Structural and functional cardiac cholinergic deficits in adult neurturin knockout mice

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Aims Previous work 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 effect of nrtn deletion on cardiac cholinergic innervation and function in the adult heart.

Methods and results Immunohistochemistry, confocal microscopy, and quantitative image analysis were used to directly evaluate intrinsic cardiac neuronal development. 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 anaesthetized mice and responses of isolated atria to muscarinic agonists. KO hearts contained only 35% the normal number of cholinergic neurons, and the residual cholinergic neurons were 15% smaller than in WT. 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 (0.013 ± 0.004 vs. 0.050 ± 0.011 pmol/μg protein; P < 0.02) as expected from cholinergic neuron and nerve fibre deficits. Maximum bradycardia evoked by vagal stimulation was reduced in KO mice (38 ± 6% vs. 69 ± 3% decrease at 20 Hz; P < 0.001), and chronotropic responses took longer to develop and fade. In contrast to these deficits, isolated atria from KO mice had normal post-junctional sensitivity to carbachol and betahanechol.

Conclusion 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.

KEYWORDS
Autonomic nervous system; Intrinsic cardiac neuron; Parasympathetic; Neurotrophic factors; Bradycardia

1. Introduction
Cardiac sympathetic neurons have a life-long requirement for nerve growth factor (NGF), which is a target-derived protein.1 Retrograde transport and signalling 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 remodelling 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,2,3 whereas elevated NGF levels around infarcted myocardium were associated with regional sympathetic hyperinnervation.4–6 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 (i.e. intrinsic cardiac neurons, ICNs), but recent findings suggest that a protein named neurturin [neurotrophic factor 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.7,8 Members of this family signal through receptors that contain a specific ligand binding protein (GDNF family receptor α1-α4, GFRα1-α4) and the transmembrane tyrosine kinase, Ret. GDNF and NRTN exhibit preferential binding to GFRα2, respectively, but some crosstalk can occur at higher concentrations of neurotrophic factor, allowing GDNF to signal through GFRα2/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.9 Ret
knockout (KO) mice die shortly after birth, but their hearts contain about half the number of cholinergic neurons present in wild-type (WT) mice. Deletion of gfrα2 produces a non-lethal phenotype, and hearts from these mice have significantly fewer cholinergic nerve fibres compared with hearts from WT mice. Collectively, these experiments provide indirect evidence that NRTN has an essential role in establishing cholinergic innervation of the heart.

Adult nrtn KO mice were used in this study to determine the requirement of NRTN for cholinergic innervation of the heart. KO and WT mice were evaluated for number and size of 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 anaesthetized mice, and post-junctional 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.

2. Methods

2.1 Experimental animals

Adult male C57BL/6 mice (16 weeks old; 24–30 g; Harlan, Indianapolis, IN, USA) and 16-week-old male nrtn KO mice15 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.

2.2 Tissue preparation for histochemistry

Animals were deeply anaesthetized 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, USA). Six sets of 16 μm short-axis sections were collected from each specimen, beginning at the most anterior aspect of the heart and ending at a level well into the ventricular myocardium. Serial sections were collected on separate slides such that sequential sections on each slide were separated by 80 μm in situ. Each set of slides was boxed separately, wrapped in aluminium foil, and stored at −80°C.

2.3 Immunohistochemistry

Slide-mounted tissue sections were immunostained at room temperature as described previously. Briefly, sections were washed in 0.1 M 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, USA), 1% BSA, and 0.4% Triton X-100. Tissues were then incubated for 15–18 h with two primary antisera generated in different species, washed several times with 0.1 M PBS, and incubated for 2 h with two species-specific donkey secondary antibodies conjugated to Alexa Fluor 488 or 555 (Molecular Probes, Eugene, OR, USA). Primary antibodies used include goat anti-choline acetyltransferase (ChAT) (1:50; Millipore, AB144P), rabbit anti-tyrosine hydroxylase (TH) (1:1000; Pel-Freez, P40101-0), and goat anti-vesicular ACh transporter (VACHT) (1:2000; Millipore, AB1578). 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, USA) 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.

2.4 Confocal microscopy and image analysis

Labelled tissue sections were viewed initially using an Olympus BX41 microscope (Olympus America Inc., Center Valley, PA, USA), and the cardiac ganglia were photographed using an Optronics MagnaFire SP charge-coupled device camera. Sections that contained the SA node were identified with the Olympus microscope, and this region was evaluated 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 relative abundance of neurons in KO and WT hearts was determined from single sets of sections that were stained for the cholinergic marker ChAT, which was previously determined to be present in virtually all ICNs of the mouse. Each set of sections was collected in a manner that provided a representative sample spanning the entire region where ganglia occur and precluded double counting of cells since adjacent sections on the slides were separated by 80 μm in situ. Cell counts and cross-sectional areas were performed using Stereo Investigator/Workstation software (MicroBrightField, Williston, VT, USA). Cross-sectional area was measured only for neurons with the nucleus visible. Cholinergic and noradrenergic nerve fibres were identified by dual staining for the VACHT and TH, respectively. Quantitative evaluation of nerve fibre density was accomplished using Image J Software (National Institutes of Health, Bethesda, MD, USA). Average nerve density at the SA node was determined from maximum projection confocal images obtained from two to three sections from a set of slides for each animal.

2.5 Histochemical staining for acetylcholinesterase

Tissue sections were stained for acetylcholinesterase (AChE) according to the histochemical method of Koelle. Sections were first pre-incubated with 1 μM tetraisopropyl pyrophosphoramide for 30 min at 37°C to inhibit pseudocholinesterase. This was followed by incubation with acetylthiocholine substrate for 2 h at 37°C and visualization of the reaction product with ammonium sulfide. Photomicrographs of the SA node region were collected (two to three sections per animal), and the abundance of AChE-stained nerves was quantified using Image J as already described for VACHT.

2.6 Acetylcholine and choline assay

Mice were anaesthetized with 5% isoflurane, euthanized by cervical dislocation, and quickly irradiated for 5 s with microwaves (1.45 kW, 2450 MHz) to denature proteins and preclude enzymatic changes in ACh and choline levels. 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, USA) 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.

2.7 Vagal nerve stimulation

Mice were anaesthetized 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. pre-amplifier (Grass Technologies, West Warwick, RI, USA), a PowerLab/8SP (ADInstruments, Colorado Springs, CO, USA), and a computer running Chart software version 5.2 (ADInstruments). 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, USA). 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.

2.8 Concentration–response studies with isolated atrial preparations

Mice were deeply anaesthetized 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, 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). 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 0.3–0.5 g using an ML224 Bridge Amplifier (ADInstruments), a PowerLab/8SP, and a computer running Chart. Post-junctional 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 10–15 min for recovery before evaluating responses to the second agonist. Data were analysed using Prism software version 4.01 (GraphPad Software, La Jolla, CA, USA) to determine the concentration of agonist that decreased heart rate by 50% (EC50).

2.9 Heart rate evaluation in conscious mice

The ECG of conscious KO and WT mice was recorded non-invasively using an ECGenie apparatus (Mouse Specifics, Inc., Boston, MA, USA). This device is a PowerLab-based system that acquires signal through disposable footpad electrodes located in the floor of a 6.5 cm × 7 cm recording platform. Several minutes or recording were collected from each mouse at weekly interval from 8 through 16 weeks of age. Segments of 2–3 s of recording (20–30 P-Q-R-S-T complexes) were chosen for analysis. Raw ECG signals were analysed using eMOUSE software (Mouse Specifics). Heart rate was determined from the average of R–R interval, and short-term heart rate variability determination was based on the standard deviation of the R–R intervals. Both parameters were expressed in beats per minute. For each animal, average heart rate and heart rate variability were calculated for the 8-week study interval.

2.10 Statistical analysis

Data are presented as the mean ± SEM (n). Pairwise 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.

2.11 Drugs

Bethanechol chloride (C5259), carbachol chloride (C4382), and tetraethylammonium pyrophosphoramidate (T1505) were obtained from Sigma-Aldrich (St Louis, MO, USA).

3. Results

3.1 Immunohistochemical, histochemical, and biochemical analysis

Gross cardiac morphology appeared identical in nrtn KO and WT mice, but KO mice had about 65% fewer ICNs than WT mice, and the cross-sectional area of these neurons was significantly less (Figure 1; n = 6 per group, P < 0.05). In accord with this deficit of ICNs, we found that cholinergic innervation of the SA node was reduced drastically in nrtn KO mice compared with WT (Figures 2A and B and 3A; n = 6 per group, P < 0.05). Furthermore, visual examination of VACH-T-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 (Figure 3B; n = 6 for WT and n = 5 for KO, P < 0.05). Choline, a precursor of ACh, was unaffected. The ACh-degrading enzyme, AChE, is primarily associated with cholinergic nerves and neurons, and the density of AChE-stained nerves was also reduced drastically at the SA node of nrtn KO mice (WT: 13.7 ± 1.5% area; KO: 14 ± 0.4% area; n = 6 per group, P < 0.0001). 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 (Figures 2C and D and 3A).

3.2 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 anaesthetized KO and WT mice (WT: 594 ± 14 b.p.m., n = 6; KO: 640 ± 21 b.p.m., n = 5; P > 0.05), and both strains had frequency-dependent decreases in heart rate during right vagal nerve stimulation (Figure 4). However, the maximum response to vagal stimulation was substantially smaller in KO mice (F₁,₆₃ = 21.41, P = 0.0012). We also observed that negative chronotropic

![Figure 1](https://academic.oup.com/cardiovascres/article-abstract/82/1/93/399208)

**Figure 1** Relative abundance and size of intrinsic cardiac neurons in 16-week-old male nrtn knockout and wild-type mice. (Left) Knockout mice had significantly fewer neurons, which were identified by immunolabelling for the cholinergic marker choline acetyltransferase. 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 intrinsic cardiac neurons was smaller in hearts from knockout mice. n = 6 per group. *P < 0.05 vs. wild-type.
responses to vagal stimulation and recovery from stimulation appeared slower in KO mice compared with WT (Figure 5A–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 with WT mice (Figure 5E; \( n = 5 \) per group, \( P < 0.05 \)).

### 3.3 Conscious heart rate analysis

*Nrtn* KO mice had slightly lower heart rate than WT mice (768 ± 4 b.p.m. for WT vs. 755 ± 2 for KO, \( n = 7 \) per group, \( P < 0.01 \)). Heart rate variability was substantially lower in conscious KO mice compared with WT controls (7.5 ± 0.8 b.p.m. for WT vs. 3.7 ± 0.4 for KO, \( n = 7 \) per group, \( P < 0.005 \)).

### 3.4 Post-junctional cholinergic sensitivity

Post-junctional 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 and those from WT mice (Figure 6). Carbachol had a higher potency than bethanechol in both strains (carbachol \(-\log EC_{50}\): 6.1 ± 0.1 for WT vs. 6 ± 0.2 for KO, \( n = 6 \) each, \( P > 0.05 \); bethanechol \(-\log EC_{50}\): 4.7 ± 0.1 for WT vs. 4.9 ± 0.1 for KO, \( n = 6 \) each, \( P > 0.05 \)).

### 4. 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 pre-ganglionic 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 post-junctional atrial sensitivity to muscarinic agonists was altered.

#### 4.1 Neurotrophic factors and cholinergic innervation of the heart

Little was known about the trophic factor requirements of ICNs, but the present findings and previous work with *ret* and *gfra2* KO mice\(^9\) 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 controlling cholinergic innervation of the heart since a smaller subpopulation of ICNs and a reduced number of cholinergic nerves still developed in hearts from \textit{nrtn} KO mice and retained some ability to modulate heart rate. Previous studies of hearts from \textit{ret} and \textit{gfra2} KO mice provide neuroanatomical support for this conclusion.\textsuperscript{9} Deletion of \textit{gfra1} and \textit{gfra3} mRNAs were localized only to non-neuronal cells in cardiac parasympathetic ganglia of neonatal and 3-week-old rats.\textsuperscript{9} Collectively, this evidence points to NRTN as the sole member of the GDNF family that affects the development of cardiac cholinergic innervation. NRTN is expressed at moderate levels in mouse atria from E14 through E18\textsuperscript{17} and is still expressed in adult atrium.\textsuperscript{11} This evidence supports the suggestion that NRTN is a target-derived trophic factor for ICNs, but its precise role remains untested. Although 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{18} 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 the 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 with 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} Other neurotrophic factors that control the development and maintenance of cardiac cholinergic neurons are unknown, but members of the neurotrophin family are among the candidates. The heart contains appreciable levels of neurotrophin-3\textsuperscript{19} and transcripts for the neurotrophin-3 receptor (i.e. trkC) are present in developing and adult rat ICNs.\textsuperscript{20} Additionally, we found that adult
4.2 Impaired cholinergic control of heart rate in nrtn knockout 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 heart rate also 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. Although ACh is normally inactivated rapidly by AChE that hydrolyzes ACh, this process may be prolonged in KO mice due to the loss of AChE associated with cholinergic nerves. AChE is localized primarily to cholinergic nerves in the heart, and we found a dramatic loss of AChE-positive nerves in the SA node of nrtn KO mice. 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. Additionally, it is conceivable that neurotransmitter synthesis, storage, and release might be impaired in the small population of cholinergic nerves present in atria of nrtn KO mice. Each of these defects might also lead to delayed onset of vagal bradycardia.

Interestingly, post-junctional 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 post-junctional supersensitivity due to a chronic reduction of cholinergic signalling at the SA node. Lack of cholinergic supersensitivity might be a consequence of cardiac sympathetic dominance in mice as opposed to parasympathetic innervation in larger animals. 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.

4.3 Cardiovascular and other phenotypic traits of nrtn knockout mice

Deletion of nrtn had no apparent effect on gross cardiac morphology. Likewise, no cardiac abnormalities were reported for ret or gfra2 KO mice, so NRNT and its receptor are not required for normal development of the heart. Our results with isolated atria, where autonomic tone is absent, further suggest that intrinsic heart rate is normal in nrtn KO mice. We observed that resting heart rate was slightly lower in conscious nrtn KO mice compared with WT, but this change was very small and probably had no functional significance. Since resting heart rates for both of our groups were higher than reported for telemetric recordings from C57BL6 mice, our values were probably elevated due to stress associated with handling of the mice. We anticipated that resting heart rate would be elevated in nrtn KO mice due to their deficit of cardiac cholinergic nerves and preservation of noradrenergic innervation of the SA node. This condition should have favoured noradrenergic dominance and a consequent elevation of resting heart rate. However, stress from handling may have masked such an effect in our experiments. Telemetric monitoring of heart rate would be needed to resolve this issue. Conscious nrtn KO mice also had substantially lower heart rate variability compared with WT mice. This parameter is a gross indicator of cardiac parasympathetic control of heart rate, so decreased heart rate variation was an expected outcome based on our observations of reduced cholinergic nerve density and impaired responses to vagal stimulation. Since impaired vagal control of heart rate is a common characteristic of many cardiovascular diseases, future studies should determine if nrtn KO mice are more prone to disorders such as hypertension, cardiac arrhythmias, and heart failure.

Previous work has shown that NRNT is also a crucial neurotrophic factor for many other parasympathetic ganglia and for subpopulations of sensory neurons in the dorsal root and trigeminal ganglia. Deficits in parasympathetic innervation were identified in the lacrimal glands, salivary glands, and throughout the bowel of nrtn KO mice. Deficient lacrimal gland innervation was suggested as the possible cause for crustating discharge around the eyes of KO mice. Reduced innervation of the intestine was associated with impaired motility. Sensory ganglia of NRNT-deficient mice showed marked reductions in the number of GFRa2-positive neurons. The functional consequences of the latter defect and the impact that NRNT-deficiency has on other parasympathetic ganglia require further study.

Conclusions

Our findings provide clear evidence that NRNT is essential for the development of normal cholinergic innervation of the heart and cholinergic control of heart rate. However, the specific role of NRNT in development of this system remains unknown. Is NRNT required for establishing the cholinergic phenotype in neuronal precursor cells, innervation of atrial muscle, and survival of ICNs (i.e. target-derived trophic factor), or does it serve all of these functions? Our findings also show that NRNT 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 fibres in the adult heart. Beyond these issues, the presence of well-defined structural and functional cholinergic deficits in the heart of NRNT KO mice should make this strain a valuable tool for evaluating the role of cardiac parasympathetic nerves in disease.

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