and density of cholinergic nerves in mouse heart was an integral part of this goal. Analysis of RNA from adult C57BL/6 mouse hearts demonstrated NRTN expression in atrial and ventricular tissue. Virtually all neurons in the cardiac parasympathetic ganglia exhibited the cholinergic phenotype, and over 90% of these cells contained both components of the NRTN receptor, Ret tyrosine kinase and GDNF family receptor α2 (GFRα2). Cholinergic nerve fibers, identified by labeling for the high affinity choline transporter, were abundant in the sinus and atrioventricular nodes, ventricular conducting system, interatrial septum, and much of the right atrium, but less abundant in the left atrium. The right ventricular myocardium contained a low density of cholinergic nerves, which were sparse in other regions of the working ventricular myocardium. Some cholinergic nerves were also associated with coronary vessels. GFRα2 was present in most cholinergic nerve fibers and in Schwann cells and their processes throughout the heart. Some cholinergic nerve fibers, such as those in the sinus node, also exhibited Ret immunoreactivity. These findings provide the first detailed mapping of cholinergic nerves in mouse heart and suggest that the neurotrophic influence of NRTN on cardiac cholinergic innervation continues in mature animals.
Introduction

Autonomic regulation of the heart is accomplished through noradrenergic sympathetic nerves and cholinergic parasympathetic nerves that innervate the myocardium (Ardell 2004). Cardiac sympathetic nerve fibers project from noradrenergic neurons located in specific paravertebral ganglia (e.g., stellate ganglia), while parasympathetic fibers come from cholinergic neurons located in the intracardiac ganglia (Ardell 2004; Hoover et al. 2004; Parsons 2004). Sympathetic neurons are known to have a lifelong requirement for nerve growth factor (NGF), which is supplied by target tissues (e.g., myocardium) and transported back to cell bodies in the ganglia via noradrenergic nerve fibers (Gorin and Johnson 1980; Thoenen et al. 1987). In adult animals, NGF plays a crucial role in regulating the density of cardiac sympathetic innervation; a deficiency of NGF causes the loss of noradrenergic nerve fibers, whereas excessive amounts of NGF cause hyperinnervation (Gorin and Johnson 1980; Hassankhani et al. 1995; Qin et al. 2002; Ieda et al. 2004). Recent studies have demonstrated that such changes can occur in cardiac disease and produce adverse remodeling of noradrenergic innervation (Cao et al. 2000; Kaye et al. 2000). Less is known regarding the trophic factor requirements of intracardiac neurons (i.e., parasympathetic neurons), but neurturin (NRTN) is a promising candidate (Heuckeroth et al. 1999; Rossi et al. 1999; Hiltunen et al. 2000; Airaksinen and Saarma 2002). NRTN is a member of the glial-cell-line-derived neurotrophic factor (GDNF) family, which also includes GDNF itself, persephin, and artemin (Airaksinen and Saarma 2002). These factors differ substantially from the neurotrophins (e.g., NGF) in structure and signaling mechanism but share the attributes of being target-derived proteins and of supporting
the development, survival, and maintenance of specific populations of peripheral neurons. Members of the GDNF family signal through multicomponent receptors that contain the transmembrane tyrosine kinase, Ret, and one of four GDNF family receptors (GFRα1–GFRα4). Each neurotrophic factor of the GDNF family has been shown to signal preferentially through a specific GFRα. For example, GDNF preferentially signals through GFRα1/Ret and NRTN via GFRα2/Ret, but some crosstalk can occur in vitro at higher ligand concentrations (Airaksinen and Saarma 2002). Specifically, NRTN can signal through GFRα1/Ret, and GDNF can signal via GFRα2/Ret. However, current evidence suggests that each of the trophic factors interacts specifically with its preferred receptor in vivo (Leitner et al. 1999; Airaksinen and Saarma 2002; Paveliev et al. 2004).

Several investigators have included the heart in studies involving in situ hybridization to define the regional expression of NRTN, Ret, and GDNF family receptors in the central nervous system and periphery of mice (Widenfalk et al. 1997; Golden et al. 1999). These experiments have provided evidence that NRTN is expressed in the myocardium from embryonic day 12 through the first postnatal week, but no signal has been detected in adult heart. Although intracardiac ganglia have not been included in these studies, another group has reported that high levels of Ret and GFRα2 mRNAs occur in parasympathetic ganglion neurons of E18, P21, and P60 rat hearts (Hiltunen et al. 2000). Ret expression is exclusive to intracardiac neurons, whereas low levels of GFRα2 mRNA are also associated with adventitial and valvular cells. These results suggest that both components of the NRTN receptor are present in intracardiac neurons of embryonic and adult rats. Neither GFRα1 nor GFRα3 has been detected in intracardiac neurons, but low to moderate levels are associated with glial
cells in the parasympathetic ganglia. Further studies conducted with ret knockout mice, which die shortly after birth, have shown that their hearts contain approximately half the number of neurons as wild type mice (Hiltunen et al. 2000). In addition, cholinergic innervation of the conducting system is absent or clearly reduced in ret knockout mice. Significant decreases in the density of cholinergic nerves in the right ventricular myocardium and the conducting system also occur in adult gfra2 knockout mice. Collectively, these findings suggest that NRTN is required for the normal development of cardiac parasympathetic ganglia and the normal cholinergic innervation of the heart (Hiltunen et al. 2000).

Whereas current evidence supports a role for NRTN in the development of cardiac parasympathetic ganglia and cholinergic innervation of specific regions of the heart, little is known about NRTN expression in the adult heart or about the extent of NRTN receptor association with intracardiac cholinergic neurons and nerve fibers. Likewise, the distribution of cholinergic nerves within the mouse heart has not been mapped in detail. Therefore, we have used reverse transcription with the polymerase chain reaction (RT-PCR) to evaluate nrtn expression in atrial and ventricular tissue from adult mice. The regional localization of cholinergic neurons and nerve fibers in adult mouse heart and the association of GFRα2 and Ret with cholinergic innervation have been determined by fluorescence immunohistochemistry.
**Materials and methods**

*Animals*

Hearts (n=15) were obtained from adult male C57BL/6 mice (Harlan, Indianapolis, IN) (24-30g) anesthetized with sodium pentobarbital (90mg/kg, i.p.). Animal protocols were approved by the East Tennessee State University Animal Care and Use Committee and conformed to guidelines of the National Institutes of Health published in the *Guide for the Care and Use of Laboratory Animals* (NIH publication No. 85-23, revised 1996).

*RNA Extraction and Quantification*

Total RNA from entire atria (including right and left atrium and interatrial septum) and left ventricular free wall was extracted using the RNeasy Fibrous Tissue kit and DNase I treatment according to the manufacturer’s protocol (QIAGEN Inc., Valencia, CA). The RNA yield and purity were determined by measuring the absorbance ratio at 260/280 nm (>1.9) on an Eppendorf BioPhotometer.

*RT-PCR analysis*

First-strand cDNA was synthesized from 1 µg of total RNA and 0.5 µg anchored oligo(dT)$_{20}$ (Integrated DNA Technologies, Inc., Coralville, IA) using Superscript II reverse transcriptase (Invitrogen, Carlsband, CA). Commercially available first-strand cDNAs from total heart and stomach of 8 week old normal mice (BioChain Institute, Inc., Hayward, CA) were used as positive control templates. Control experiments were performed in the absence of reverse transcriptase to exclude the possibility of
amplification from contaminating genomic DNA. NTRN cDNA was amplified using the sense primer 5’-CTCCCTG CTATCTGTCTGGATGTGC-3’ and antisense primer 5’-TCTCATCCGACGTGTA GCCCAG-3’ to yield a 320 bp PCR product. Each PCR reaction (25 µL) consisted of 2.5 µL 10X Taq buffer, 2.5 µL 2.0 mM dNTPs, 1.25 µL DMSO, 20 pmol of primers, 1µL cDNA template, and 0.25µL MasterTaq DNA Polymerase (Eppendorf, Westbury, NY). PCR was performed under the conditions of one cycle at 94°C for 2 min, 35 cycles at 94°C for 15 s, 55°C for 30 s, and 72°C for 45 s, followed by a final extension at 72°C for 9.5 min. β-actin was used as an internal standard along with commercially available primers (BioChain Institute, Inc., Hayward, CA). PCR products were electrophoresed on 1.5% agarose gels and visualized using ethidium bromide staining. Band intensity was quantified on a BioRad FX phosphorimager using Quantity One software (Bio-Rad Laboratories, Inc., Philadelphia, PA). The identity of the PCR products was confirmed by sequencing on both strands.

_Tissue processing for histology_

Hearts were was obtained from deeply anesthetized mice and placed briefly in phosphate buffered saline (PBS) (pH 7.4) to remove blood. They were then fixed for 2 hours in cold PBS containing 4% paraformaldehyde and 0.2% picric acid, cryoprotected for 2 days in cold 20% sucrose/PBS, and sectioned in an IEC cryostat/microtome. In a few cases, the ascending aorta was cannulated to enable brief perfusion fixation of the heart before it was placed in cold fixative. Serial 30µm short-axis sections were collected in three sets beginning at the most anterior aspect of the heart and ending at a level well into the ventricular myocardium. For a few hearts, six sets of 15µm sections
were collected. Each set of slides was boxed separately, wrapped in aluminum foil, and stored at -80°C.

**Immunohistochemistry**

Slide mounted tissue sections were immunostained at room temperature. Briefly, sections were washed in 0.1M PBS (pH 7.3), permeabilized with 0.4% Triton X-100 in PBS containing 0.5% bovine serum albumin (BSA), and blocked for 2 hours in PBS containing 10% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA), 1% BSA, and 0.4% Triton X-100. Tissues were then incubated for 15-18 hours with two primary antisera generated in different species (Table 1), washed several times with 0.1M PBS, and incubated for 2 hours with species specific donkey secondary antibodies conjugated to Alexa Fluor 488 or 555 (Molecular Probes, Eugene, OR). All secondary antibodies were used at a dilution of 1:200. After washing the sections several times with PBS, coverglasses were attached using Citifluor mounting medium (Ted Pella, Inc., Redding, CA) and sealed with clear nail polish. Some sections were routinely processed without primary antibodies as a negative control.

**Image Analysis and Confocal Microscopy**

Labeled tissue sections were viewed and photographed using an Olympus BX41 microscope equipped with an Optronics MagnaFire SP CCD camera and regions of interest selected for further evaluation by confocal microscopy. Fluorescence microscopic images of intracardiac ganglia were evaluated using Stereo Investigator/Workstation software (MicroBrightField, Inc., Williston, VT) to quantify
labeled neurons. The total number of neurons present in each section was determined based on staining for the pan-neuronal marker, protein gene product 9.5 (PGP 9.5). The percentage of these neurons that also stained for Ret or GRFα2 was determined in double labeling experiments. Confocal images of selected tissue regions were collected using a Leica TCS SP2 confocal microscope system. Specimens were scanned sequentially to avoid crosstalk between fluorochromes, and a maximum projection image was obtained from each series. Negative controls for each fluorochrome were also scanned using the same parameter settings. Images were exported into Corel Draw 11 and adjusted for brightness and contrast.
Results

Presence of NRTN mRNA in adult mouse heart

To begin investigation of NRTN expression in the adult mouse heart, we performed PCR analysis using cDNA from whole heart as well as stomach, an established positive control tissue (Golden et al. 1999). As shown in Fig. 2.1 A, PCR produced identically sized amplimers from both tissues. Sequence analysis of the products revealed that they matched a 320 base pair region of murine NRTN (392-712bp; GenBank accession NM00873), thus confirming their identity. Based upon these findings, we next evaluated regional expression of NRTN. RT-PCR was carried out on total RNA isolated from total atrium and the left ventricular free wall. Comparison of band intensities normalized to β-actin levels demonstrated that the abundance of NRTN mRNA in atria did not differ significantly from that in left ventricular wall (Fig. 2.1 B,C). Comparable results were obtained when the same cDNA samples were evaluated using mouse beta-2-microglobulin mRNA levels for normalization. Neither β-actin nor beta-2-microglobulin mRNA levels varied appreciably between samples (data not shown).

Fig. 2.1. NRTN is expressed by adult mouse heart. PCR analysis of whole heart (H) cDNA and stomach (S) cDNA (positive control) from an adult mouse resulted in a 320bp product with a sequence identical to 392-712bp of murine NRTN (A). RT-PCR analysis of mRNAs obtained from total atrium (A) and left ventricular (LV) wall established that NRTN expression occurred in both regions of the heart (B). NRTN band intensities were expressed as a percentage of beta-actin band intensities for the same samples (C). Values for atria and left ventricle (mean ± SE, n=3) are not significantly different (paired t-test, P>0.05).
**Localization of GFRα2 & Ret to neuronal cell bodies in intracardiac ganglia**

Parasympathetic ganglia were identified initially in sections of mouse heart immunostained for PGP 9.5 to visualize all neurons and for choline acetyltransferase (ChAT) to identify cholinergic neurons. These ganglia were located primarily in epicardial connective tissue along the posterior surface of the atria. A vast majority of the PGP 9.5-positive somata in the intracardiac ganglia exhibited ChAT immunoreactivity (Fig. 2.2 A,B), indicating that they are cholinergic neurons. Similar staining for PGP 9.5 and each NRTN receptor component showed that most intracardiac neurons exhibit GFRα2 and Ret immunoreactivity (Fig. 2.2 C-F).

Quantitative evaluation of double-labeled sections from three hearts demonstrated that 94 ± 1% (mean ± SE) of intracardiac neurons (534 ± 168 PGP 9.5 labeled neurons counted per heart) contained GFRα2 immunoreactivity and 93 ± 3% stained for Ret (487 ± 184 PGP 9.5 labeled neurons counted per heart). Therefore, most intracardiac neurons of the mouse contain the specific cholinergic marker ChAT and both components of the NRTN receptor.
Fig. 2.2. Most neurons of adult mouse intracardiac ganglia contain ChAT, GFRα2 and Ret. Sections of mouse heart were double immunolabeled for the pan-neuronal marker PGP 9.5 and either ChAT (cholinergic marker), GFRα2 or Ret. Panels show sequentially scanned confocal images of intracardiac ganglia. The vast majority of PGP 9.5 immunoreactive neuronal cell bodies also demonstrate ChAT immunoreactivity (A, B), GFRα2 immunoreactivity (C, D) and Ret immunoreactivity (E, F). Images are single optical sections. Scale bars = 80 μm
Localization of GFRα2 to cholinergic nerve fibers and Schwann cells.

The localization of GRFα2 immunoreactivity to a high proportion of intracardiac cholinergic neurons suggests that this component of the NRTN receptor should be present in cholinergic nerves throughout the heart. This premise was tested by evaluating the colocalization of GFRα2 and high affinity choline transporter (CHT) immunoreactivity in short-axis sections of heart beginning at a level anterior to the sinus node and ending in the ventricular myocardium about 2 mm below the atroventricular (AV) valves. Confocal analysis of these sections established that GFRα2 immunoreactivity was highly associated with CHT-positive cholinergic nerve fibers throughout their distribution in the atria, the AV conducting system, and ventricular tissue. Highest densities of CHT/GFRα2 positive nerve fibers occurred in the sinus node (Fig. 2.3 A-C), interatrial septum (Fig. 2.3 D-F), AV node (Fig. 2.3 G-I), ventricular conducting system and atrial muscle near the AV junction (Fig. 2.4 A-C). Other regions of right atrial myocardium contained a high to moderate density of dual-labeled fibers (Fig. 2.4 D-F), and the overall abundance of cholinergic nerve fibers in left atrium was less than in the right (Fig. 2.4, compare D-F to G-I). A low density of CHT/GFRα2 positive nerve fibers was seen in right ventricular myocardium (Fig 2.5 A-C) but cholinergic innervation was sparse in the ventricular septum and left ventricular free wall (Fig. 2.5 D-F). Lastly, some CHT/GFRα2 positive nerve fibers occurred in atrial and ventricular epicardium, AV valves and around coronary arteries of various sizes (Figs. 2.4 D-F and 2.5 A-C).

While there was extensive colocalization of GFRα2 and CHT in many nerve fibers, a few cholinergic nerves did not show GFRα2 immunoreactivity (Fig. 2.4 D-F). It
was noted also that staining for GFRα2 often extended beyond the cholinergic nerve fibers that were identified by labeling for CHT (e.g., Fig. 2.3 & 2.4, solid arrowheads). Furthermore, GFRα2 immunoreactivity was evident in some non-cholinergic fiber bundles (e.g., asterisks in Fig. 2.4) and processes. Such labeling was very evident in the left ventricular wall where cholinergic nerves were sparse (Fig. 2.5D-F). Since GFRα2 has been identified in Schwann cells in other tissues (Rossi et al., 2003; Wanigasekara et al. 2004), additional double labeling experiments were done using antibodies to the Schwann cell marker S100 and to GFRα2. These experiments showed that Schwann cells and their processes were common in the left ventricular wall (Fig. 2.6 A-F) and in other regions of atrial and ventricular myocardium. A vast majority of these glial cells and their processes also stained for GFRα2 (Fig. 2.6 A-F). Double labeling for S100 and the vesicular ACh transporter (VACHT), another cholinergic marker, further demonstrated that Schwann cells and their processes often occurred adjacent to cholinergic nerve fibers (Fig. 2.6 G-I). Satellite cells of the cardiac parasympathetic ganglia also stained intensely for S100 but did not exhibit GFRα2 immunoreactivity (not shown).
Fig. 2.3. GFRα2 immunoreactivity associated with dense cholinergic innervation of cardiac nodal tissue and the interatrial septum. Panels show sequentially scanned confocal images of tissue sections that were double immunolabeled for CHT (red) and GFRα2 (green). Extensive colocalization of these markers (yellow in right panels) was observed in the sinus node (A-C), interatrial septum (D-F) and AV node (G-I). Arrows indicate examples of colocalization. Arrowheads indicate examples of probable Schwann cell elements labeled for GFRα2 only. Panels A-F are maximum projection images obtained from 26 optical sections spanning a tissue thickness of 10 µm. Panels G-I are single optical sections. Scale bars = 80 µm.
Fig. 2.4. GFRα2 immunoreactivity associated with cholinergic innervation of right and left atrium. Confocal images were obtained by sequential scanning of tissue sections that were double immunolabeled for CHT (red) and GFRα2 (green). Many cholinergic nerves (CHT positive) are present throughout the right atrium (RA, A and D) and are especially abundant near the annulus of the tricuspid valve (TV, A). Left atrium (LA) contains fewer cholinergic nerves than right atrium (compare D and G). Extensive colocalization of CHT and GFRα2 (yellow in right panels) was observed throughout the right atrium (A-C, level of TV; D-F more anterior level) and the left atrium (G-I). A few CHT immunoreactive fibers did not exhibit GFRα2 immunoreactivity (arrows). Open arrowhead in D indicates CHT innervation along blood vessel. Solid arrowheads indicate examples of probable Schwann cell elements labeled for GFRα2 only. Asterisks indicate fiber bundles stained for GFRα2 only. Maximum projection images were obtained from 26 optical sections spanning a tissue thickness of 10 µm. Scale bars = 80 µm.
Fig. 2.5. GFRα2 immunoreactivity associated with cholinergic nerves in ventricular tissue. Confocal images were obtained by sequential scanning of tissue sections that were double immunolabeled for CHT and GFRα2 (A-F). Low density of cholinergic nerves typical of right ventricular myocardium (RV) is seen in panel A along with cholinergic innervation of a coronary artery (*). GFRα2 is colocalized with CHT in most of these fibers (A-C). GFRα2 immunoreactivity is associated with the sparse cholinergic innervation of mouse left ventricle (LV, D-F) but occurs also in some non-cholinergic processes (arrowheads). Maximum projection images were obtained from 26 optical sections spanning a tissue thickness of 10 µm. Scale bar = 80 µm.
Fig. 2.6. GFRα2 immunoreactivity localized to S100 positive Schwann cells and their processes in left ventricular wall (A-F) and S100 positive cell process located next to a VACHT labeled cholinergic nerve fiber in the interatrial septum (G-I). Confocal images were obtained by sequential scanning of tissue sections that were double labeled for S100 and either GFRα2 or VACHT. GFRα2 and S100 immunoreactivity were extensively colocalized to Schwann cells (arrows) and their processes (arrowheads) in the left ventricular wall (A-F). Insert in A-C is a high magnification scan (63X oil objective) of the process indicated by a small box in the larger image. Areas of colocalization are evident as a yellow to orange color in the merged images (C and F). High magnification scan of atrial cholinergic nerve fiber (G-I) shows that VACHT and S100 occur in very close proximity but are not colocalized as indicated by the near absence of yellow to orange areas in the merged image (I). All panels are single optical sections. Asterisks mark the lumen of a blood vessel. Scale bar = 80 µm in A-C; 20 µm in D-F; 5 µm in G-I and inserts.
Identification of Ret-immunoreactive cardiac nerve fibers

Immunolabeling of cardiac nerve fibers for Ret was weaker than that observed for GFRα2 in fibers and more difficult to detect than Ret labeling of neuronal cell bodies. Nevertheless, colocalization of Ret immunoreactivity with CHT was clearly observed at a few atrial sites including the sinus node (Fig. 2.7) and AV node (not shown). This staining was obtained using tissue from hearts that were processed by brief perfusion with fixative prior to immersion fixation.

Fig. 2.7. Localization of Ret to cholinergic nerves in the sinus node. Sequentially scanned confocal images show significant colocalization of Ret (green) with CHT (red) in cholinergic nerves of the mouse sinus node (A-C). Maximum projection images were obtained from 26 optical sections spanning a tissue thickness of 10 µm. Scale bar = 80 µm.
Discussion

Our findings demonstrate that expression of NRTN continues in the adult mouse heart and that NRTN receptors are highly associated with intracardiac cholinergic neurons. A vast majority of the neurons in parasympathetic ganglia of the mouse heart exhibited the cholinergic phenotype (i.e., positive staining for ChAT), and over 90 percent of these cells expressed GFRα2 and Ret. Our dual staining for CHT and GFRα2 provides the first detailed mapping of cholinergic innervation in the mouse heart and establishes that most cholinergic nerve fibers contain GFRα2 immunoreactivity. Some of these cholinergic nerve fibers, particularly those in the sinus and AV nodes, also exhibited Ret immunoreactivity, thereby presenting the possibility that NRTN could act at these sites to activate retrograde signaling mechanisms, which are characteristic of neurotrophic factors. Additionally, immunolabeling for GFRα2 was detected in numerous Schwann cells that were associated with cholinergic and non-cholinergic nerves in the adult mouse heart. This finding suggests that these support cells may be an additional target for NRTN in the mature heart.

The expression of NRTN was detected by RT-PCR in the present study, and the identity of the PCR reaction product was confirmed by sequence analysis. This finding is consistent with the initial report on NRTN, which demonstrated NRTN mRNA in adult rat heart by RT-PCR analysis (Kotzbauer et al. 1996). Previous reports that adult mouse heart lacks NRTN mRNA were based on evidence from in situ hybridization (Widenfalk et al. 1997; Golden et al. 1999), which has a lower sensitivity than RT-PCR. Nevertheless, the sensitivity of in situ hybridization was sufficient to detect NRTN mRNA in mouse atria from E12-E18 (Golden et al. 1999), which suggests
that a higher level of expression occurs during development of the intracardiac nervous system. This conclusion is consistent with the observation that Ret-/- mice, which die soon after birth, have approximately half the normal number of intracardiac neurons and reduced cholinergic innervation of the AV conducting system compared to wild-type controls (Hiltunen et al. 2000). Our observation that NRTN is expressed by atrial tissue of adult mice supports the premise that this neurotrophic factor continues to influence cholinergic function in the mature heart since all cardiac cholinergic somata and a vast majority of their nerve fibers occur at this site. In contrast, the detection of comparable NRTN expression in left ventricular myocardium was unexpected since this area of the mouse heart contains relatively few cholinergic nerves. However, our discovery that cardiac Schwann cells and their processes exhibit GFRα2 immunoreactivity suggests that NRTN also might have a trophic influence on these non-neuronal support cells, which are present in the left ventricular wall and throughout the remainder of the heart.

Cholinergic nerve fibers were identified in this study using an antibody to CHT, a protein that is specifically localized to cholinergic neurons (Kuhar et al. 1973; Suszkiw and Pilar 1976; Ferguson et al. 2003; Hoover et al. 2004). This marker was used previously to map the cholinergic innervation of guinea pig hearts, where the vast majority of CHT immunoreactive nerve fibers occur in the sinus and AV nodes, conducting system and specific regions of right atrium (Hoover et al. 2004). We found that cholinergic innervation of mouse heart was similar to that of guinea pig in that CHT immunoreactive nerves were most abundant in the sinus node and atrioventricular conducting system but sparse in working myocardium of ventricular septum and left ventricular wall. However, the mouse differed from guinea pig by having more
cholinergic nerves in non-specialized regions of right and, to a lesser extent, left atrial myocardium, a low but significant number of cholinergic nerves in the right ventricular wall, and cholinergic innervation of blood vessels in atrial and ventricular myocardium. The distribution and relative regional abundance of GFRα2 immunoreactive nerve fibers paralleled the pattern of cholinergic innervation, and dual staining established that these markers were usually localized to the same processes throughout the heart and around coronary blood vessels. These observations provide direct evidence for the association of GFRα2 with cholinergic nerve fibers in the mouse heart and expand on an earlier report that cholinergic nerve density is reduced in right ventricle and AV conducting system of Gfrα2 -/- mice (Hiltunen et al. 2000). Based on our findings, we predict that the cholinergic deficit of Gfrα2 -/- mice extends beyond these regions and likely includes the sinus node. It should be noted that the widespread colocalization of GFRα2 and CHT to cholinergic nerve fibers in cardiac tissue is consistent with the presence of GFRα2 in a vast majority of ChAT immunoreactive neurons in the cardiac parasympathetic ganglia. Collectively, these observations imply that most cholinergic nerve fibers and cell bodies in the adult mouse heart retain the ability to specifically bind NRTN.

The presence of Ret is required for signaling by NRTN (Airaksinen and Saarma 2002; Enomoto 2005) and our findings demonstrate that this crucial protein is present in over 90% of mouse intracardiac neurons. We have also detected Ret immunoreactivity associated with some intracardiac nerve fibers. Although this staining was weaker than that observed for the neuronal somata, it was specifically localized to intracardiac nerve fibers and was most prominent in the sinus node, an important region not evaluated in
previous studies, and in the AV node. These results, when combined with our findings for GFRα2, demonstrate that both components of the NRTN receptor (i.e., ligand binding and signaling) are associated with cholinergic nerves that are known to regulate chronotropic and dromotropic functions of the heart.

While GFRα2 was commonly associated with cholinergic nerve fibers, a small population of intracardiac nerves stained for only CHT. The absence of GFRα2 staining in some cholinergic nerve fibers is consistent with the fact that about 5% of intracardiac neurons that stained for PGP 9.5 did not exhibit immunoreactivity for the NRTN receptor. Thus, a small population of intracardiac cholinergic neurons may be insensitive to NRTN.

The presence of additional GFRα2 immunoreactivity in cardiac Schwann cells was established by demonstrating its colocalization with the glial cell marker S100. This finding is consistent with previous reports that GFRα2 is present in glial cells located in the mucosal and muscle layers of mouse small intestine (Rossi et al., 2003) and in glial cells located in several mouse pelvic organs (Wanigasekara et al. 2004). Results from our double labeling experiments indicate that GFRα2 positive cardiac Schwann cells can be associated with cholinergic and non-cholinergic nerve fibers. Likewise, previous investigators found that GFRα2 positive Schwann cells were located near cholinergic and non-cholinergic nerve fibers in pelvic organs (Wanigasekara et al. 2004). Since Schwann cells and their processes occur in close proximity to some cholinergic nerve fibers and both of these cell types exhibit GFRα2 immunoreactivity, it is possible that NRTN affects some cholinergic neurons directly through neuronal receptors and indirectly through receptors on adjacent glial cells.
The continued expression of NRTN by adult mouse heart and the localization of NRTN receptors to most intracardiac neurons suggest that this neurotrophic factor is likely to have a tonic influence on cholinergic innervation of cardiac muscle and coronary vessels. Specific effects of NRTN on established cardiac innervation require evaluation. However, if NRTN affects cholinergic innervation in a fashion analogous to the influence of NGF on cardiac sympathetic nerves, NRTN might be required to maintain the structure, neurochemical phenotype and function of cholinergic neurons and their processes. Support for this scenario comes from a recent study of sacral parasympathetic ganglion neurons of the rat (Wanigasekara and Keast 2005). These investigators demonstrated that NRTN stimulated growth of dissociated neurons from this ganglion and initiated concentration dependent outgrowth and branching of neurites. Based on these considerations, it is possible that altered expression of NRTN might play a role in the development of cholinergic dysfunction, which often occurs in cardiac disease (Eckberg et al. 1971; La Rovere et al. 1998; Kuo et al. 1999; Singh et al. 2000).
Acknowledgements

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References


Widenfalk J, Nosrat C, Tomac A, Westphal H, Hoffer B, Olson L (1997) Neurturin and glial cell line-derived neurotrophic factor receptor-β (GDNFR-β), novel proteins related to GDNF and GDNFR-α with specific cellular patterns of expression suggesting roles in the developing and adult nervous system and in peripheral organs. J Neurosci 17:8506-8519
CHAPTER 3

STRUCTURAL AND FUNCTIONAL CARDIAC CHOLINERGIC DEFICITS IN ADULT NEURTURIN KNOCKOUT MICE

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Abstract

Aims Previous work has provided indirect evidence that the neurotrophic factor neurturin (NRTN) is required for normal cholinergic innervation of the heart. This study used nrtn knockout (KO) and wild-type (WT) mice to determine the impact that nrtn deletion has on cardiac cholinergic neurons and nerve fibers and on cholinergic function in the adult heart.

Methods Immunohistochemistry, confocal microscopy, and quantitative image analysis were used to evaluate cardiac cholinergic structure directly. Atrial acetylcholine (ACh) levels were determined as an indirect index of cholinergic innervation. Cholinergic function was evaluated by measuring negative chronotropic responses to right vagal nerve stimulation in anesthetized mice and negative chronotropic responses of isolated atria to muscarinic agonists.

Results KO hearts contained only 35% the number of cholinergic neurons in WT hearts, and the residual cholinergic neurons in KOs were 15% smaller in cross-sectional area. Cholinergic nerve density at the sinoatrial node was reduced by 87% in KOs, but noradrenergic nerve density was unaffected. Atrial ACh levels were substantially lower in KO mice (0.050 ±0.011 vs. 0.013 ± 0.004 pmol/μg protein; P<0.02) as expected from cholinergic neuron and nerve fiber deficits. The maximum bradycardia evoked by vagal stimulation was reduced in KO mice (38 ± 6% vs. 69 ± 3% decrease at 20Hz; P<0.001), and negative chronotropic responses in KOs took longer to develop and fade. In contrast to these deficits, isolated atria from KO mice had normal postjunctional sensitivity to carbachol and bethanechol.
**Conclusions**  These findings demonstrate that NRTN is essential for normal cardiac cholinergic innervation and cholinergic control of heart rate. The presence of residual cardiac cholinergic neurons and vagal bradycardia in KO mice suggests that additional neurotrophic factors may influence this system.
Introduction

Cardiac sympathetic neurons have a life-long requirement for nerve growth factor (NGF), which is a target-derived protein. Retrograde transport and signaling by this neurotrophic factor are essential for survival of developing sympathetic neurons and for maintaining the structure and function of sympathetic nerves in the adult heart. The latter requirement was manifest in recent findings implicating NGF in the remodeling of cardiac noradrenergic nerves that occurs in heart disease. Specifically, reduced NGF levels in failing hearts were linked to the loss of sympathetic nerves and reduced norepinephrine re-uptake, while elevated NGF levels around infarcted myocardium were associated with regional sympathetic hyperinnervation. Loss of sympathetic nerves contributes to inotropic dysfunction in the failing heart, while sympathetic hyperinnervation increases risk for ventricular arrhythmias. Far less is known about trophic factor requirements of cardiac parasympathetic neurons, but recent findings suggest that a protein named neurturin (NRTN) could serve as a crucial neurotrophic factor for these cells.

NRTN is a member of the glial-cell-line-derived neurotrophic factor (GDNF) family, which contains three other ligands including GDNF. Members of this family signal through two-component receptors that comprise a specific ligand binding protein (GDNF family receptor \( \alpha \), GFR\( \alpha \)) that couples to the transmembrane tyrosine kinase, Ret. GDNF and NRTN exhibit preferential binding to GFR\( \alpha 1 \) and GFR\( \alpha 2 \), respectively, but some crosstalk can occur at higher concentrations of neurotrophic factor, allowing GDNF to signal through GFR\( \alpha 2 \)/Ret complexes. Both components of the NRTN receptor are expressed by cardiac parasympathetic neurons during development, and
deletion of the gene for either component (i.e., *ret* or *gfra2*) disrupts development of cholinergic parasympathetic innervation of the heart. *Ret* knockout mice die shortly after birth, but their hearts contain about half the number of cholinergic neurons present in wild-type (WT) mice. Deletion of *gfra2* produces a non-lethal phenotype, and hearts from these mice have significantly fewer cholinergic nerve fibers compared to hearts from WT mice. Collectively, these experiments provide indirect evidence that NRTN has an essential role in establishing cholinergic innervation of the heart.

The requirement of NRTN for cholinergic innervation of the heart was assessed directly in this study by using *nrtn* (-/-) mice. Hearts from adult *nrtn* knockout (KO) and WT mice were evaluated for number and size of intrinsic cardiac neurons (ICNs), cholinergic and noradrenergic nerve densities at the sinoatrial (SA) node, and concentration of acetylcholine (ACh) in the atria. Negative chronotropic responses to vagal nerve stimulation were measured in anesthetized mice, and postjunctional cholinergic responses were evaluated using spontaneously beating isolated atria. Our findings delineate structural and functional cholinergic deficits that are present in the hearts of adult *nrtn* KO mice.
Material and methods

Experimental animals

Adult male C57BL/6 mice (16 weeks old; 24-30 g; Harlan, Indianapolis, IN) and 16-week-old male nrtn KO mice 10 were used for this study. Animal protocols were approved by the East Tennessee State University Animal Care and Use Committee and conformed to guidelines of the National Institutes of Health as published in the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996). Animals were used at the minimum numbers required for reaching statistically valid conclusions.

Tissue preparation for immunohistochemistry

Animals were deeply anesthetized with 5% isoflurane and euthanized by cervical dislocation. Hearts were removed rapidly and perfused through the ascending aorta with 3 ml of phosphate buffered saline (PBS) (pH 7.4, room temperature) followed by 3 ml of cold fixative comprising 4% paraformaldehyde and 0.2% picric acid in PBS. Tissues were post-fixed another 2 h at 4°C, cryoprotected for 2 days in cold 20% sucrose/PBS, and sectioned in a Leica CM3050S cryostat (Leica Microsystems Inc., Bannockburn, IL). Serial 16 μm short-axis sections were collected in six sets beginning at the most anterior aspect of the heart and ending at a level well into the ventricular myocardium. Each set of slides was boxed separately, wrapped in aluminum foil, and stored at -80°C.
**Immunohistochemistry**

Slide-mounted tissue sections were immunostained at room temperature as described previously. Briefly, sections were washed in 0.1M PBS (pH 7.3), permeabilized with 0.4% Triton X-100 in PBS containing 0.5% bovine serum albumin (BSA), and blocked for 2 h in PBS containing 10% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA), 1% BSA, and 0.4% Triton X-100. Tissues were then incubated for 15-18 h with two primary antisera generated in different species (Table 3.1), washed several times with 0.1M PBS, and incubated for 2 h with two species specific donkey secondary antibodies conjugated to Alexa Fluor 488 or 555 (Molecular Probes, Eugene, OR). Secondary antibodies were used at a dilution of 1:200. After washing the sections several times with PBS, coverglasses were attached using Citifluor mounting medium (Ted Pella, Inc., Redding, CA) and sealed with clear nail polish. Representative sections were routinely processed without primary antibodies (negative control). In all cases, these negative control sections showed only background fluorescence.

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**Confocal microscopy and image analysis**

Labeled tissue sections were viewed initially using an Olympus BX41 microscope (Olympus America Inc., Center Valley, PA), and regions of interest were selected for further evaluation by laser scanning confocal microscopy with a Leica TCS SP2 confocal microscope system. Specimens were scanned sequentially to avoid crosstalk between fluorochromes, and a maximum projection image was obtained from each series. Negative controls for each fluorochrome were also scanned using the same parameter settings. Images were exported into Corel Draw 11 and adjusted for brightness and contrast. The total number of neurons present in each section was determined based on staining for the cholinergic marker choline acetyltransferase (ChAT), which was previously determined to be present in virtually all ICNs of the mouse. Cell counts and cross-sectional areas were performed using Stereo Investigator/Workstation software (MicroBrightField, Williston, VT). Cross-sectional area was measured only for neurons with the nucleus visible. Cholinergic and noradrenergic nerve fibers were identified by dual staining for the vesicular ACh transporter (VACht) and tyrosine hydroxylase (TH), respectively. Quantitative evaluation of nerve fiber density was accomplished using Image J Software (National Institutes of Health, Bethesda, MD). Average nerve density was determined from maximum projection confocal images obtained from 2 to 3 sections for each animal.

**ACh and choline assay**

Mice were anesthetized with 5% isoflurane, euthanized by cervical dislocation, and quickly irradiated for 5 sec with microwaves (1.45 KW, 2450 MHz) to denature
proteins and preclude enzymatic changes in ACh and choline levels\textsuperscript{13}. The atria were removed, frozen in powdered dry ice, and weighed. Frozen tissue samples were sent to the Center for Molecular Neuroscience Neurochemistry Core Lab at Vanderbilt University (Nashville, TN) for determination of ACh and choline concentrations by high performance liquid chromatography with detection using a post-column enzyme reactor. ACh and choline values were normalized to the protein concentration in samples.

\textit{Vagal nerve stimulation}

Mice were anesthetized with 2\% isoflurane, and needle electrodes were inserted subcutaneously into the right anterior and left posterior leg regions. The electrocardiogram (ECG) was recorded using a Grass P55 A.C. preamplifier (Grass Technologies, West Warwick, RI), a PowerLab/8SP (ADIInstruments, Colorado Springs, CO), and a computer running Chart software version 5.2 (ADIInstruments, Colorado Springs, CO). The right vagus nerve was isolated in the neck region, secured with a suture tie, and cut anterior to the tie. A bipolar platinum electrode was placed on the distal end of the vagus nerve and kept in place with Kwik-Cast silicon sealant (World Precision Instruments, Sarasota, FL). A Grass SD9 stimulator was used for nerve stimulation (10 ms, 1 V) at increasing frequencies from 0.5 to 20 Hz, allowing time for recovery between each 10 s stimulus train. Baseline heart rate, minimum rate during stimulation, and times required to reach minimum rate and return to baseline after ending stimulation were measured.
**Concentration-response studies with isolated atrial preparations**

Mice were deeply anesthetized with 5% isoflurane and euthanized by cervical dislocation. Hearts were removed and placed in oxygenated (95% O₂, 5% CO₂), cold (4°C) Krebs-Ringer bicarbonate buffer (pH 7.35 to 7.4) of the following composition (in mM): 120 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 25 NaHCO₃, 2.5 CaCl₂, 1.2 MgCl₂, and 11.1 D-glucose. The entire atrium was dissected from the ventricles, and each side was impaled with a small metal hook (#28 trout hooks) with attached 5-0 suture. The left atrium was anchored to the bottom of a vertical support rod in a 10 mL tissue bath, and the right atrium was attached to a 25-g force transducer (World Precision Instruments, Sarasota, FL). Krebs-Ringer buffer in the tissue bath was oxygenated continuously and maintained at 37°C. Spontaneous atrial contractions were recorded at a resting tension of 0.3 to 0.5 g using a ML224 Bridge Amplifier (ADInstruments, Colorado Springs, CO), a PowerLab/8SP, and a computer running Chart. Postjunctional cholinergic sensitivity was evaluated by measuring changes in heart rate caused by cumulative additions of a muscarinic receptor agonist to the bath. Concentration-response data were collected for two agonists, carbachol and bethanechol, in random order. Atria were washed several times with buffer after testing the first agonist and given 10 to 15 min for recovery before evaluating responses to the second agonist. Data were analyzed using Prism software version 4.01 (GraphPad Software, La Jolla, CA) to determine the concentration of agonist that decreased heart rate by 50 percent (EC50).
**Statistical analysis**

Data are presented as the mean ± SEM (n). Pair wise comparisons of means were done using the Student’s t-test. Frequency-response data from vagal stimulation were evaluated by two factor analysis of variance with repeated measures. *P*<0.05 was considered significant.

**Drugs**

Bethanechol chloride (C5259) and carbachol chloride (C4382) were obtained from Sigma-Aldrich (St. Louis, MO).
Results

Immunohistochemical and biochemical analysis

Nrtn KO mice had about 65% fewer ICNs than WT mice, and the cross-sectional area of these neurons was significantly less (Fig. 3.1). In accord with this deficit of ICNs, we found that cholinergic innervation of the SA node was reduced drastically in nrtn KO mice compared to WT (Fig. 3.2 A,B and Fig. 3.3 A). Furthermore, visual examination of VACHT-stained sections showed a widespread deficit of cholinergic nerves in KO hearts, and this impression was supported by measurements of total atrial ACh concentration, which was reduced by 74% in KOs (Fig. 3.3 B). Choline, a precursor of ACh, was unaffected. In contrast to the marked cholinergic deficit in KO mice, the density of TH-immunoreactive, sympathetic nerves at the SA node was unaffected by deletion of nrtn (Fig. 3.2 C,D and Fig. 3.3 A).

Figure 3.1 Relative abundance and size of ICNs in 16 week old male nrtn KO and WT mice. (Left) KO mice had significantly fewer neurons, which were identified by immunolabeling for the cholinergic marker ChAT. Data were obtained from one set of sections per mouse (i.e., one sixth of total sections), so the actual number of neurons per heart would be larger. (Right) The cross-sectional area of ICNs was smaller in hearts from KO mice. n=6 per group. *P<0.05 vs. WT.
Figure 3.2  Representative images from confocal scans of SA nodes that were stained simultaneously for VACHT and TH to label cholinergic and noradrenergic nerves, respectively.  (A and B) VACHT-positive cholinergic nerve fibers were abundant in the SA node of WT mice but sparse in KO mice.  (C and D) TH staining of the same sections showed that noradrenergic nerves were less abundant than cholinergic nerves in the SA node but their density was similar in WT and KO mice.  Each panel shows a maximum projection image compiled from 21 optical sections that spanned an 8 μm thickness of tissue.  Scale bars = 150 μm.
Figure 3.3  Analysis of cholinergic and noradrenergic nerve fiber density in the SA node (A) and atrial ACh & choline concentrations (B) in nrtn KO and WT mice.  (A) Labeled tissue sections were scanned sequentially to obtain images of cholinergic (VACHT) and noradrenergic (TH) nerves.  Nerve fiber density was measured with Image J software and expressed as a percentage of the total nodal area.  n=6 per group.  *P<0.05 vs. WT.  (B) n=5 per group.  *P<0.05 vs. WT.
Negative chronotropic response to vagal nerve stimulation in vivo

Negative chronotropic responses to right vagal stimulation were evaluated to assess the function of cardiac parasympathetic nerves in KO and WT mice. No significant difference in baseline heart rate occurred between anesthetized KO and WT mice (WT: 594 ± 14 beat per min, n=6; KO: 640 ± 21 beats per min, n=5; P>0.05), and both strains had frequency-dependent decreases in heart rate during right vagal nerve stimulation (Fig. 3.4). However, the maximum response to vagal stimulation was substantially smaller in KO mice. We also observed that negative chronotropic responses to vagal stimulation and recovery from stimulation appeared slower in KO mice compared to WT (Fig. 3.5 A-D). Specifically, KO mice took longer for heart rate to reach a nadir during vagal nerve stimulation and longer for return to baseline heart rate after stopping stimulation. We quantified these effects by measuring the time for reaching minimum heart rate after vagal stimulation was initiated (onset) and the time for return of heart rate to baseline after vagal stimulation was stopped (offset). Both the “onset” and “offset” were significantly longer in KO mice compared to WT mice (Fig. 3.5 E).

![Figure 3.4](image.png) Negative chronotropic responses to right vagal nerve stimulation in anesthetized nrtn KO and WT mice. n=6 for WT and 5 for KO. $F_{1,63}=21.41$ for treatment effect (KO versus WT). $P=0.0012$. 
Figure 3.5 Time course of negative chronotropic responses to vagal stimulation. (A-D) Representative recorder tracings showing the time course of heart rate responses to 10 sec intervals of vagal stimulation at 8 and 20 Hz in WT and KO mice. Vertical broken lines indicate the start and stop of vagal stimulation in each panel. (E) Comparison of onset and offset times for vagal responses in WT and KO mice. Onset was defined as the time from start of stimulation to reaching minimum rate. Offset is the time required for return of heart rate to baseline after stopping stimulation. n=5 per group. *P<0.05 vs. WT.
Postjunctional cholinergic sensitivity

Postjunctional muscarinic receptor sensitivity was evaluated in spontaneously beating isolated atria from KO and WT mice. Cumulative concentration-response curves were constructed for two muscarinic receptor agonists (bethanechol and carbachol), and no differences were observed between atria from KO mice compared to WT mice (Fig. 3.6). Carbachol had a higher potency than bethanechol in both strains (carbachol –log EC50: 6.1 ± 0.1 for WT versus 6 ± 0.2 for KO, n=6 each; bethanechol –log EC50: 4.7 ± 0.1 for WT versus 4.9 ± 0.1 for KO, n=6 each).

Figure 3.6 Cumulative concentration-response curves for carbachol and bethanechol in isolated atria from nrtn KO and WT mice. No difference in response occurred between atria from WT and KO mice. Carbachol had a greater potency in both strains. Baseline heart rates were not significantly different between groups (WT: 351 ± 21 beats per min; KO: 332 ± 5 beats per min; n=6 per group; P>0.05)
Discussion

This work provides definitive evidence that NRTN plays a major role in the development of cardiac parasympathetic ganglia and cholinergic innervation of the heart. Deletion of the NRTN gene produced a drastic reduction in the number of ICNs and corresponding decreases in total atrial ACh concentration and cholinergic nerve density at the SA node. These structural and neurochemical deficits were accompanied by impaired negative chronotropic responses to direct activation of preganglionic cholinergic nerves by vagal stimulation. Autonomic effects of NRTN deficiency at the heart were specific for cholinergic neurons since neither noradrenergic nerve density at the SA node nor postjunctional atrial sensitivity to muscarinic agonists was altered.

Neurotrophic factors and cholinergic innervation of the heart

The neurotrophin NGF has a vital role in the development and maintenance of cardiac noradrenergic innervation. Little was known about the trophic factor requirements of ICNs, but the present findings and previous work with ret and gfra2 KO mice established that NRTN is essential for the development of a majority of ICNs and for normal cholinergic innervation of the mouse heart. However, NRTN cannot be the sole neurotrophic factor affecting ICNs since a smaller subpopulation of ICNs and a reduced number of cholinergic nerves still developed in all three strains of KO mice (i.e., nrtn, ret, and gfra2). Contributions from other GDNF family ligands seems unlikely since gfra1 and gfra3 mRNAs were localized only to non-neuronal cells in cardiac parasympathetic ganglia of neonatal and three week old rats. Also, hearts from E18 ret KO mice did not show larger deficits in number of ICNs than we observed in adult
nrtn KO mice\textsuperscript{9}, although it remains possible that neuronal loss would have been greater if these mice survived. Neurotrophin-3 might serve as a neurotrophic factor for ICNs since transcripts for the neurotrophin-3 receptor (i.e., trkC) were identified in developing rat ICNs\textsuperscript{14}. Additionally, we found that adult mouse ICNs exhibit trkA and p75 receptor immunoreactivity\textsuperscript{12}, suggesting that these cells may respond to NGF and other neurotrophins.

While cardiac sympathetic neurons in adults require NGF, it is unclear whether ICNs have a similar, life-long requirement for trophic support. Evidence from studies of adult rat sacral parasympathetic neurons suggested that they retained their dependence on NRTN\textsuperscript{15}. These parasympathetic neurons decreased in size after axotomy was performed to preclude retrograde transport of target-derived trophic factor. In contrast, treatment of cultured sacral parasympathetic neurons with NRTN caused an increase in cross-sectional area of somata and stimulated neurite outgrowth. Thus, NRTN still had a trophic action on adult sacral parasympathetic neurons. The smaller size of KO ICNs compared to WT in the present study might be a consequence of NRTN deficiency. This possibility is supported by our previous work, which showed that \textit{nrtn} is expressed in adult mouse atria and that adult mouse ICNs have both components of the NRTN receptor\textsuperscript{11}.

\textit{Impaired cholinergic control of heart rate in nrtn KO mice}

The substantial cholinergic nerve deficit that we observed in KO mice was associated with a marked impairment of negative chronotropic responses to right vagal nerve stimulation. Not only was the magnitude of bradycardia reduced at each
stimulation frequency, but also the heart rate took longer to reach a nadir during nerve stimulation and to return to baseline after stimulation ended in the KO mice. Delayed onset of maximum bradycardia could be a consequence of reduced cholinergic nerve density and the corresponding need for ACh to activate muscarinic receptors at a greater distance from the release sites. While ACh is normally inactivated rapidly by acetylcholinesterase (AChE) that hydrolyzes ACh\(^{16}\), this process may be prolonged in KO mice due to loss of AChE associated with cholinergic nerves. Since AChE is localized primarily to cholinergic nerves in the heart\(^{17,18}\), reduced density of cholinergic nerves would also cause a deficit of AChE. The same factor might explain the delayed recovery of heart rate in KO mice. Decreased regional abundance of AChE within the SA node could prolong the survival of ACh and its ability to stimulate atrial muscarinic receptors. In this case, diffusion of ACh could be important for terminating the bradycardia.

Interestingly, postjunctional sensitivity to muscarinic agonists was not affected in KO mice in spite of major deficits in cholinergic innervation and neurally-mediated cholinergic effects on heart rate. It was possible that this condition might have triggered postjunctional supersensitivity due to a chronic reduction of cholinergic signaling at the SA node\(^{19}\). Lack of cholinergic supersensitivity might be a consequence of cardiac sympathetic dominance in mice as opposed to parasympathetic dominance in larger animals\(^{20}\). From this perspective, the reduced cholinergic innervation present in KO hearts may be sufficient to meet their needs. It is also possible that C57BL/6 mice might have enough “spare” atrial muscarinic receptors to maintain cholinergic neurotransmission at a level sufficient to preclude development of supersensitivity.
Conclusions

Our findings provide clear evidence that NRTN is essential for the development of normal cholinergic innervation of the heart and cholinergic control of heart rate. However, the specific role of NRTN in development of this system remains unknown. Is NRTN required for migration of cholinergic precursor cells to the heart, innervation of atrial muscle (i.e., target-derived trophic factor), or does it serve both functions? Our findings also show that NRTN cannot be the sole neurotrophic factor for development of cardiac cholinergic innervation. Additional work is needed to identify other neurotrophic factors that influence the development of ICNs and determine if neurotrophic factors are required for the maintenance of cholinergic neurons and nerve fibers in the adult heart. Beyond these issues, the presence of well-defined structural and functional cholinergic deficits in the heart of NRTN knockout mice should make this strain a valuable tool for evaluating the role of cardiac parasympathetic nerves in disease.
Funding

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Acknowledgements

We are very grateful to Dr. Eugene Johnson, Jr., (Washington University, St. Louis, MO) for generously providing breeding pairs of \textit{nrtn} (-/-) mice that were essential for this study. Thanks also to Dr. John C. Hancock for valuable instruction in methods for vagal nerve stimulation.

Conflict of Interest: none declared
References


CHAPTER 4

STRUCTURAL AND FUNCTIONAL CARDIAC CHOLINERGIC REMODELING IN STREPTOZOTOCIN-INDUCED DIABETIC MICE

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Keywords: autonomic nervous system; intrinsic cardiac ganglia; parasympathetic; streptozotocin; diabetes; electrocardiogram
ABSTRACT

Background: Cardiac autonomic neuropathy (CAN) occurs as a complication of diabetes mellitus (DM), resulting in imbalance of sympathetic and parasympathetic control of the heart. This imbalance causes abnormalities in heart rate control and increases the risk of serious cardiac arrhythmias and sudden death. Several studies have examined the impact of diabetes on the cardiac sympathetic nervous system, but little is known about the effects on the cardiac parasympathetic system.

Methods and Results: In vivo electrocardiogram (ECG) recordings were obtained weekly from conscious control and streptozotocin (STZ)-induced diabetic mice for up to 16 weeks post-injection. Over the 16 week time course, these recordings showed progressively decreased heart rate, increased short term heart rate variability, and increased duration of ST and rate corrected QT (QTc) intervals in diabetic mice. ECG recordings were supplemented with pharmacological testing using atropine and atenolol, which showed evidence of autonomic imbalance. Immunohistochemical analysis of atrial tissue stained for vesicular acetylcholine transporter revealed increased cholinergic nerve density at the sinoatrial node in diabetic mice compared to controls (14.9±1.2 vs. 8.9±0.8; p<0.01). Responses to right vagal nerve stimulation were blunted in 16-week diabetic mice with no change in postjunctural sensitivity of isolated atria to muscarinic agonists.

Conclusions: STZ-induced diabetes causes structural and functional remodeling of the cardiac parasympathetic nervous system in mice and these changes increase susceptibility to cardiac rhythm disturbances. The present study improves our
understanding of the impact that type 1 diabetes has on the cardiac parasympathetic nervous system and potential causes of arrhythmias associated with DM.
Introduction

Diabetes is a major cause of disability and mortality in the United States and worldwide. It is a pervasive disease that causes a host of serious complications resulting from prolonged exposure to high levels of glucose and lipids (11). Diabetes can have a particularly devastating impact on the heart by causing cardiac pathology alone and in synergism with other diseases such as hypertension and atherosclerosis (34). Additionally, diabetes can further impair cardiac function by affecting the autonomic nerves that control heart rhythm (36). The presence of such cardiac autonomic neuropathy in diabetic patients increases their risk for life threatening ventricular arrhythmias and sudden death (23). Several epidemiological studies have provided evidence that diabetes also increases the risk for atrial fibrillation, which often impairs quality of life and can lead to stroke (23; 28). The mechanisms underlying these associations are undoubtedly complex and most likely involve pathophysiological remodeling of the autonomic nervous system and cardiac tissue.

Several clinical studies have used heart rate variability (HRV) measurements to assess parasympathetic and sympathetic control of the sinus node in patients with and without DM (6; 27; 39). In a majority of these studies it was found that diabetics with decreased HRV were at increased risk for ventricular fibrillation and sudden death compared to non-diabetic patients and diabetic patients with normal HRV (27). Power spectral analysis of HRV has proven useful in evaluating cardiovascular autonomic function in several diseases, including diabetes (4; 6). Decreased HRV in the high frequency (HF) range indicates that there is a relative deficiency of parasympathetic
(cholinergic) input to the sinus node, and this change is sometimes viewed as reflecting sympathetic dominance throughout the heart (5).

The autonomic nervous system and remodeling of atrial structure are both thought to play a role in the pathogenesis of atrial fibrillation. Assessing the impact of cholinergic remodeling on atrial function in diabetes requires consideration of structural and functional changes in cholinergic neurons and nerve fibers, postjunctional changes that influence response to acetylcholine (ACh), and the progressive nature of the disease. A substantial body of evidence suggests that cholinergic mechanisms can have a particularly strong proarrhythmic influence on the atria (9; 37). Vagal stimulation and muscarinic (cholinergic) agonist drugs increase susceptibility to AF because they decrease the relative refractory period and action potential duration of atrial myocytes (43). Furthermore, ultrastructural changes in diabetic rabbit atria were accompanied by reduced sinus nodal automaticity, slowed conduction throughout the atria, and inhomogeneity of conduction (38). Such conditions could form the substrate for atrial arrhythmias. While these findings are intriguing, effects of diabetes on atrial innervation were not evaluated.

This study investigates the hypothesis that diabetes causes remodeling of the atrial cholinergic nervous system, which could make the atria more susceptible to arrhythmias. Experiments within this study provide a thorough analysis of the cardiac parasympathetic nervous system in mice with type 1 diabetes.
Materials and Methods

Animals

Adult male C57BL/6 mice (8 weeks old; 24-30g; Harlan, Indianapolis, IN) were used for this study. Animal protocols were approved by the East Tennessee State University Committee on Animal Care and conformed to guidelines of the National Institutes of Health as published in the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996).

Induction of diabetes

Diabetes was induced in adult male C57BL/6 mice at 8 weeks of age by multiple injections of streptozotocin (STZ, 50mg/kg; Sigma-Aldrich) according to a modified method based on work by Qi et al (31). STZ was dissolved in 0.1M sodium citrate buffer (pH 4-5) and administered by intraperitoneal (i.p.) injection within 10 min of preparing the solution. This time limit is crucial since STZ is very unstable in solution. Injections were given on five consecutive days, at the same time each day, after mice had been fasted for at least 6 h. Fasting for a minimum of 6 h is crucial for optimal effectiveness of STZ. STZ enters beta cells of the pancreas through the glucose transporter (35; 44); therefore, any glucose present at the time of injection would compete with STZ for the transporter and reduce STZ entry. Control mice received injections of citrate buffer vehicle only. Animal weights and non-fasting blood glucose levels were determined weekly to monitor progression of diabetes.
**Insulin treatment**

Effects of insulin were evaluated in one experiment via insulin therapy, beginning 3 weeks after STZ injections. Half of the diabetic mice in this experiment were treated twice daily by subcutaneous injection of Novolin N insulin (0.5-2U; Novo Nordisk Inc., Princeton, NJ) for a total of 5 weeks. The remaining diabetic mice and non-diabetic controls were treated with diluent only (Eli Lilly and Company, Indianapolis, IN). Treatments were given at 7:00AM and 7:00PM daily based on non-fasting blood glucose levels. Insulin treated diabetic mice with non-fasting blood glucose of ≤100mg/dL at time of treatment were not given insulin until the next scheduled treatment time. In the event of hypoglycemia (non-fasting blood glucose of ≤60 mg/dL), corn syrup was administered orally to bring blood glucose levels quickly back to the normal range.

**Blood collection and analysis**

Blood for glucose measurements was obtained by pricking the tip of the tail with a lancet between the hours of 7:00AM-10:00AM. The blood was collected directly onto a glucose testing strip and analyzed with a OneTouch Ultra2 glucose monitor (Life Scan Inc., Milpitas, CA). Larger quantities of blood (at least 8μL) were required for glycosylated hemoglobin A1c (HbA1c) measurement. Blood for analysis of HbA1c was drawn prior to termination by pricking the facial vein with a lancet and collected in heparin-coated Microcuvette CB300 tubes (Sarstedt, Newton, NC). These samples were analyzed by the Mouse Metabolic Pathophysiology Core at Vanderbilt University (Nashville, TN).
Tissue processing for histology

Animals were deeply anesthetized with 5% isoflurane and euthanized by cervical dislocation. Hearts were removed rapidly and perfused through the ascending aorta with 3 mL of phosphate buffered saline (PBS) (pH 7.4, room temperature) followed by 3 mL of cold fixative comprising 4% paraformaldehyde and 0.2% picric acid in PBS. Tissues were post-fixed another 2h at 4°C, cryoprotected for 2 days in cold 20% sucrose/PBS, and sectioned in a Leica cryostat/microtome. Serial 16μm short-axis sections were collected in six sets beginning at the most anterior aspect of the heart and ending at a level well into the ventricular myocardium. Each set of slides was boxed separately, wrapped in aluminum foil, and stored at -20°C.

Immunohistochemistry

Slide mounted tissue sections were immunostained at room temperature. Briefly, sections were washed in 0.1M PBS (pH 7.3), permeabilized with 0.4% Triton X-100 in PBS containing 0.5% bovine serum albumin (BSA), and blocked for 2 h in PBS containing 10% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA), 1% BSA, and 0.4% Triton X-100. Tissues were then incubated for 15-18 h with two primary antisera generated in different species (Table 4.1), washed several times with 0.1M PBS, and incubated for 2 h with two species specific donkey secondary antibodies conjugated to Alexa Fluor 488 or 555 (Molecular Probes, Eugene, OR). Secondary antibodies were used at a dilution of 1:200. After washing the sections several times with PBS, coverglasses were attached using Citifluor mounting medium (Ted Pella, Inc., Redding, CA) and sealed with clear nail polish. Representative sections
were routinely processed without primary antibodies (negative control). In all cases, these negative control sections showed only background fluorescence.

**Table 4.1** Primary antibodies used for immunohistochemistry

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<td>P40101-0</td>
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**Confocal Microscopy and image analysis**

Labeled tissue sections were viewed initially using an Olympus BX41 microscope and regions of interest were selected for further evaluation by laser scanning confocal microscopy with a Leica TCS SP2 confocal microscope system. Specimens were scanned sequentially to avoid crosstalk between fluorochromes, and a maximum projection image was obtained from each series of optical sections. Colocalization of immunolabels was evaluated in overlay images obtained from these scans. Negative controls for each fluorochrome were also scanned using the same settings. Images were exported into Corel Draw 11 and adjusted for brightness and contrast. The total number of neurons present in each section was determined based on staining for the cholinergic marker choline acetyltransferase (ChAT), which was previously determined to be present in all intrinsic cardiac neurons of the mouse (21). Total cell counts and cross-sectional neuronal areas were measured using Stereo Investigator/Workstation software (MicroBrightField, Williston, VT). Cholinergic and noradrenergic nerve fibers were identified by staining for vesicular ACh transporter (VACHT) and tyrosine hydroxylase (TH), respectively. Quantitative evaluation of nerve fiber density at the SAN in maximum projection images obtained from confocal microscopy was accomplished.
using Image J Software (National Institutes of Health, Bethesda, MD). The SAN was
outlined within each of 3 images obtained per animal, from which nerve density was
analyzed by measuring the total number of pixels per image, and then isolating pixels
containing nerve fibers only based on a threshold fluorescence level. Final values for
nerve fibers are expressed as percent cholinergic/noradrenergic pixels out of total pixels
(\% total area).

Electrocardiogram (ECG) acquisition and in vivo pharmacological testing

Lead II ECGs were obtained non-invasively using the ECGenie apparatus
(Mouse Specifics, Inc., Boston, MA). The ECGenie is a PowerLab-based system that
acquires signal through disposable footpad electrodes located in the floor of a recording
platform (8). Contact with two of three electrodes is required to obtain an ECG and 2-3
seconds of recording (20-30 P-Q-R-S-T complexes) are sufficient for analysis. Several
minutes of recording were collected from each mouse at every recording session with a
constant segment of 20-30 complexes used for analysis. Raw ECG signals were
analyzed using e-MOUSE software (Mouse Specifics, Inc., Boston, MA), which employs
processing algorithms for peak detection, digital filtering, and correction of baseline for
motion artifacts. ECG was acquired weekly throughout the duration of the experiment.
Heart rate (HR) was determined from the average of R-R intervals and short term heart
rate variability (HRV) was based on the standard deviation of the R-R intervals. Power
spectral analysis of ECG in the frequency domain was accomplished using low
frequency (LF) and high frequency (HF) ranges that were previously published for
C57BL/6 mice (16). LF power range detection limits were 0.15-1.5 Hz and HF at 1.51-5.0 Hz.

Assessment of autonomic control of the heart was accomplished through conscious ECG acquisition in conjunction with pharmacological testing. ECG was recorded with the ECGenie apparatus before and after each of the following drug injections when mice were 8 and 16 weeks post-STZ injection. To block autonomic receptors and determine parasympathetic/sympathetic tone, atropine sulfate (5 mg/kg) or atenolol (5 mg/kg) was injected i.p. with at least 24 h between injections. Atropine and atenolol were also given in combination on a separate day in order to determine intrinsic HR. For atropine and atropine/atenolol combination, ECGs were measured when the drug effect had reached a maximum (30 min). For atenolol only, ECGs were measured at 20, 30, and 40 min post-injection. All drug doses and times to maximum effect were determined based on preliminary investigations. All injections were performed at the same time each day (8:00AM-10:00AM).

Vagal nerve stimulation

Diabetic and control mice were anesthetized with 2% isoflurane, and needle electrodes were inserted subcutaneously in the right anterior and left posterior leg regions. ECG was recorded using a Grass P55 A.C. preamplifier (Grass Technologies, West Warwick, RI), a PowerLab/8SP (ADInstruments, Colorado Springs, CO), and a computer running Chart software version 5.2 (ADInstruments, Colorado Springs, CO). The right vagus nerve was isolated in the neck region, secured with a suture tie, and cut anterior to the tie. A bipolar platinum electrode was placed on the distal end of the
vagus nerve and kept in place with Kwik-Cast silicon sealant (World Precision Instruments, Sarasota, FL). A Grass SD9 stimulator was used for nerve stimulation (10ms, 1-2V) at increasing frequencies from 0.5 to 20 Hz, allowing time for recovery between each 10s stimulus train. Baseline HR, ECG, and minimum HR during vagal stimulation were measured.

**Concentration-response studies with isolated atrial preparation**

Diabetic and control mice were deeply anesthetized with 5% isoflurane and euthanized by cervical dislocation. Hearts were removed and placed in oxygenated (95% O₂, 5% CO₂), cold (4°C) Krebs-Ringer bicarbonate buffer (pH 7.35-7.4) of the following composition (in mM): 120 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 25 NaHCO₃, 2.5 CaCl₂, 1.2 MgCl₂, 11.1 D-glucose. Atria were dissected from the ventricles and impaled with small metal hooks (#28 trout hooks) with attached 5-0 suture. The left atrium was anchored to the bottom of a vertical support rod in a 10mL tissue bath, and the right atrium was attached to a 25-g force transducer. The tissue baths contained oxygenated Krebs-Ringer solution maintained at 37°C with a thermostatically controlled circulating-water bath (Isotemp 2013S; Fisher Scientific, Pittsburg, PA). Atria were placed under a resting tension of 0.5g and force of contraction was measured using a 25-g force transducer (World Precision Instruments, Sarasota, FL). Spontaneous atrial contractions were recorded using a ML224 Bridge Amplifier (ADInstruments, Colorado Springs, CO), a PowerLab/8SP, and a computer running Chart. Postjuncttional cholinergic sensitivity was evaluated by measuring changes in HR caused by cumulative additions of a muscarinic receptor agonist to the bath. Concentration
response data were collected for two agonists, carbachol and bethanechol, in random order. Atria were washed several times with buffer after testing the first agonist and given 10 to 15 min for recovery before evaluating responses to the second agonist. Data were analyzed using Prism software version 4.01 (GraphPad Software, La Jolla, CA) to determine the concentration of agonist that decreased HR by 50 percent (EC50).

**Drugs**

Atenolol (A-7655), atropine sulfate (A-0257), bethanechol chloride (C-5259), carbachol chloride (C-4832) and STZ (85882) were from Sigma-Aldrich (St. Louis, MO).

**Statistical Analysis**

Statistical analyses were performed using GraphPad Prism 4.01 (GraphPad Software, La Jolla, CA,). Values are presented as mean ± SEM, unless otherwise specified. Values for n represent the number of animals/hearts used. A Student’s t-test was used for between group comparisons of HbA1c and nerve fiber density. A two-way ANOVA with repeated measures was used to compare all other differences between groups over time. This was followed by LSD *post hoc* testing when appropriate. Differences were considered significant at P<0.05.
Results

General characteristics of STZ-treated mice

Average body weight and blood glucose measurements did not differ between groups prior to injections and were monitored weekly to assess effects of diabetes. Mice injected with STZ had significantly reduced body weight gain when first measured at 2 weeks post-injection (Control 25.5±0.5 grams vs. STZ 24.2±0.4 grams; P<0.05; Fig 4.1 A). Mice with diabetes gained body weight slower than control mice for most of the study (2-way ANOVA; P<0.05). Non-fasting blood glucose levels were significantly increased in diabetic mice at 2 weeks (Control 144±5 mg/dL vs. STZ 376±15 mg/dL; P<0.05) (Fig 4.1 B) and subsequently remained over 400mg/dL for the entire experiment. HbA1c levels (%) were significantly increased in diabetic mice when measured at 8 and 16 weeks (Fig 4.1 C).

Figure 4.1: Characteristics of STZ treated mice. Effects of STZ on body weight (A) and blood glucose (B) over 16 weeks (Control: n=12; STZ: n=14). HbA1c levels (C) at 8 and 16 week-post injection (n=6 for all). *P<0.05 vs. control at same time.

ECG analysis

HR fell significantly within 2 weeks after administration of STZ (Control 803±4 vs. STZ 749±8; P<0.0001) and continued to decline throughout the experiment (2-way ANOVA P<0.0001; Fig 4.2 A). Analysis of the ECG waveform revealed that the ST and
rate corrected QT (QTc) intervals were significantly increased in diabetic mice (2-way ANOVA P<0.05 for both; Fig. 4.2 B,C). QT dispersion was also significantly increased in diabetic animals (2-way ANOVA P<0.05; data not shown). Raw ECG waveforms from diabetic mice (Fig. 4.2 E) showed increased short term HRV compared to control (Fig. 4.2 D), suggesting the presence of sinus arrhythmias. Power spectral analysis of the ECG showed that diabetic mice had significantly decreased HF power (Fig. 4.2 F) and increased LF power (Fig. 4.2 G) when first examined 2 weeks post-STZ treatment (Student’s t-test P<0.01 for both). These changes were evident at most subsequent time points as well (2-way ANOVA P<0.0001 for both; Fig. 4.2 F,G).

**Figure 4.2:** ECG analysis of heart rate. ECG derived heart rate (A), ST segment length (B) and QTc interval (C). Representative ECG from non-anesthetized 14 week post-injected control (D) and diabetic (E) mouse. Power spectral analysis of HF (F) and LF (G) power. (Control: n=12; STZ: n=14).
Pharmacologic assessment of HR control

Effects of treatment with the muscarinic antagonist atropine on HR and HRV at 8 weeks were no different in control and diabetic mice (Student’s t-test; P>0.05). At 16 weeks, there was no change in overall HR with atropine (Student’s t-test; P>0.05), but short term HRV (defined in bpm as standard deviation of RR interval), which was significantly elevated in diabetic animals at baseline (5.93±0.5 vs. 16.3±4.7 bpm, P<0.05), was significantly decreased in diabetic mice 30 min after the injection (P<0.05) (Fig. 4.3 A). At 8 and 16 weeks, diabetes enhanced effects of β₁-adrenergic receptor blockade on HR and HRV with atenolol (2-way ANOVA P<0.01) (Fig. 4.3 B). Effects of combined blockade with atropine and atenolol on HR (Fig. 4.3 C) and HRV (not shown) were not different at 8 or 16 weeks, indicating no change in intrinsic HR at either time.
Figure 4.3: Pharmacological assessment of HR control. HR and HRV responses in control and diabetic mice at 16 weeks to pharmacological challenge with atropine (A) and atenolol at 16 weeks (B). HR responses (intrinsic heart rate) to combination blockade of atropine and atenolol at 8 and 16 weeks (C). (A: n=16 and B,C: n=9 per group; *P<0.05 compared to control at same time point; #P<0.05 compared to baseline).
**Effect of STZ on intracardiac neurons and nerve fibers**

Intracardiac ganglia (ICG) are located primarily in epicardial connective tissue along the posterior surface of the atria and in interatrial septum. These ganglia are innervated by the vagus nerve and send projections to discrete areas of the heart, including the sinoatrial node (SAN). Quantitative evaluation of ICG in 16-week post-injected control and STZ-treated mice showed that all neurons exhibited ChAT immunoreactivity. The total number of intracardiac neurons did not differ between the two groups. However, the cross-sectional area of neurons in STZ-treated mice was about 10% smaller compared to controls (249±7μm² versus 223±4μm²; n=6 each; P<0.05).

Quantitative evaluation of staining at the SAN revealed increased cholinergic nerve fiber density in STZ-treated mice compared to controls at both times studied (Fig. 4.4). Insulin therapy prevented this increase in 8 week STZ-treated mice (Fig 4.4 C, F). There was no significant change in SAN noradrenergic fiber density at either of the times studied (8 weeks: Control 3.7±0.7 vs. STZ 3.5±0.5; 16 weeks: Control 2.5±0.4 vs. STZ 3.0±0.8; both P>0.05 Student’s t-test).
Figure 4.4: Cholinergic nerve fiber density in the SAN is increased in STZ-treated mice. Maximum projection confocal images of sections of SAN immunostained for VACHT in (A) 8wk control, (B) 8wk STZ, (C) 8wk insulin-treated STZ, (D) 16wk control and (E) 16wk STZ hearts. (F) Quantitative analysis of cholinergic fiber density in the SAN at 8 and 16 weeks post-injection. Maximum projections images were obtained from 21 optical sections spanning a tissue thickness of 8µm. Scale bar 150µm. *P<0.05 vs. Control at same time; #P<0.05 vs. STZ at same time (n=6 per group).
**Effects of Vagal Nerve Stimulation**

Negative chronotropic responses to right vagal nerve stimulation were measured in control and diabetic mice at 8- and 16-week post-injection to assess the function of cardiac parasympathetic nerves. There was no difference in baseline HR between groups before vagal stimulation at either time studied (8wk: 561±21 bpm vs. 570±29 bpm, P>0.05; 16wk: 572±31 bpm vs. 581±22 bpm, P>0.05). Vagal nerve stimulation caused frequency dependent decreases in HR in all animals (Fig. 4.5). There was a trend toward suppression of vagal nerve response in diabetic animals at 8 weeks (2-way ANOVA P>0.05; Fig 4.5 A), which became significant with 16 weeks of diabetes (Fig 4.5 B; P<0.03). Specifically at 16 weeks, responses to stimulation frequencies of 1-12Hz were significantly suppressed in diabetic mice (P<0.05).

**Figure 4.5:** Negative chronotropic responses to right vagal nerve stimulation in anesthetized 8 (A) and 16 week (B) post-injected control and STZ-induced diabetic mice. (n=6 per group).
Concentration-response studies with isolated atrial preparations

Cumulative concentration-response curves were constructed for the direct-acting cholinergic agonists at 8 and 16 weeks post-injection. No difference in intrinsic rate amongst isolated atria was observed among any of the groups prior to addition of agonists (data not shown; ANOVA, P>0.05). No differences in HR response to cumulative additions of either agonist were observed at 8 or 16 weeks (Fig 4.6). Carbachol had a higher potency than bethanechol in both groups at each time tested (carbachol: 8 weeks –log EC50: 6.1±0.1 for control vs. 6.1±0.1 for STZ, 16 weeks –log EC50: 5.8±0.2 for control vs. 6.1±0.1, for STZ; bethanechol: 8 weeks –log EC50: 4.6±0.2 for control vs. 4.8±0.2 for STZ, 16 weeks –log EC50: 4.5±0.2 for control vs. 4.6±0.1 for STZ; n=6 each).

Figure 4.6: Cumulative concentration-response curves for carbachol and bethanechol in isolated atria from 8 (A) and 16 week (B) post-injected control and STZ-induced diabetic mice.
Discussion

This study represents the first evaluation of the impact of STZ-induced type 1 DM on the cardiac parasympathetic nervous system in mice. Our results demonstrate that structure and function of this system are remodeled when challenged with STZ-induced diabetes. In mice with severe type 1 diabetes, cholinergic nerves within the SAN undergo sprouting, which can be prevented with insulin treatment. Short-term HRV was increased in diabetic mice which could be attenuated by muscarinic receptor blockade. Removal of sympathetic contribution to heart rate with $\beta_1$-adrenergic blockade resulted in lower heart rate in diabetic mice. In spite of this evidence for enhanced cholinergic function, diabetic mice had a decreased response to direct vagal nerve stimulation with no change in postjunctival sensitivity of atria to muscarinic agonists. Power spectral analysis of the diabetic ECG revealed an imbalance in autonomic control of heart rate. Altogether, these data suggest that type 1 diabetes causes either a defect at the intracardiac ganglia or further upstream, including transmission from the central nervous system.

Using STZ as a model of type 1 diabetes in rodents

Previous studies using animal models of type 1 diabetes have most commonly been performed in STZ-induced rats (7; 10; 12; 14; 15). Our model of STZ-induced diabetes produces more severe hyperglycemia than in rats, thus preventing the mice from living long enough to examine the long-term effects of the disease. Therefore, comparisons of studies within the literature have somewhat conflicting conclusions. The current study employs a multiple low-dose (50mg/kg) STZ-induced model in male
C57BL/6 mice. In our model, non-fasting blood glucose levels rose to nearly 500 mg/dL within 8 weeks and remain above this level. Severity of diabetes was also evident from HbA1c measurements, which were consistently around 5% for controls, but were almost 12% in diabetic mice at 8 weeks and over 14% by 16 weeks. By 16 weeks, these mice are beginning to show a decline in overall physical condition from the severe hyperglycemia, which was evident by significant weight loss and reduced activity and grooming. The standard diabetes induction method in rats, a single dose of 50-65 mg/kg STZ, produces only moderately high levels of blood glucose ranging from 267-465 mg/dL by 10 weeks (12; 15) and around 356 mg/dL after almost 1 year (7).

**Preganglionic cholinergic function**

In human diabetics, decreased HRV is considered to be the earliest sign of cardiac dysfunction. This, in combination with decreased cardiac baroreflex observed in type 1 diabetics, has been attributed to cardiac parasympathetic denervation (42). In human atrial appendage, ACh release and choline uptake were decreased in diabetic patients with late complications (neuropathy, nephropathy), compared to those without, indicating time dependency of pathological changes (30), which would be consistent with a loss of nerves (22; 42). In our study with severe type 1 diabetic mice, we found reductions in responses to VNS at 16, but not 8, weeks of diabetes with increased sprouting of cholinergic nerves in the SAN at both times. This implies that postganglionic nerves are preserved during early diabetes, suggesting a defect in preganglionic transmission that could be related to loss of those nerves. Intracardiac neuronal cell bodies appear normal in diabetic mice, but are ~10% smaller in size than
control. In rats with a year of STZ diabetes, neurons were still present and appeared normal, but nerves, likely preganglionic, were showing degeneration (17). Electron microscopic studies on alloxan-treated rats showed a loss of atrial post-ganglionic cholinergic nerves and their function after 7-8 months of diabetes (41). These data suggest there are progressive changes in the neural axis with postganglionic function being preserved early on in diabetes, with deficits primarily residing upstream. From other studies in animals with less severe forms of diabetes, it also appears that a central defect occurs earlier, since responses to vagal stimulation are preserved but baroreflex sensitivity is decreased (10; 13; 24-26).

Postganglionic cholinergic function

Postganglionic cholinergic nerve function was not directly evaluated in type 1 diabetic mice, but two lines of evidence suggest this is largely intact. First of all, we observed increased short term HRV in 16-week diabetic mice that was normalized by blockade of muscarinic receptors with atropine. Additionally, treatment with atenolol, to block sympathetic effects on heart rate, resulted in a significantly lower heart rate in diabetic mice at 8 and 16 weeks. These results, if anything, suggest enhanced cholinergic function and parasympathetic tone, which is consistent with the cholinergic hyperinnervation we observed in sections of SAN from 8- and 16-week diabetic mouse hearts. Furthermore, the increase in cholinergic innervation in diabetic SAN is supported by evidence from a study using STZ rats that where found to have increased levels of ChAT and ACh in atrium at 8 weeks (1). Secondly, responses of the SAN to direct stimulation of post-junctional muscarinic receptors were no different in normal mice and
severe diabetic mice. Previous studies in long-term human diabetics theorized that postjunctional supersensitivity might be linked to decreased baroreflex response that would be expected to result in a compensatory increase in muscarinic receptors (32), but there is no actual evidence for this increase. There is, however, evidence that such changes may occur with long-term experimental diabetes (10; 19). These data suggest as diabetes progresses there may be a compensatory increase in muscarinic receptor sensitivity. If there were a decrease in cholinergic signaling to the heart or a decrease in nerve function, then compensatory supersensitivity of post-junctional muscarinic receptors would have been expected, but this did not occur in our model. Additionally, in our model, there is no driving force for these types of changes because functional evidence suggests that receptors are getting activated, if anything more than normal.

**HR, HRV and potential for arrhythmias**

Diabetic mice examined in this study had decreased baseline HR with increased short term HRV and sinus arrhythmias. Decreased HR has been documented in STZ diabetic rats, which was attributed in part to decreased body temperature because it and HR were normalized with insulin treatment (14). This decrease in rate was accompanied by increased short term HRV and respiratory sinus arrhythmias in a majority of diabetic mice in our model. As mentioned previously, the increased HRV and sinus arrhythmias were cholinergic in nature because they were blocked with atropine. Decrease in heart rate and arrhythmias were only observed in the *in vivo* experiments and not in isolated atrial preparations, suggestive of asynchronous input into the system as apposed to dysfunction in pacemaker or conduction through the atria. In addition,
results from power spectral analysis of the ECG were indicative of autonomic imbalance based on a shift in high and low frequency parameters. However, there were limitations to the ECG measurements contained within this study in that they were only representative of short-term changes in HR and HRV, as compared to those measured long term (over a 24 hour time period).

Diabetic patients also have a higher incidence of ventricular arrhythmias and sudden cardiac death, especially when combined with cardiac autonomic neuropathy (3). Clinical assessment of risk for ventricular arrhythmias involves examination of the ECG recording, particularly the QT segment. Abnormal prolongation of the QT or any variant of the QT interval, including QTc and QT dispersion, has been associated with higher risk for developing ventricular arrhythmias (2; 3; 20; 33; 40). The presence of a prolonged QT interval reflects an increase in myocyte recovery phase (46) following excitation of the ventricles (2). Significant increases in QT, QTc and QT dispersion were observed in diabetic mice in the present study as well as in rats and rabbits with STZ-induced diabetes (18; 29; 45; 46).

**Conclusions**

The data presented in this study show that type 1 diabetes impacts structure and function of the cardiac parasympathetic nervous system. Multiple components of this system appear to be affected, suggesting disconnect between cholinergic activity at the heart level and central control of heart rate. Parasympathetic remodeling of the atria is likely to result in nerves firing in a less synchronous fashion which may contribute to the autonomic dysfunction and increased atrial arrhythmogenesis commonly associated
with diabetes. Further studies into the origination of the mechanisms responsible for the cholinergic remodeling will assist in determining how to prevent the remodeling from occurring.
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References


CHAPTER 5

SUMMARY

This study represents the first detailed mapping of cholinergic neuroanatomy of the mouse heart based on immunohistochemical staining. Cholinergic innervation of the mouse heart is largely focused in the atrium and conducting system, which is consistent with rat (Schafer et al. 1998; Xu et al. 2006) and guinea pig (Hoover et al. 2004). In contrast to larger animals and humans, there is very little amount of cholinergic innervation in the ventricles of the mouse heart. This work definitively demonstrates that NRTN plays a major role in the development of cardiac parasympathetic ganglia and cholinergic innervation of the mouse heart. The presence of NRTN and its receptors in adult mouse atria suggests a continued requirement for this neurotrophic factor in the adult heart. We’ve also shown that the cardiac cholinergic nervous system in mouse undergoes structural and functional remodeling when challenged with STZ-induced diabetes. Cholinergic nerves in diabetic hearts undergo extensive sprouting at the SAN with no change in the number of intracardiac neurons. Diabetic cholinergic function appears to be enhanced, based on pharmacological testing, despite decreased response to direct vagal nerve stimulation. Evidence also suggests that diabetic mice have an imbalance in autonomic control of HR. The latter findings suggest that disruption of central input into intrinsic cardiac ganglia also contributes to the neuropathology of type 1 diabetes.
Species Dependent Variation in the Distribution of Cholinergic Nerves and their Role in Cardiac Regulation

Cholinergic innervation of the mouse heart is similar to that of guinea pig in that the cholinergic nerves are more abundant in the sinus node and AV conducting system but sparse in ventricles. However, the mouse differs from guinea pig by having more cholinergic nerves in non-specialized regions of the right and, to a lesser extent, left atrial myocardium, a low but significant number of cholinergic nerves in the right ventricular wall, and cholinergic innervation of the blood vessels in the atrial and ventricular myocardium. Anatomical evidence of cholinergic innervation in larger animals and humans is fairly sparse with the majority of studies focusing on parasympathetic function. In larger animals, such as dogs, and in humans, which have a lower resting heart rate, functional studies suggest there is more cholinergic innervation in the ventricles. Even within different strains of mice we have observed variation in cholinergic nerve density. For example, NON LtJ mice, which are an inbred white mouse control strain, have significantly less cholinergic nerve fiber density at the SAN compared to C57BL/6 mice (see Appendix A1). The decreased cholinergic innervation parallels their increased atrial postjunctional muscarinic sensitivity (see Appendix A2).

Neurotrophic Influences on Cholinergic Neurons

Cardiac sympathetic neurons in adults require NGF; however, it is unclear whether ICNs have a similar, life-long requirement for trophic support. The present findings and previous work with ret and gfra2 KO mice (Hiltunen et al. 2000) established
that NRTN has an essential role in the development of a majority of ICNs and for normal cholinergic innervation of the mouse heart. However, NRTN cannot be the sole neurotrophic factor influencing ICNs because a small number of ICNs and cholinergic nerves still developed in all three strains of KO mice (i.e., \textit{nrtn}, \textit{ret}, and \textit{gfra2}).

Neurotrophin-3 might serve as a neurotrophic factor for ICNs because expression of its receptor, \textit{trkC}, was found in rat ICNs (Hiltunen et al. 1996). Additionally, we found that adult mouse ICNs exhibit \textit{trkA} and \textit{p75} receptor immunoreactivity (Hoard et al. 2008), suggesting that these cells may respond to NGF and other neurotrophins.

The Nature of Cholinergic Remodeling in Diabetes and How it Affects Function

In our study we found no evidence for the loss of intracardiac cholinergic neurons as a result of type 1 diabetes in mice, which is consistent with results obtained with one-year STZ-treated rats showing that cardiac neurons appeared normal (Kamal et al. 1991). STZ-treated rats also had degeneration within preganglionic cholinergic nerve fibers surrounding the cardiac neurons (Kamal et al. 1991). Alloxan-treated 7-8 month diabetic rats exhibited cardiac nerve degeneration as well (Tomlinson and Yusof 1983). These observations were similar to those seen in studies where NGF was depleted from sympathetic neuronal ganglia, resulting in a loss in nerve processes but not in neurons (Ruit et al. 1990), and suggests possible involvement of neurotrophic factors.

Our study is the first to use true cholinergic markers to examine the effect of diabetes on murine cardiac parasympathetic innervation. We found cholinergic hyperinnervation in the SA node of diabetic mouse atrium at 8 and 16 weeks, with no change in adrenergic innervation. However, it is possible that a longer term of diabetes
with chronic severe glucose impairment may be required for cardiac cholinergic innervation in the atrium to be lost, as suggested by studies in rat (Kamal et al. 1991; Tomlinson and Yusof 1983). Unfortunately, we were unable to examine mice that had been diabetic for longer than 4 months due to the severity of the disease in this model; therefore, the duration of cholinergic hyperinnervation seen in diabetic mice is unknown. The status of cholinergic innervation in the atrium of diabetic rats is unknown early on in diabetes, but biochemical evidence of increased ChAT and ACh at 8 weeks in diabetic rat atrium (Akiyama et al. 1989) is consistent with the hyperinnervation found in our studies. The mechanism for the parasympathetic hyperinnervation is uncertain, but increased availability of neurotrophic factors seems likely based on previous studies with NGF and sympathetic cardiac innervation (Ieda et al. 2006).

Diabetic mice in our studies exhibited evidence of cholinergic functional remodeling as well. Short term HRV was increased in diabetic mice indicating enhanced cholinergic tone. This finding was further supported by pharmacological evaluation wherein HRV was normalized with atropine and HR was further decreased with atenolol, which is consistent with our observed hyperinnervation of the atrium. On the other hand, ECG power spectral analysis of HF power and responses to direct vagal nerve stimulation were both decreased indicating preganglionic nerve dysfunction. The apparent incompatibility of these results suggests that cholinergic activity is enhanced at the heart level, but central regulation of heart rate is impaired. A substantial body of evidence suggests that cholinergic mechanisms can have a particularly strong proarrhythmic influence on the atria (Coumel 1996; Schuessler et al. 1992). Furthermore, diabetics have been shown to have increased risk for atrial arrhythmias
(Movahed et al. 2005). Therefore, our findings suggest that structural and functional remodeling of cardiac cholinergic innervation could be a contributing factor to the increased arrhythmogenesis seen in diabetic patients. Additionally, it is well known that the intracardiac nervous system has a high degree of complexity, which could be a factor which enables activity independent of central input to the heart.

**Topics for Future Studies**

Like any research study, this one has identified topics for further investigation. The following are some of the problems that still need to be resolved:

- Identify other neurotrophic factors that influence the development of intracardiac neurons and determine if neurotrophic factors are required for the maintenance of cholinergic neurons and nerve fibers in the adult heart.
- Investigate mechanisms responsible for cardiac cholinergic hyperinnervation observed in type 1 diabetic mouse atrium, including involvement of NRTN or other neurotrophic factors.
- Investigate central remodeling occurring with type 1 diabetes to clarify the disconnect between cholinergic activity at the heart level and central control of heart rate.
- Determine if more extensive remodeling occurs with longer duration of type 1 diabetes in mouse because studies in diabetic rats have shown evidence for preganglionic cholinergic nerve degeneration and increased postjunctional muscarinic supersensitivity.
- Determine if type 1 diabetic mice have increased susceptibility to atrial tachycardia through use of tissue stimulation studies in isolated atrium.
- Examine cholinergic remodeling in mice with Type 2 Diabetes because this type of diabetes is more prevalent.
REFERENCES


Hoard JL, Hoover DB, Mabe AM, Blakely RD, Feng N, Paolocci N. Cholinergic neurons of mouse intrinsic cardiac ganglia contain noradrenergic enzymes, norepinephrine


Szkudelski T. The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. Physiol Res 2001; 50:537-546

Thayer JF, Lane RD. The role of vagal function in the risk for cardiovascular disease and mortality. Biol Psychol 2007; 74:224-242


Widenfalk J, Nosrat C, Tomac A, Westphal H, Hoffer B, Olson L. Neurturin and glial cell line-derived neurotrophic factor receptor-α (GDNFR-α), novel proteins related to GDNF and GDNFR-α with specific cellular patterns of expression suggesting roles in the developing and adult nervous system and in peripheral organs. J Neurosci 1997; 17:8506-8519.


Appendix

Comparison of NON LtJ and C57BL/6

Figure A1: Comparison of cholinergic nerve fiber density at the SAN in NONLtJ and C57BL/6 mice. (*P<0.05 n=7 each)

Figure A2: Comparison of cumulative concentration-response curves for carbachol in isolated atria from NONLtJ and C57BL/6 mice. (-log EC50s: NONLtJ 6.4±0.08 (n=4) and C57BL/6 5.713±0.07 (n=6); P<0.05).
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Schoborg RV, Kintner J, Mabe A, Khanna R, Lajoie D. Commonly prescribed beta-lactams induce C. trachomatis persistence in culture at physiologically relevant concentrations. Accepted for 2007 Chlamydia Basic Research Society meeting in Louisville, KY.

Mabe AM, Hoover DB. Structural and functional cardiac cholinergic deficits in adult neurturin knockout mice. Abstract #3445 Experimental Biology meeting abstracts 2008.

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