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Original Article

Age-related attenuation of parasympathetic control of the heart in mice

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Abstract: The autonomic nervous system maintains homeostasis through the balance of the sympathetic nervous system (SNS) and parasympathetic nervous system (PSNS). Especially evident in the heart, maintenance of this balance is important for the control of heart rate, conduction, and contractility. It is known that aging, similar to various cardiovascular diseases, results in an increase in SNS activity and a decrease in PSNS activity, which may contribute to age-related cardiac dysfunction and remodeling. Intracardiac ganglia relay and integrate the PSNS signals to the heart. Therefore, this study investigated whether altered function of intracardiac ganglia is involved in age-related parasympathetic dysfunction and the potential role of the major cholinergic components of intracardiac ganglionic transmission in the process. This study utilized two age groups of mice, the younger mice at 1-2.5 months of age, and the older mice at 11-12 months of age. The results show that the older mice exhibit diminishment of both baroreflex sensitivity and response to rostral-severed vagal stimulation but preserved response to administration of muscarinic acetylcholine receptor agonist, bethanechol. Analysis of whole atrial lysate revealed significant diminishments in choline acetyltransferase (ChAT) and the upper band of vesicular acetylcholine transporter (VAchT). In contrast, the upper band of the high affinity choline transporter (CHT) was significantly upregulated in the older group. Further analysis showed that the soluble but not insoluble fraction of CHT protein is significantly increased in the older group. This implicates a potential reduction of acetylcholine synthesis and/or release and an improper compensatory change of CHT may be responsible for the PSNS dysfunction exhibited in this model.

Keywords: Aging, parasympathetic, heart rate, cholinergic, ganglia

Introduction

As a branch of the autonomic nervous system, the parasympathetic nervous system (PSNS) negatively regulates heart rate, conduction, and, to a lesser extent, contraction in the heart. Closely interacting with the sympathetic nervous system (SNS), the PSNS plays an important role to maintain normal cardiac function in response to the external and internal stresses of the body [1]. Since parasympathetic innervation to the heart is concentrated in the atria and atrio-ventricular (AV) node areas, the PSNS is particularly important for regulation of heart rate and rhythm. The declined regulation of the heart by the PSNS has been known for decades to be present in cardiovascular diseases (CVD) [2-5] and aging [6-9]. Lack of parasympathetic balance is involved in incidence of arrhythmia and sudden death [10-14]. The decline of PSNS control of the heart in aging is of particular interest given the morbidity and mortality associated with cardiac deaths in aging [15-17]. The aging patient exhibits a blunted cardiovagal-baroreflex sensitivity (BRS) as evidenced in human studies in which phenylephrine (PE)-induced elevation in blood pressure prompts less diminished reflex slowing of the heart in the aged than the young [18]. Consistently in healthy subjects the parameters of heart rate variability (HRV), which indicates parasympathetic effect on the heart, progressively decline with age [8]. The central control mechanism of the abnormal PSNS cardiac regulation in CVD has been documented [7]. In addition, recent research points toward the alteration of the cholinergic system within the heart as playing a potential role in the decline of the PSNS control of the heart. For example, in a dog model with pacing-induced heart failure, the reduced response to vagal stimulation was likely due to an intra-ganglionic but not post-ganglionic

mechanism [19]. A human study found that isolated human heart atrium exhibited diminished acetylcholine (Ach) release in aging [20]. Currently, however, this peripheral mechanism of reduced PSNS control of the heart remains to be fully understood.

PSNS signals coming from the central nervous system (CNS) to the heart via the vagal nerve are relayed in the intracardial ganglia. Acetylcholine (Ach) is the primary neurotransmitter in the PSNS ganglionic transmission. Ach is synthesized from Acetyl Co-enzyme A and choline by choline acetyltransferase (ChAT) in neuron cytosol, and then transported into synaptic vesicles by vesicular acetylcholine transporters (VAChT). Released into the synaptic clefts, Ach binds and activates muscarinic receptors on the post synaptic membrane and is then quickly degraded by acetylcholinesterase (AchE) into acetates and choline. It is believed that choline for Ach *de novo* synthesis in cholinergic neurons is predominantly dependent on the acute uptake of extracellular choline [21], which is primarily carried out by high affinity choline transporter (CHT) [21-23]. Therefore, CHT-mediated choline reuptake is considered as a rate limiting step for Ach synthesis. Conceivably, any changes of these key proteins that are involved in the synthesis, packaging/release, and reuptake of Ach may affect PSNS function.

In this study, we compared the PSNS effect on the heart between two age groups of mice and examined the function of the intracardial ganglia and potential alteration of cholinergic proteins in the ganglia. We postulate that altered cholinergic transmission within parasympathetic intracardiac ganglia could contribute to the PSNS dysfunction observed in aging.

Materials and methods

Animals

Male ICR (outbred strain) mice aged 11-12 months (designated as older group) and mice aged 1-2.5 months (designated as younger group) were utilized. Mice were obtained from Harlan Laboratories (Indianapolis, IN.) Animals were housed in standard micro-isolator units, fed rodent chow *ad libitum*, and exposed to 12 hour light/dark cycles. Animals utilized and all experimental protocols conducted in this study were approved for use by the Institutional Animal Care and Use Committee of The University of South Dakota and conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Acute physiologic recordings

Animals from each of the two groups of ICR mice (younger and older) were subjected to non-recovery anesthetic, Urethane (2-2.5 g/kg IP). Mice were orally intubated to maintain patent airway and body temperature was maintained via a thermostatically controlled heated pad. A short piece of stretched tubing (PE 20) that connected to a fluid-type blood pressure transducer (MLT0670 AD Instruments, Colorado Springs, CO) was inserted into the left common carotid artery to enable the recoding of blood pressure (BP) and heart rate (HR) via the Powerlab System (ADInstruments, Colorado Springs, CO.). The left jugular vein was then cannulated to facilitate administration of test substances and subsequent recordings of their effects on HR and BP. 0.1 ml of 0.9% saline was administered to each animal to replace fluid balance and test patency of the intravenous cannula. There was a fifteen minute stabilization period prior to beginning baseline recordings and administration of test drugs. Upon delivery of test substances, the subsequent responses were recorded and values for analysis extracted from these recordings.

Baroreflex sensitivity (BRS)

The alpha-1 adrenergic receptor (α1-AR) agonist, PE, was administered intravenously into the cannulated jugular port. Three incremental doses of 0.1 ml, 0.2 ml, and 0.4 ml at a concentration of 0.1 mg/ml each were administered with a five minute period of recording between each dose to allow BP and HR return to the basal levels. Responses in BP and HR to each dose of PE were recorded and the change values from each baseline were measured.

Vagal stimulation (VS)

Some of the animals from each age group were subjected to fluid-transducer placement, jugular cannulation, and isolation of the right vagal nerve. The right vagal nerve was tied and severed rostral to the tie. The vagus was then stimulated with rectangular pulses (1 V in amplitude and 1 ms in duration) at incremental hertz (2, 4,

6, and 8 Hz) levels to produce a frequency dependent and step-wise bradycardic HR response. The change values of HR responses to each frequency were measured.

Responses to muscarinic receptor (mAchR) stimulation

Specific mAchR agonist, bethanechol (Beth), was administered intravenously in the cannulated jugular port at the end of the physiologic experiments to assess mAChR function. Three incremental doses of 0.1 ml, 0.2 ml, and 0.4 ml at a concentration of 0.1 mg/ml each were given with a five minute period of recording between each dose. The changes value of HR responses to each dose was measured.

Assessment of cardiac hypertrophy

At the end of each experiment, hearts were collected by thoracotomy followed by cardiac excision from Urethane (2-2.5 g/kg IP) anesthetized or cervical-dislocated mice and placed in room temperature PBS to facilitate pumping and removal of blood. Heart weight (HW) to body weight ratios (BW) were measured as an indicator of hypertrophy.

Assessment of key cholinergic proteins in atria

Following thoracotomy and cardiac excision, hearts were placed in room temperature PBS to facilitate pumping and removal of blood. Both the left and right atrium with the surrounding fat pad was plucked from the ventricles using forceps and the tissues were rapidly frozen on the surface of dry ice and then stored at -70°C until use. Proteins of interest were evaluated either in whole tissue lysate or in soluble versus insoluble fractions.

Whole tissue lysis

Using RIPA lysis buffer (1.25% sodium deoxycholate, 1.25% NP40, 0.0125 M sodium phosphate, 2 mM EDTA, 50mM sodium fluoride, 0.05% SDS) with the addition of 100× protease and phosphatase inhibitor (Pierce, #PI-78445), the atrial tissue with fat pad was homogenized. After centrifugation at 4°C for ten minutes at 10,000× g, the supernatant was recovered and subjected to protein assay using BCA Protein Assay Kit (Pierce, #PI-23225.) After adjustment of the protein concentrations, samples were prepared utilizing a beta-mercaptoethanol-based loading buffer and boiled for 10 minutes.

Soluble and insoluble fractionation

Utilizing 200 µl of non-detergent lysis buffer (100 mM Tris-HCl, 5 mM EGTA, 5 mM EDTA), with the addition of 100× protease and phosphatase inhibitors, the atrial tissue was homogenized. After centrifugation at 10,000× *g* and 4°C for 10 min, the supernatant was then removed and transferred to a fresh tube and centrifuged at 4°C and 25,000× g for 20 minutes. The supernatant was then recovered and transferred to a tube designated as the soluble fraction. Next, 100 µl of RIPA buffer (1.25% sodium deoxycholate, 1.25% NP40, 0.0125 M sodium phosphate, 2 mM EDTA, 50 mM sodium fluoride, 0.05% SDS) with the addition of 100× protease and phosphatase inhibitors was added to the pellet in the original tube to enable lysis of the insoluble fraction. This tube was then homogenized and allowed to set at room temperature for one-half hour to facilitate complete lysis. The sample was then centrifuged at 10,000× g for 10 minutes and all supernatant was transferred to a fresh tube and designated as the insoluble fraction.

Proteins of interest in whole lysate or fractions were assessed using standard Western Blot techniques and fluorescent conjugated secondary antibodies as previously described [24]. Immunoblotted membranes were scanned utilizing a LI-COR Imager (LI-COR Biosciences, Lincoln, NE.) Bands were analyzed utilizing ImageJ analysis software (NIH). Primary antibodies of interest included: CHT (Custom Made by GeneMed Synthesis Inc., rabbit polyclonal 1:400), ChAT (Santa Cruz Biotechnology, #sc-55557, 1:250), VAChT (Santa Cruz Biotechnology, #sc-15315, 1:500), and pan-Actin (Santa Cruz Biotechnology, #sc-1616-R 1:10000. Secondary antibodies included: Goatanti-mouse 680 (Alexa-Fluor #A21057, 1:5000) and Goat-anti-rabbit 680 (Thermo Fisher Scientific, Inc. #35568, 1:5000).

Statistical analysis

Data was compiled in Microsoft Excel and basic analysis completed within this software. Subsequent analysis to determine significance was performed utilizing StatMost (DataMost, Inc.). Repeated measures ANOVA was used to establish initial significance for physiological data, followed by SNK t-test. For analysis of Western Blots, ANOVA was used initially then Kruskal-Wallis non-parametric distribution free

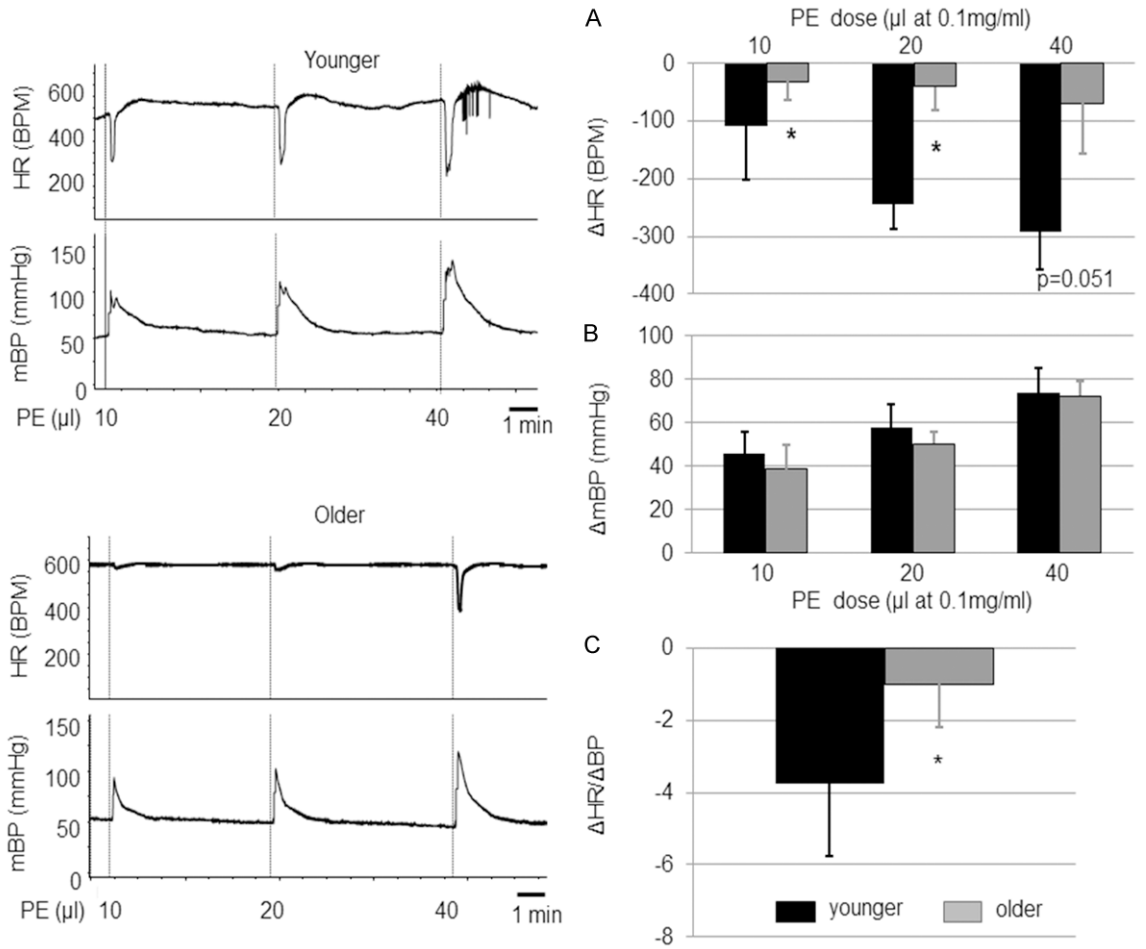

Figure 1. Reduced PSNS responses in baroreflex in older mice. The left panel shows representative raw record tracings of responses in HR and mean BP to injections of 3 doses of PE in a younger (top) and an older (bottom) mouse. The right panels are quantification and comparison of mean change values of HR (A) and mean BP (B) in response to PE administration from two age groups. C is the quantification of baroreflex sensitivity, calculated as the ratio of change HR vs. change mBP, from two age groups. "*"indicates $P < 0.05$, n = 7 younger, 4 older.

tests were used to compare bands between groups. All bands were compared as ratios to pan-Actin housekeeping control for that given sample. ImageJ (NIH) software was utilized for optimization and analysis of bands. In all experiments, group data are represented as mean ± SD and a $P < 0.05$ was considered statistically significant and was designated with an asterisk (*) in the graphs.

Results

Baseline physiologic data

Baseline physiologic data were measured in the younger (1-2.5 month old) and the older (11-12 month old) animals. First, baseline heart rate was significantly lower in the older (467.14±78.78 BPM) compared to the younger animals (527.29±53.25 BPM). Likewise, baseline mean blood pressure was significantly lower in the older (42.98±7.18 mmHg) compared to the younger (53.25±9.41 mmHg) group. However, heart weight to body weight ratios were not significantly different between the two groups (older: 5.41±0.85 younger: 5.58±0.71), indicating that there was no cardiac hypertrophy at the age of 11-12 months in this strain.

Baroreflex sentitivity (BRS)

Baroreflex sensitivity was assessed and compared utilizing the administration of three incremental doses of PE in the younger and older mice. While the change in mean blood pressure was similar at each dose in the younger and older groups (**Figure 1B**), the PSNS-

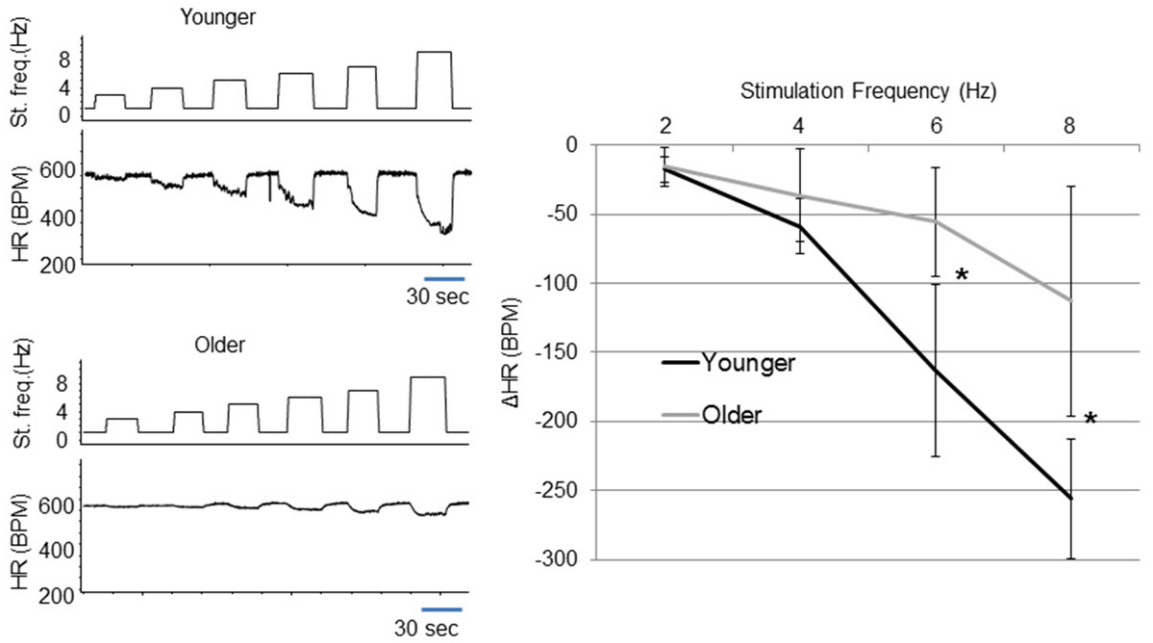

Figure 2. Reduced bradycardic response to vagal stimulation in older mice. The left panel shows representative raw tracings of stimuli and HR responses in a younger (top) and an older (bottom) mouse. The right panel is the quantification and comparison of mean change values of HR in response to various stimulating frequency in the younger and older groups. "*"indicates $P < 0.05$, n = 5 younger, 6 older.

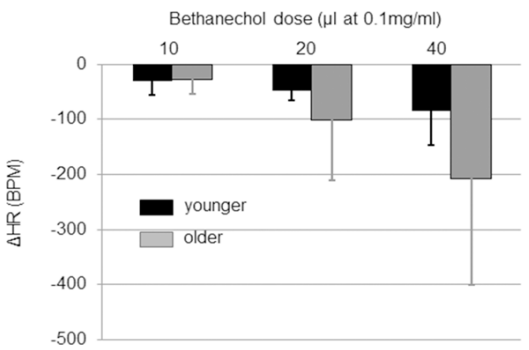

Figure 3. Effect of direct mAChR stimulation with bethanechol. The bar graph shows the quantification of mean change values of HR in response to intravenous injections with various doses of bethanechol in younger and older mice. N = 4 younger and 4 older.

mediated bradycardia in response to this rapid increase in blood pressure was significanly reduced in the older group (**Figure 1A**). To quantify the baroreflex sensitivity (BRS), **Figure 1C** depicts the combined responses to all of the doses of PE as a ratio of mean change in heart rate to mean change in mean blood pressure (ΔHR:ΔBP). There is a statistically significant difference in the BRS between younger and older mice, depicting a nearly 75% decline in the BRS in these aging mice.

Response to acute rostral-severed vagal stimulation (VS)

To determine whether any peripheral mechanisms are responsible for the reduced PSNS control of the heart seen in this aging model, animals were assessed for response to vagal stimulation. The right vagal nerve was severed rostral to the point of stimulation to eliminate the effect of stimulation on the CNS and the consequent input from the brain. Stimulation of the vagus therefore allowed evaluation of the specific activity of the peripheral component of the PSNS to the heart. As exemplified in **Figure 2**, the younger mice exhibited a normal ability of the heart to respond to the stimulation in that an increased stimulation resulted in an increased bradycardic response. However the older mice exhibit a severely blunted ability of the heart to respond to vagal stimulation.

Response to muscarinic acetylcholine receptor (mAchR) stimulation

The PSNS activity induced bradycardia is primarily mediated by mAchRs in the membrane of the cardiomyocytes. Diminished mAchR function could in theory be contributing to the decline of BRS and VS response seen in this aging mouse model. To determine the function

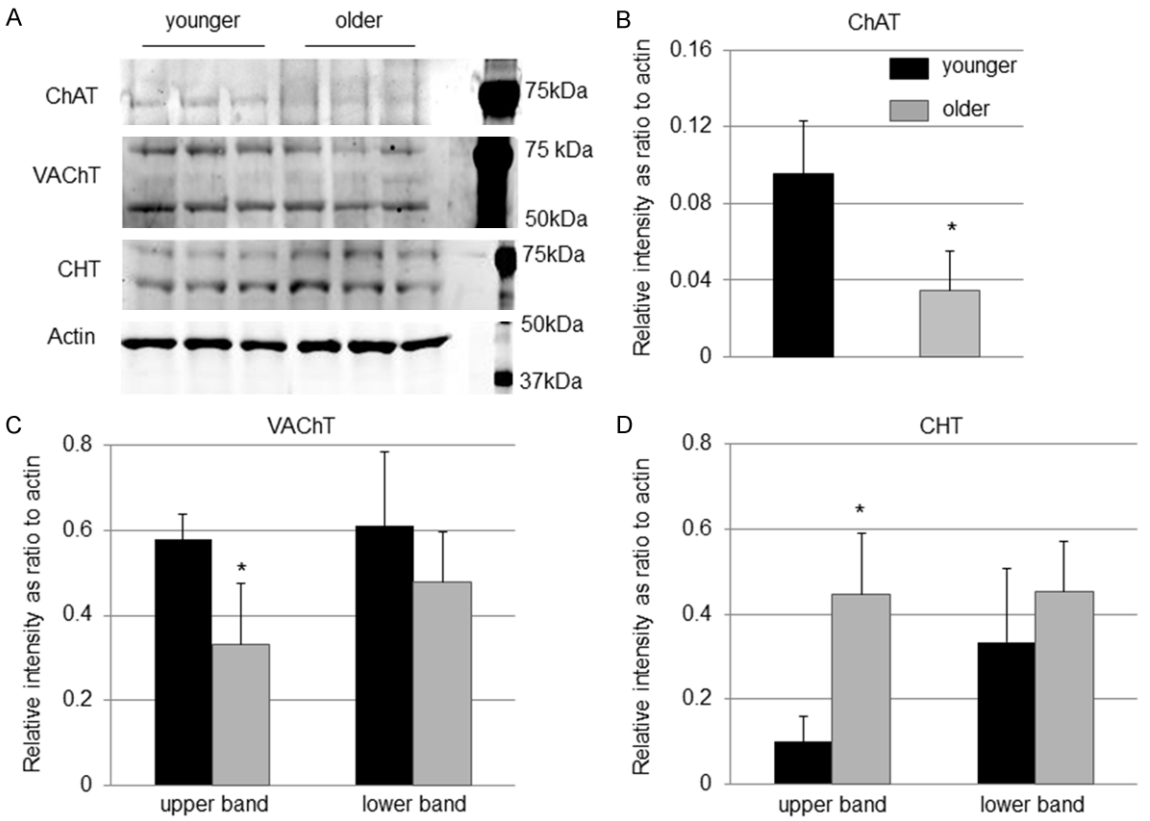

Figure 4. Altered expression of key cholinergic proteins in atria tissues in older mice. (A) Images of Western blot of ChAT, VAChT, CHT, and Actin with atrial samples from the younger and older mice. (B) to (D) are the quantification of relative intensity of Western blot bands of ChAT (B), VAChT (C), and CHT (D) against actin. "*"indicates $P < 0.05$, $n = 3$ for each group.

of these mAchRs, specific agonist Beth was administered. **Figure 3** shows that Beth induced bradycardic responses in a dose-dependent manner. This response was not diminished in the older mice. In fact, although not statistically significant due to variability, the older mice actually exhibit a trend towards an increase in mAchR function compared to younger mice. The findings of a bradycardic response to vagal nerve stimulation, but not to mAchR stimulation in these older mice, suggest a possible altered mechanism related to the neurotransmitter Ach in the PSNS intracardiac ganglionic neurons.

Expression of key cholinergic proteins in atrial tissues

To investigate if the alterations of PSNS activity in the heart seen in aging mice are associated with the possible alteration of Ach function within intracardiac ganglia, the major cholinergic proteins, ChAT, VAChT, and CHT in the atria were detected and compared between the two age groups using SDS Page Gels/Western Blots. Proteins evaluated as tissue whole lysate are shown in **Figure 4**. ChAT protein expression was significantly diminished in the older animals. Expression of the upper VAChT (~75 kDa) band was significantly diminished while the lower VAChT (~55 kDa) band was unchanged in the older compared with the younger. In contrast, there was a statistically significant increase in the upper band (75 kDa) of CHT in the older. Additionally, the lower band of CHT (65 kDa) was tending towards an increase in these older mice, although this was not significant due to variability.

CHT undergoes a dynamic trafficking between membrane and cytosol [22, 25]. We then further determined whether the increased CHT in whole tissue lysate represents an increase of CHT in membrane portion in the older group. Surprisingly, compared to the younger group, the CHT protein in atrial tissues of older mice was mainly increased in the soluble (cytosol) fraction but not in the membrane-enriched

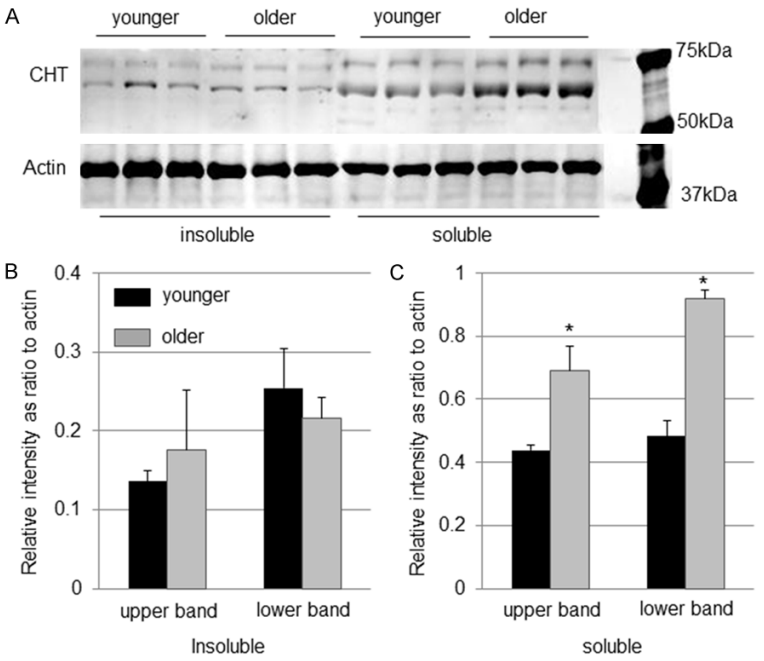

Figure 5. Altered distribution of CHT protein in soluble and insoluble fractions in older mice. (A) Raw images of Western blot of CHT protein in insoluble and soluble fractions of atria samples from younger and older mice. (B) and (C) are quantification of relative intensity of Western blot bands of CHT in insoluble (B) and soluble fractions against action. "*"indicates $P < 0.05$, $n = 3$ for each group.

insoluble fraction (**Figure 5**), suggesting a possibility that the trafficking of CHT resurfacing from cytosol to plasma membranes of atrial cholinergic neurons may be attenuated in older mice.

Discussion

Declined PSNS regulation of the heart has been reported in humans and animals [8, 9, 18, 26] but underlying mechanisms remained to be defined. In humans, it has been found that PSNS parameters of HRV are attenuated progressively with age, starting from a young age [8]. Using two groups of mice at ~2-month and ~12 month of age, this study confirmed the age-dependent reduced PSNS regulation of the heart rate, indicated by the reduced baroreflex bradycardic response in the older group. Then the study further investigated the possible peripheral mechanism underlying the reduced PSNS control of the heart in this model. The major findings include: 1, the response in heart rate to rostral severed vagal stimulation was reduced in the older mice, suggesting reduced peripheral action of the PSNS to the heart in these mice; 2, the bradycardic response to the direct mAchR stimulation was preserved in these older mice, suggesting that the reduced peripheral PSNS function is unlikely due to the reduced post-synaptic receptors on the cardiac muscles in these mice; and 3, the key proteins that are involved in Ach synthesis and release are altered in the atria of older mice, suggesting that altered Ach function in atria, likely at PSNS intracardial ganglia, may be involved in the age-related reduction of the PSNS control of the heart.

PSNS innervation of the heart is relayed at the intracardial ganglia which are mainly localized in atria [27-29]. It is known that peripheral neuronal ganglia, including PSNS ganglia are important sites to integrate signals to the target. Moreover, this integrative function of ganglia can be altered in physiological and pathophysiological conditions [30, 31]. It has been previously reported that reduced PSNS ganglionic transmission may be involved in a dog model of heart failure [19, 32]. Furthermore, it is reported that nitric oxide (NO) plays a role in PSNS ganglia in the heart and reduced NO is involved in the reduced PSNS control of the heart in hypertensive mice [33]. In this study, the heart rate response to vagal stimulation is reduced but the response to direct mAchR stimulation is preserved in the older mice, indicating that the ganglionic relay of the PSNS control of the heart is reduced with age. This provides further evidence that the plasticity of the PSNS ganglia has an important physiological and pathophysiological role in the PSNS regulation of the heart.

The majority of ganglionic neurons of the PSNS in the heart are cholinergic [29]. Ach synthesis and release is critical in the PSNS ganglionic relay to the heart. Results of this study show that the protein level of ChAT, the Ach synthase, is reduced in the atria of the older mice. This result suggests the possibility that Ach synthe-

sized and released from the PSNS pre- and/or post-ganglionic nervous terminals may be reduced, leading to attenuation of the PSNS negative chronotropic effect in the atria in the older mice. This possibility is further supported by the trend of increased bradycardic response to the mAchR agonist bethanechol, indicating a compensatory upregulation of mAchR in response to the reduced endogenous Ach stimulation.

The VAChT transports the synthesized Ach into synaptic vesicles and therefore is important for neurotransmitter packaging and release. Western blot detected two bands (~55 kDa and ~75 kDa) of VAChT protein in atrial samples, consistent with previous reports [34, 35]. Our data show that the upper band, but not the lower band, of VAChT protein was reduced in the atria of the older group. According to the previous study, the upper band of VAChT represents the protein form with post-translational modification and is the major form that is associated with regulation of VAChT subcellular distribution in neurons [34, 35]. Therefore, the specific reduction of this form of VAChT in the atria of older mice suggests that the altered Ach packaging may also contribute to the reduced Ach function in atria in the aging condition.

CHT is responsible for choline reuptake into neurons, which is a rate-limiting step for Ach synthesis. Surprisingly, the CHT protein level is increased, instead of decreased, in the atria of the older mice. On the one hand, this seems contradictory to the functional change. On the other hand, this may also be a compensatory change attempting to increase choline supply to boost the reduced Ach synthesis. Noticeably, the increased CHT is mainly present in the soluble fraction but not in the insoluble fraction. At this time, the functional significance of this change in CHT distribution is not clear. It is known that CHT undergoes dynamic trafficking between the cytosol and membrane of neurons [22, 25, 36]. Our data suggest a possibility that the trafficking of CHT protein resurfacing from cytosol to plasma membrane may be attenuated in aging. Theoretically, it is the membrane CHT that carries out choline reuptake. Therefore the increased CHT in soluble, i.e. cytosolic fraction found in the atria in the older mice may suggest an ineffective compensatory response. It would be interesting and important to further investigate why CHT distribution is altered in aging and whether this alteration contributes to the reduced Ach function.

A central mechanism of age-dependent reduction of PSNS cardiac regulation has been reported [7]. This study cannot exclude the possible reduction of central activity of PSNS in the older mice used in this study. In fact, it would be conceivable to postulate that the altered PSNS peripheral function and protein components are the secondary response to the reduced input from the PSNS center. Given the activity-dependent property of synapses, the reduced input stimulation from the PSNS center may result in an altered function of peripheral PSNS synapses. On the other hand, it is also possible that altered peripheral PSNS function is a primary change directly caused by the various age-related hormonal factors, such as inflammatory cytokines, reactive oxidative species, and angiotensin II. Indeed, the impacts of these factors on the function and plasticity of peripheral ganglionic neuron has been observed [33, 37, 38].

The limitations of this study should be discussed. For example, this study was conducted in anesthetized mice. Anesthesia is known for its negative effect on BP and baroreflex sensitivity. It is noted that both groups of mice exhibited a low basal BP. We attribute the low BP to the use of Urethane as the anesthetic. Changes in BP in response to PE are the same in two groups, indicating the same hemodynamic conditions of the animals. Therefore, the data from comparison of the two groups are valid and reflect the age-related changes in PSNS function. Certainly the experiment in conscious animals will be important in future study to confirm the phenomenon observed in anesthetized animals in this study. Second, Ach levels in the atria were not measured in this study. Such data will indicate whether the altered cholinergic proteins found in this study results in an effective compensatory response. Third, cholinergic proteins were measured in whole atria tissues that contained intracardial ganglia. Although these proteins are mainly present in PSNS ganglionic neurons, the approach used in this study could not exclude non-neuronal cholinergic sources [39].

Nevertheless, this study provides novel evidence suggesting that altered peripheral PSNS cholinergic function in the atria may be involved

in the age-related decline of PSNS regulation of the heart. The reduced PSNS control of the heart is an independent risk factor of cardiac incident in aging [6]. Therefore, preventing and restoring the decline of PSNS function is clinically important in age-related cardiac dysfunction and remodeling. Further identification of the mechanisms and factors that are involved in the age-related alteration of peripheral PSNS function may suggest new therapeutic strategies for age-related cardiac diseases.

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Disclosure of conflict of interest

None.

Authors' contribution

Jessica Freeling composed this paper and performed the surgical/laboratory work and data analysis. Yifan Li is the principle investigator and implemented the study design as well as performed laboratory work.

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