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Authors: Dalia Ranceviene, Kristina Rysevaite-Kyguoliene, Hermanas Inokaitis, Inga Saburkina, Khrystyna Plekhanova, Deimante Sabeckiene, Ignas Sabeckis, Joana Azukaite, Dainius H. Pauza and Neringa Pauziene

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EARLY STRUCTURAL ALTERATIONS OF INTRINSIC CARDIAC GANGLIONATED PLEXUS IN SPONTANEOUSLY HYPERTENSIVE RATS

Dalia Ranceviene, Kristina Rysevaite-Kyguoliene, Hermanas Inokaitis, Inga Saburkina, Khrystyna Plekhanova, Deimante Sabeckiene, Ignas Sabeckis, Joana Azukaite, Dainius H. Pauza and Neringa Pauziene

Institute of Anatomy, Lithuanian University of Health Sciences, Mickeviciaus st. 9, Kaunas LT-44307, Lithuania

*Correspondence to: Prof. Neringa Pauziene, Institute of Anatomy, Faculty of Medicine, Lithuanian University of Health Sciences, A. Mickeviciaus Street 9, Kaunas LT-44307, Lithuania.

e-mail: neringa.pauziene@lsmuni.lt;

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Short title: Intracardiac nerve plexus in spontaneously hypertensive rats
ABSTRACT

Persistent arterial hypertension leads to structural and functional remodeling of the heart resulting in myocardial ischemia, fibrosis, hypertrophy, and eventually heart failure. Previous studies have shown that individual neurons composing the intracardiac ganglia are hypertrophied in the failing human, dog, and rat hearts, indicating that this process involves changes in cardiac innervation. However, despite a wealth of data on changes in intrinsic cardiac ganglionated plexus (GP) in late-stage disease models, little is known about the effects of hypertension on cardiac innervation during the early onset of heart failure development. Thus, we examined the impact of early hypertension on the structural organization of the intrinsic cardiac ganglionated plexus in juvenile (8-9 weeks) and adult (12-18 weeks) spontaneously hypertensive (SH) and age-matched Wistar-Kyoto (WKY) rats. GP was studied using a combination of immunofluorescence confocal microscopy and transmission electron microscopy in whole-mount preparations and tissue sections. Here, we report intrinsic cardiac GP of SH rats to display multiple structural alterations: (i) a decrease in the intracardiac neuronal number, (ii) a marked reduction in axonal diameters and their proportion within intracardiac nerves, (iii) an increased density of myocardial nerve fibers, and (iv) neuropathic abnormalities in cardiac glial cells. These findings represent early neurological changes of the intrinsic ganglionated plexus of the heart introduced by early-onset arterial hypertension in young adult SH rats.
LIST OF ABBREVIATIONS

ad. – adult
DOPA – dihydroxyphenylalanine
GP – ganglionated plexus
juv. – juvenile
LD – left dorsal
PBS – phosphate-buffered saline
PFA – paraformaldehyde solution
SH – spontaneously hypertensive
WKY – Wistar-Kyoto
1. Introduction

Persistent arterial hypertension leads to structural and functional alterations of the heart resulting in myocardial ischemia, fibrosis, and hypertrophy (Kuroda, 2015). Previous studies have shown that individual neurons composing the intracardiac ganglia are hypertrophied in the failing hearts of human, dog, and rat (Singh et al., 2013). It was associated with increased nerve growth factor levels found in the advanced stage failure hearts (Ueyama et al., 1992; Zettler and Rush, 1993; Singh et al., 2013). However, little is known about hypertension-induced effects on cardiac innervation during the early onset of HF development.

The intrinsic cardiac nervous system integrates neural signals from the central vagal neurons, sensory afferents, sympathetic preganglionic neurons and is proposed to involve local intracardiac interneurons (Armour, 2008, 2004). The spontaneously hypertensive (SH) rat was initially developed by Okamoto et al. (1963) as an animal model for studying chronic, persistent hypertension. It is an inbred strain established by selecting for high arterial blood pressure in the Wistar Kyoto (WKY) rat strain. Due to that, WKY rats are widely used as a control group for studying SH rats. In this model, dysregulation of cardiac sympathetic neurotransmission arises before the onset of hypertension, as shown by augmented sympathetic nerve activity already present early in postnatal life (Simms et al., 2009).

Similarly, intracellular calcium transient in the stellate and the superior cervical ganglionic neurons was shown to be increased in response to neuronal depolarization in prehypertensive (4 week old) SH rats (Li et al., 2012). In addition, the reduced activity of presynaptic norepinephrine uptake transporter has also been reported in the cultured stellate ganglionic cells of the juvenile prehypertensive SH rats (Shanks et al., 2013a). This is accompanied by the fact that the resting heart rate is significantly higher in the SH rats than the age- and weight-matched WKY, despite the unchanged intrinsic contraction rate of the isolated atria and vagal influence (Shanks et al., 2013b). It is further shown that direct stimulation of the right stellate ganglion produces an increased tachycardic response in the young SH rats compared to age-matched WKY controls (Shanks et al., 2013b).

In SH rats, there is a parallel increase in the number of cholinergic neurons and adrenergic glomus cells in cardiac ganglia, a higher proportion of synaptic α7-subunit but not β2-containing nicotinic receptors, and an elevation in the number of synaptic terminals onto neuronal somata of intrinsic ganglionated plexus GP (Ashton et al., 2020). The significant structural and functional plasticity occurs in the intracardiac nervous system, and the enhanced excitability through synaptic plasticity together with the remodeling of cardiac neuron electrophysiology contribute to the substrate for atrial arrhythmia in
hypertensive heart disease (Ashton et al., 2020). The densities of noradrenaline nerve fibers in the epicardium of the right and left ventricles are higher in SH than in WKY rats from 10 to 180 days of age, those in the myocardium of the right ventricle are higher in SH than in WKY rats from 30 to 90 days of age, and those in the myocardium of left ventricle and interventricular septum in SH rats are similar to those in WKY rats (Adams et al., 1989). Thus, the hyperinnervation of the heart by noradrenaline nerve fibers in SH rats may be a primary change of the heart prior to hypertension and may be caused by hyperfunction, increased activity of lysine and DOPA uptake, and increased relative weight of the stellate ganglia that innervate the heart (Kondo et al., 1996).

Therefore, the present study aimed to identify the impact of both the early and the bloom hypertension on the structural organization of the intrinsic cardiac ganglionated plexus in SH rat model compared with WKY rats.

2. Material and methods
2.1. Animals

Juvenile (8-9 weeks) and adult (12-18 weeks) spontaneously hypertensive (SH) rats and control Wistar Kyoto (WKY) rats were used for the study. Juvenile SH rats before sexual maturity are prehypertensive in which the blood pressure only starts to climb; meanwhile, the adult SHRs, sexually matured animals of this breed, are generally considered as stably hypertensive with systolic blood pressure exceeding over 150 mm Hg (Okamoto and Aoki, 1963; Adams et al., 1989; Boluyt et al., 1995; Doggrell and Brown, 1998; Rezende et al., 2021). For immunohistochemistry, we used 8 hearts per group – juv. SH and WKY, ad. SH and WKY – 32 hearts in total. Three types of specimens were prepared from each heart: (i) whole-mount of atria, (ii) whole-mount of the basal part of the ventricles, (iii) cryosections of the ventricular middle part. In addition, a transmission electron microscope investigation was performed on adults (SH n=3, WKY n=3). All rats were male and purchased from a breeding company Charles River Laboratories (Germany). They were grown until preferred age and euthanized in the Animal Research Center of the Lithuanian University of Health Sciences under permission No. G2-137.
2.2. Wholemount preparations and immunohistochemical protocol

After euthanasia in the Animal Research Center, the rat chest was opened, and the heart was perfused in situ with 0.1 M phosphate-buffered saline (PBS) containing 0.14 M NaCl, 2.7 mM KCl, 10 mM phosphate, pH-value 7.4, via a cannula inserted into the left ventricular cavity, then prefixed with 4% paraformaldehyde solution (PFA) in 0.1 M PBS (pH=7.4). Hearts were dissected from the chest and postfixed with 4% PFA for 40 min. After washing the heart two times in PBS, the atria were dissected from ventricles and the interatrial septum. Then the atria were flattened and pinned in a Petri dish with a silicone bottom filled with cold PBS. 2-3 mm below the coronary groove, the base of ventricles was cut off along the coronary groove, separated from the interventricular septum, and pinned flat to extirpate most of the myocardium from the endocardial side. When preparation of the upper portion of ventricles became thin due to separation of myocardial tissues, it was turned epicardium up and pinned flat to a silicone dish. In order to decrease background light for a laser scanning microscope examination, tissues were dehydrated and cleared using a dimethyl sulfoxide and hydrogen peroxide solution, as reported previously (Dickie et al., 2006). Subsequently, whole-mount preparations were rehydrated through a graded ethanol series (in each for 10 min), washed 3×10 min in 0.1 M PBS containing 1% Triton X–100 (Serva, Heidelberg, Germany). Next, the non-specific binding was blocked for 2 hours in PBS containing 5% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The blocked for non-specific binding specimens were incubated with a Protein gene product 9.5 (38-1000, Invitrogen, Rockford, USA; dilution 1:500) primary antisera for 48–72 hours at 4°C. Afterward, whole mounts were washed three times for 10 min in 0.1 M PBS and incubated with donkey anti-rabbit antisera conjugated with a fluorochrome Alexa Fluor plus 488 (A32790, Invitrogen, Rockford, USA; dilution 1:500) for 4 hours at room temperature. In the last stage, specimens were washed three times for 10 min in 0.1 M PBS, mounted with a Vectashield Mounting Medium (Vector Laboratories, Inc., Burlingame, CA, USA), cover slipped, and sealed with clear nail polish.

2.3. Sectioned tissue preparations

Cardiac ventricles separated from the atria were washed in PBS and then immersed in 30% sucrose in 0.1M PBS containing 0.1% sodium azide in 4°C for 24 hours. Following cryoprotection, ventricles were frozen using a tissue-freezing medium (Triangle Biomedical Sciences, USA) for sectioning. Next, tissues were sectioned into 18 µm slices using a cryomicrotome HM 560 (Microm, Germany) at -22°C, mounted onto Superfrost Plus microscope slides (Menzel Glaser, Germany), and dried at room
temperature for 30 min. For immunohistochemical permeabilization, sections were incubated for 40 min. in a solution containing 9% DMSO and 1% Triton X-100 in 0.1M PBS. After 3x10 min washing in PBS, immunohistochemical procedures were performed as earlier described applying Protein gene product 9.5 (38-1000, Invitrogen, Rockford, USA; dilution 1:500) and secondary antisera donkey anti-rabbit, conjugated with a fluorochrome Alexa Fluor plus 488 (A32790, Invitrogen, Rockford, USA; dilution 1:500). Finally, specimens were washed three times for 5–8 min in 0.1 M PBS mounted with a Vectashield Mounting Medium (Vector Laboratories, Inc., Burlingame, CA, USA), cover slipped, and sealed with clear nail polish. Both positive and negative controls were used.

2.4. **Preparations for transmission electron microscopy**

After euthanasia, the rat chest was opened, perfusion with PBS (300 ml, 140 mmHg) and prefixation with 2.5% glutaraldehyde in 0.1 M PB pH 7.4, (300 ml, 140 mm/Hg) were performed *in situ* through the left ventricle. Afterward, the hearts were excised from the chest and placed for 40 minutes in 2.5% glutaraldehyde in 0.1 M PB (pH 7.4). Tissue samples of 1 mm³ from the definite sites of atria and ventricles were dissected using fine scissors and tweezers. The samples were fixed overnight at 4°C in 2.5% glutaraldehyde in 0.1 M PB (pH 7.4), washed twice in PB, postfixed for two hours with 1% osmium tetroxide in 0.1 M PB (pH 7.4) and dehydrated through a graded ethanol and acetone series then infiltrated into a mixture of Epon 812 and Araldite 502 resins. Tissue samples were carefully orientated for transverse sectioning in flat embedding molds under a stereoscopic microscope Stemi 2000CS (Zeiss, Gottingen, Germany) and polymerized for 48 hours at 60 °C temperature. Semi-thin sections (1 µm) were cut with a Leica EM UC7 ultra-microtome (Leica Mikrosysteme Handelsges.m.b.H., Vienna, Austria) using a glass knife, and stained with methylene blue according to Ridgway (1986). Ultrathin sections (50–70 nm) were cut using the same Leica EM UC7 ultramicrotome with DiATOME ultra 45° diamond knife (Biel, Switzerland). Following Reynolds, samples were mounted on 600-mesh thin bar support nickel grids (Agar Scientific, Essex, UK) and stained with 2% uranyl acetate and lead citrate. Finally, ultrathin sections were analyzed at 120 kV with a Tecnai BioTwin Spirit G2 transmission electron microscope (FEI, Eindhoven, the Netherlands). Images were taken with a bottom-mounted 16 mega-pixel Eagle 4K TEM CCD camera, employing specific TIA software (FEI, Eindhoven, the Netherlands). They were photographed at grid bar intersections using 2900 magnification and analyzed with AxioVision Rel. 4.8.2 (Carl Zeiss, Jena, Germany).

2.5. **Microscopy and quantitative analysis**
**Whole-mount preparations of atria** were snapped using confocal laser scanning microscope LSM 700 with the software package ZEN 2010B SP1 (Carl Zeiss, Jena, Germany). The ventricular whole-mounts were photographed using fluorescence microscope AxioImager Z1 equipped with digital camera AxioCamMRm applying a software package AxioVision (rel. 4.8.2; Carl Zeiss, Jena, Germany). The magnifications of images were at x10 (for panoramas of both preparations types) and x20 (for neural structures of atrial whole-mounts). The ganglia number per atrium, ganglion area, and neuronal somata number were counted and measured in atrial whole-mounts. Measurements were performed on 547 ganglia – juv. SH n=137, ad. SH n=143, juv. WKY n=120, ad. WKY n=147. All measurements were done manually under the same conditions. The criterion for the separation of ganglia was to have a distance of at least two neuronal bodies from the next ganglia. Neurons were identified and found in ganglia and epicardiac nerves. Neuronal bodies with prominent nuclei were counted as neurons and their somata areas were measured, whereas nerve cells without clearly noticeable nuclei were ignored during neuronal counting in serial ganglion sections. Neuronal bodies were counted and measured in separate stacks; meanwhile, the ganglia area was measured in summed stacks.

The epicardiac nerves, ≥5 µm in width, were measured manually from the right atrium left dorsal region (n=2246) and the whole-mount preparations of ventricles directly below the coronary sulcus (n=4369) in ventricular whole-mount preparations. In addition, digital images of atrial whole-mount myocardium were analyzed using a standard counting frame (150135 µm²), selecting threshold grey values and expressing the area of segmented nerve fibers in relation to the counting frame area as a percentage. Counting frames were selected manually to avoid big epicardiac or vascular nerves as artifacts.

**Sectioned tissue preparations** were snapped at x10 (for panoramas) and 20x (for neural structures) magnification using confocal laser scanning microscope LSM 700 with the software package ZEN 2010B SP1 (Carl Zeiss, Jena, Germany). Epicardiac nerves, ≥5 µm in diameter, were investigated. The whole epicardiac nerve cross-section area and area labeled for PGP 9.5 pan-neuronal marker were measured with threshold values adequate for the best visibility and expressed as the area occupied by PGP 9.5-positive neural structures in percentage. In addition, ventricles’ myocardial innervation was measured in digital images using the same method as in atrial preparations.

**In electron micrograms**, the number of axons inside unmyelinated nerve fiber were counted. Unmyelinated and myelinated axon areas, myelinated nerve fiber area, and the overall nerve area occupied by unmyelinated fibers was measured. Axon diameter and G ratio (the ratio of the axon...
diameter to the outer diameter of the myelin sheath) was calculated. Furthermore, the area occupied by unmyelinated axons in the nerve was determined and expressed as a percentage.

**Statistical analysis.** Data are presented as absolute numbers (n), percentages (%), mean (M), and standard error (SE). Statistical analysis was done with IBM SPSS 20.0. Kolmogorov-Smirnov test was used to determine the normality of the data. The parametric data samples from two groups were compared with the Student’s t-test; means of non-parametric data were compared with the Mann-Whitney U test. The Kruskal-Wallis test was employed for comparing non-parametric data of multiple groups. Differences were considered significant when the p-value was equal to or less than 0.05.

3. **RESULTS**

The intracardiac nervous system includes three distinct neural components: 1) autonomic neurons forming intracardiac ganglia on atrial and ventricular surfaces; 2) large epicardiac nerves through which nerve fibers proceed to reach their effector targets; 3) and a fine meshwork of nerve fibers encompassing the myocardium. Each of these components is considered below in detail.

3.1. **Intracardiac ganglia and neurons**

In all animals studied, intracardiac ganglia were mainly localized within the hilum of the heart. Ganglia largest in size (containing up to 1410 neurons), were composed of the defined ganglionic fields scattered predominantly on the anterior upper side of the left atrium. These ganglia were interconnected by large nerves and fine nerve fibers that together formed a ring-like plexus as they extended along the perimeter of the pulmonary vein roots (Fig. 1). Ganglia found further away from the main neuronal network of the heart hilum were smaller in size and did not form ganglionic fields. Solitary neuronal somata and neuron bodies forming small groups (average count – 22, range 2-305) were also found and often were observed within large epicardiac nerves. In contrast, no intracardiac ganglia were found residing in the cardiac ventricles.

In juvenile SHR, both the number of intracardiac ganglia and the ganglion area were slightly larger in regard to age-matched WKY controls (Table 1). This was accompanied by the significantly augmented quantity of neuronal somata per ganglion compared to juvenile WKY rats. Interestingly, in juvenile
SHR rats, the average area of individual neuron bodies composing the ganglia was found to be smaller than that in normotensive WKY counterparts.

In adult animals of both strains, all measured morphometric parameters were reduced compared to juveniles (Table 1). These age-related differences were less pronounced in the normotensive WKY group. In adult WKY rat hearts, the area of intracardiac ganglia was slightly smaller than that found in juveniles, whereas in adult SHR this was reduced by 38% compared to strain-matched juveniles. Similarly, the number of neurons per ganglion was slightly reduced in WKY adults but significantly decreased by 68% in SHR adults compared to strain-matched juveniles.

In cross-species comparison, ganglion neuron count was 44% greater in juvenile SHR compared to age-matched normotensive WKY rats; however, no differences between strains were found in ganglion neuron count in adult animals (Table 1).

Average neuron size was 10% greater in juvenile normotensive WKY compared to SHR juveniles. In adult animals, the average neuron size was still significantly smaller in SHR, yet this difference was less pronounced (6%). This might be attributed to the fact that with aging, the reduction of average neuron size was more pronounced in WKY compared with SH rats (Table 1).

Next, autonomic ganglia of adult SHR and WKY rats were examined at the cellular level by means of transmission electron microscopy (TEM). All intracardiac neuron bodies had a characteristic cytoplasmic structure at the ultrastructural level. In contrast to WKY animals, the nucleolemma of some SHR neurons was distorted with oscillating nucleic boundaries. In most cases, satellite glial cells ensheathed the intracardiac neuron body in a single cell layer, yet the neuroglial layer in some instances was much thicker and was composed of multiple layers of satellite cells. Interestingly, the glial sheath surrounding the intracardiac neurons in SHR and WKY rat hearts was oftentimes porous with deep collagen-containing invaginations of the endoneurium (Fig. 2A). Such glial formations were much more pronounced in the SHR neurons, where such porous glial sheaths were found covering large portions of the neuronal cell body (Fig. 2B). Occasionally, cell bodies of satellite cells had well-defined centrioles. Within the intracardiac ganglia, axonal terminals and/or synapses were not abundant, but they were more commonly observed in WKY rat ganglia compared to that of SHR.
3.2. Atrial and ventricular epicardiac nerves

In rats, epicardiac nerves access walls of cardiac ventricles mainly by two pathways. The first neural route proceeds throughout the arterial part of the heart hilum at the roots of ascending aorta and pulmonary trunk (Fig. 1). Epicardiac nerves from this site extend widely on the ventral side of cardiac ventricles, as described in detail by us previously (Batulevicius et al., 2003). The second neural route by which epicardiac nerves pass on the dorsal side of cardiac ventricles comes from the venous portion of the heart hilum along the left cranial vein and towards the heart apex (Fig. 1). This neural pathway contains epicardiac ganglia on the left atrium at the root of the left and middle pulmonary veins and has been named the left dorsal ganglionated subplexus (Batulevicius et al., 2003). The left dorsal subplexus supplies the dorsal walls of both cardiac ventricles and is linked by thin interconnecting nerves with the numerous nerve cells concentrated into two large clusters on the heart base in the limits of the venous part of the heart hilum. Both the number and morphologic pattern of epicardiac nerves on walls of cardiac ventricles is highly variable (Fig. 3A, B).

Next, epicardiac nerves were examined in whole-mount cardiac ventricles and atria preparations. For atrial preparation, epicardiac nerves were measured only in the left-dorsal subplexal (LD) area due to abundant and highly replicable innervation patterns within this region between different hearts. Only nerves with a width greater than 5 µm were selected for evaluation. Overall, the width of both ventricular and atrial nerves varied greatly, and some reached up to 200 µm. No differences in atrial nerve thickness were found between the juvenile SHR and WKY rats (Table 1). In contrast, the thickness of ventricular epicardiac nerves was significantly larger in SHR juveniles compared to age-matched WKY rats. The atrial nerves in adult animals compare to juveniles thickened in WKY, whereas in SHR it is thinned. At the same time, ventricular nerves stayed unchanged during aging in both groups (Table 1).

Based on the results of immunohistochemical analysis, the percentage area immunoreactive for pan-neuronal marker PGP 9.5, also known as UCHL1, within the epicardiac nerves did not differ between the juvenile SHR and WKY rats. However, this ratio changed in adult animals. In normotensive WKY rats, it increased 13% (p<0.001) during aging, while in SHR, it remained unchanged, resulting in significant 12% (p<0.001) difference between the two strains (Table 1).

Axons within the unmyelinated and myelinated nerve fibers had a characteristic ultrastructural appearance in both adult strains examined. In WKY rats, unmyelinated nerve fibers with compact
Schwann cells resulting in the smooth outline of the fiber (Fig. 2C) predominated in the epicardiac nerves. In stark contrast, most Schwann cells in SHR nerves were fractioned, with specific invaginations of the endoneurium that separated a single nerve fiber into distinguishable parts interconnected by a narrow glial fissure (Fig. 2D). Occasionally, fractionated fibers were found in the intracardiac nerves of WKY rats, especially close to intracardiac ganglia. In both strains, within the intracardiac nerves, multiple axons were incompletely covered by the glial cells and oftentimes contacted each other within a single Schwann cell groove (Fig. 2C-D).

The area of unmyelinated axons was lesser in SHR nerves compare to WKY, but tendency was different for the ventricles and atria. The area of axons was 23% lesser in the ventricular nerves and only 17% lesser in atrial (Table 2). Furthermore, classifying non-myelinated axons into 5 categories based on their diameters (<0.5; 0.5-1; 1-1.5; 1.5-2; >2 µm) revealed that small-diameter axons predominate within the SHR epicardiac nerves. Contrarily, the percentage of large-diameter axons was markedly higher in the WKY rat epicardiac nerves (Fig. 4).

The area occupied by axons in the nerve (%) was found to be significantly smaller in both the atrial and ventricular SHR epicardiac nerves (Table 2).

The area of myelinated nerve fibers differed significantly between atrial and ventricular nerves. In the atrial nerves, axon diameter was smaller, whereas myelin sheath was thicker, as indicated by a lower G ratio (axon diameter/nerve fiber diameter) within both strains. However, they were found to be similar in corresponding parts of the heart between WKY and SH rats (Table 2).

Myelinated fibers in nerves of WKY rats mainly had myelin sheath attached to the axon corresponding to its diameter and shape (Fig. 5A). However, a number of unusual ultrastructural features localized to myelinated nerve fibers were found (Fig. 5 and 6). These features can be categorized based on (1) differences attributable to Schwann cells and/or (2) differences observed in axons.

In the former case, unusual axon-myelin relations are exemplified in Fig. 5B-E. A collapsed/empty myelin sheath often enclosed an axon with its outer surface (Fig. 5B, C). Also, endoneurial collagen was observed in between the myelin sheath and the axon (Fig. 5B, D, E). In such fibers, some axons were without myelin (Fig. 5B, D), whereas others were covered by an additional layer of myelin (Fig. 5B, E). In some fibers, the cytoplasm was observed between the myelin layers, which formed
characteristic protrusions that varied in size; however, they always remained inside the Schwann cell (Fig. 5B, E). Myelin swellings (tomacula) were also found to be present (Fig. 5F).

Furthermore, some myelinated axon changes were observed. Markedly smaller axon cross-section areas compared to the perimeter of the myelin sheath enclosing them led excess myelin to form protruding folds that varied in size and structure (Fig. 6A, C-D). In addition, multiple axons were observed insulated by a single myelin sheath in some instances. This is exemplified by a myelin-covered fiber containing three distinct axons (Fig. 6B). Interestingly, individual Schwann cells were found to incorporate two (Fig. 6F) and occasionally multiple myelinated axons at once (Fig. 6E). Regularly, axons possessed a number of unusual artifacts such as an accumulation of vesicles ensheathed by a single membrane (Fig. 6F), laminated bodies, spiral membrane formations, fractionated myelin, or intra-axon myelin rings.

3.3. Innervation of myocardium

In juvenile animals, the density of nerve fibers innervating the atrial myocardium was comparable between the SHR and WKY strains (Table 1). Overall, innervation of both the atrial and ventricular myocardium increased with aging, although to different extents in hypertensive and normotensive animals. In WKY rats, myocardial innervation of the atria did not differ, whereas that of the ventricles was markedly higher in adult animals. In SHR, the density of nerve fibers innervating the atrial myocardium increased significantly, while the increase of ventricular innervation was only marginal in adult animals compared to juveniles (Table 1).

The innervation of the ventricular myocardium was not uniform. Comparison of the nerve fiber density between the right and the left ventricles and the interventricular septum revealed innervation of the right ventricle to be higher than that of the left, while interventricular septum innervation was rather sparse. These results were found in all studied groups (Fig. 7, Table 1).

The density of fine neural meshwork innervating the right ventricle was higher by about 30% in both juvenile and adult SHR compared to age-matched WKY rats (Fig. 7). An opposite result was found for the left ventricle in which the myocardial innervation was ~10% less dense in SHR compared to aged-matched normotensive controls. Although the density of innervation in the left ventricle was markedly
lower than the right one in all groups, comparatively most remarkable innervation changes during aging appeared in the left ventricle of SH rats (Table 1).

4. DISCUSSION

Here, we investigate the influence of early-onset arterial hypertension on the structural organization of the intrinsic cardiac ganglionated plexus in the SHR. Primary findings of the present study are that: (i) innervation of the heart exhibits a number of morphological alterations in association with the development of arterial hypertension in adult SHR; (ii) age-related differences in cardiac innervation are present in both SHR and normotensive WKY rats and should be taken into consideration when assessing hypertension-related effects on cardiac function. These conclusions are based on our data showing that in young adult SHR hearts: (i) the neuronal number per intracardiac ganglion is significantly decreased; (ii) axonal diameters and the axonal area within epicardiac nerves is reduced; (iii) the density of myocardial nerve fibers is higher, but significantly decreases with aging, and (iv) glial cells in intracardiac ganglia and nerves display a number of ultrastructural alterations indicative of cardiac neuropathy.

Whereas a wealth of data is available on the remodeling of the intrinsic cardiac nervous system related to chronic hypertension in the development of ventricular hypertrophy and heart failure, data on the effects of early-onset of hypertension on the cardiac autonomic innervation, related specifically to cardiac neuromorphology, is lacking, thus providing the rationale behind the present study. Wistar Kyoto (WKY) and Spontaneously Hypertensive Rats (SHR) are known to have divergent hemodynamic and behavioral features despite being highly similar genetically (Huang et al., 2016). Compared to age-matched normotensive WKY rats, SHR display lower resting heart rate, significantly elevated arterial blood pressure, higher left ventricular mass to body weight ratio, reduced ejection fraction and fractional shortening, prolonged deceleration time, and lowered E/A ratio, among a host of other alterations (Kokubo et al., 2005). It was shown that an increased number of inflammatory cells might play a role in peripheral sympathetic hyperactivity observed in the early stages of hypertension (Neely at al., 2022). Our data indicate that 8 week old prehypertensive SHR animals, i.e., SHR that do not exhibit arterial blood pressure elevation yet, already possess a range of distinct morphological alterations related to cardiac innervation compared to age-matched WKY controls. With aging, some of these features change to a similar extent in both SHR and WKY strains, e.g., intracardiac neuron size proportionally decreases in adult animals compared to juvenile hearts. For example, many strain-
related changes only occur in association with the development of arterial hypertension in adult SHR animals.

The intracardiac ganglia in both studied rat strains formed a ring-like plexus around the entry of the pulmonary veins and were interconnected by a series of fine nerve fibers and were similar to that previously studied in Sprague-Dawley (Richardson et al., 2003) and outbred Wistar rats (Batulevicius et al., 2003). Our data indicate that the number of intracardial ganglia and ganglionic neuron counts decreases in adult animals in both WKY and SHR strains. This contrasts with previous studies reporting an overall increase in neuron number in the intracardiac ganglionic plexus in the SHR (Ashton et al., 2020). Although this warrants further investigation, this discrepancy might be attributed to differently aged animals used in the study – 60-72 weeks compared to 12-18 weeks used in this study. Alternatively, this could be attributed to a different method of neuron quantification, as here, we employed manual nuclei counting within the intrinsic ganglia compared to neuron count estimation based on a fixed area. However, it is worth mentioning that the general trend observed in our and Ashton et al. (2020) study in the SHR matches our previous observations in outbred Wistar rats where young adult hearts (8-12 weeks) had fewer intracardiac neurons compared to juvenile (3-4 weeks) rats, whereas hearts of aged animals (>48 weeks) contained the greatest number of intracardiac neurons compared to the other two age groups (Batulevicius et al., 2003). Together, this might represent the plasticity and the dynamic nature of the intracardiac nervous system throughout the ontogenesis that needs to be considered when assessing changes related to (or induced by) specific pathological states.

Recently, it was demonstrated that abnormal intracellular calcium homeostasis may cause autonomic dysfunction characterized by suppressed parasympathetic and increased sympathetic activity in young prehypertensive SHR (Li and Paterson, 2019). This dysregulation of presynaptic neuronal Ca\(^{2+}\) handling contributes presumably to hypertension, among other cardiac diseases. As a consequence of Ca\(^{2+}\) overexpression, abnormalities of mitochondria and endoplasmic reticulum take place in stellate ganglia sympathetic neurons of prehypertensive SHR (Shanks et al., 2017). These recent findings confirm the earlier implications that prehypertension may impact the structural changes within intracardiac neurons as has been implied analyzing hypertrophied intracardiac neurons in hearts of the human, canine, porcine, and rabbit following myocardium ischemia or experimentally induced heart failure syndrome (Meerson and Krokhina, 1965; Richardson et al., 2003; Singh et al., 2013). Since SHR develops compensatory ventricular hypertrophy attributable to increased cardiac load due to chronic and severe hypertensive phenotype as early as 6 months postnatally (Singh et al., 2013), we attempted to explore whether this would lead to size alterations of ganglionic neuronal somata reported
in other species. Our data suggest that short-term hypertension in the SHR, i.e., occurring between 12-18 weeks of life, is not yet associated with neuronal hypertrophy as indicated by the significantly smaller neuronal size found in both SHR and WKY adult rats compared to strain matched-juveniles. It is possible that smaller neurons could reflect parasympathetic inhibition or yet unmanifested compensatory parasympathetic sensitization in the progression of compensatory hypertrophy in the SHR (Singh et al., 2013; Ashton et al., 2020). This assumption is based on the fact that the intracardiac neurons located within the rat’s heart are almost exclusively immunoreactive for choline acetyltransferase (ChAT) – a well-established marker of parasympathetic neurons (Richardson et al., 2003). However, this hypothesis requires further investigation into the neurotransmitter phenotypes of the intracardiac neurons between SHR and WKY hearts.

Regarding the epicardiac nerves, both our ultrastructural data and immunofluorescence labeling with PGP 9.5 indicated a possible axonal loss associated with the onset of arterial hypertension in the SHR, as no age-related changes were detected in the WKY rats. This is an interesting finding that relates to a previous (Adams et al., 1989) study, which reports tissue noradrenaline (NE) concentration in the left ventricle and interventricular septum of the SHR to be nearly twice as that found in age-matched WKY controls aged 4 to 14 weeks. While in aged, 20 weeks and older, NE tissue levels were similar in both strains (Adams et al., 1989). It might be due to the loss of myocardial nerve fibers observed in this study. In the juvenile group (8-9 weeks), the percentage density of myocardial nerve fibers was 52% greater in the SHR (WKY 0.65% vs. SHR 1.35%), while this difference decreases to 23% in adult animals (12-18 weeks, WKY 1.17% vs. SHR 1.51%). Together, this suggests that autonomic activation happens: (i) already at a young age, with (ii) a greater extent at younger than older age and (iii) prior to the manifestation of arterial hypertension. Another difference to consider between our and Adams et al. findings is that we assessed fiber densities in both right and left ventricles and found the right ventricle to exhibit a greater degree of innervation. Similarly, Kondo et al. studied the densities of myocardial noradrenergic nerves and found increased right ventricle innervation in stroke-prone SHR at the age of 30 and 90 days (Kondo et al., 1996). This result might be explained by myocardial hypertrophy in adult animals that affects the left ventricle to a greater extent and is not accompanied by an increase in innervation. However, differential innervation of left and right ventricles in young animals remains an open question.

The importance of glial cells in the functioning of the central nervous system is commonplace as glial cells actively shape synaptic properties, regulate neuronal firing, and are essential for metabolic support, ionic homeostasis, and neurovascular modulation (Simard and Nedergaard, 2004; Attwell et
al., 2010; Araque et al., 2014). In addition, glial signaling plays an active role in modulating the excitatory circuits in the peripheral (enteric) nervous system (McClain et al., 2015), whereas recent evidence has highlighted the central role of enteric glia cells in the regulation of gut homeostasis as well as their implication in digestive and extra-digestive diseases (Neunlist et al., 2014). In contrast, little is known about the role of glial (Schwann and satellite) cells in the intracardiac nervous system (Tedoldi et al., 2021). For this, our results on intracardiac glial cells can only be discussed in the context of autonomic plexi in other organs.

It is well established that Schwann cells rarely divide during adult life in health. The rate of Schwann cell proliferation in adult life increases noticeably under various experimental conditions (e.g., after axonal section and following the administration of certain chemical substances) or some pathological conditions (Pannese, 2015). Interestingly, centrioles within Schwann cells were observed at many instances in our study, indicating increased mitotic activity of this cell population associated with arterial hypertension. It has been suggested that satellite glial and Schwann cells can be activated by inflammatory responses, supporting a more active role of these cells other than just being a support network for neuronal cells (Tay et al., 1984). Thus, our finding of multilayer and porous satellite cells within intracardiac ganglia could indicate an activated state which might be evoked by an ongoing inflammatory/pathological process.

Furthermore, myelin alterations, like those observed in the present study, have been previously described in the context of various neuropathies. In the common peroneal nerve of dystrophic Bar Harbor mice, abnormal features of myelination - such as redundant myelin loops, thinly myelinated axons, few myelinated axons enclosed by the cytoplasm of one Schwann cell, Schwann cells that lacked an axon but contained myelin debris in the cytoplasm can be found (Jaros and Bradley, 1979). Authors interpret these findings as an indication of a metabolic disorder of Schwann cells. Furthermore, a formation by a single Schwann cell of myelin around two axons of different diameters has been reported in the sural nerve of a 45-year-old man with mononeuritis multiplex (Kusaka et al., 1992). In a more recent mechanistic study, it has been shown that loss of Fbxw7, an E3 ubiquitin ligase component, enhances the myelinating potential of Schwann cells in that in these mice Schwann cells produce thicker myelin sheaths and sometimes appear to myelinate multiple axons (Harty et al., 2019). Moreover, myelin abnormalities in various neuropathies, including infoldings/outfoldings and tomacula, have been previously associated with Akt activation that seems to enhance wrapping and sorting but not myelination of axons in Remak Schwann cells (Domènech-Estévez et al., 2016). Various demyelinating neuropathies are also associated with the formation of tomacula in the sural...
nerve biopsies of humans (Sander et al., 2000). As many of the aforementioned abnormalities related to Schwann cells have been detected in adult SHR hearts, this suggests that the cardiac nerves’ neuropathic processes may relate to chronically elevated arterial blood pressure in this model.

Lastly, in the present study, we analyzed G-ratios, which we found to be unusually high for peripheral myelinated nerves, indicating thin myelin sheath. Our data show that the G-ratio in WKY rats was relatively high (>0.7), while it was even higher in the SHR hearts. G-ratios found in the central nervous system fibers fall within a range of 0.72–0.81, whereas for peripheral axons, it can reach 0.6, indicating a thicker myelin layer (Chomiak and Hu, 2009). It is known that some peripheral axon g-ratio values tend to be lower than central axon g-ratio values (Chau et al., 2000), although the underlying mechanism remains poorly understood. In humans, peripheral nerve g-ratio values lower than 0.4 indicate degenerated nerve fibers with abnormal thickening of the myelin sheath, whereas values higher than 0.7 indicate either regenerated fibers with thinner myelin sheath or demyelinated nerve fibers (Ugrenović et al., 2016). To the best of our knowledge, no other available study reports g-ratios of cardiac nerve fibers in rats, making it impossible to draw any comparative conclusions. Thus, further investigation of cardiac nerve fibers g-ratios is needed.

Finally, it is worth mentioning that deranged quality of the cardiac ganglia and nerves due to hypertension demonstrated in this and recent other studies along with ischemic neuronal degeneration of the vagal nodose ganglia may substantially contribute to the substrate for atrial and ventricular arrhythmia, heart failure, and shortened survival (Aydin et al., 2011, 2019; Ashton et al., 2020). Therefore, the demonstrated extent to which the intrinsic cardiac plexus undergoes structural changes due to hypertension has to be considered as clinically crucial for all kinds of cardiac interventions - cardiac surgery including heart transplantation, coronary artery bypass grafting, valvuloplasty, valve replacement, and arrhythmia treatment via catheter ablations.

Nevertheless, it is important to consider several limitations of the current investigation. For one, it is important to acknowledge that inbred WKY rats, used as controls in the present study, themselves display elevated blood pressure compared to outbred Wistar rats. For this reason, a further analysis comparing SH, WKY, and outbred Wistars rats might provide a more accurate picture of the undergoing processes. Another limitation is that here we only describe morphological differences between SH and WKY rat hearts, and whether these would translate to any detectable functional differences remains to be established.
5. CONCLUSIONS

Our study provides evidence on (i) the structural differences in cardiac innervation between SH and WKY rats; (ii) the effects of early-onset hypertension on intracardiac neuron and nerve loss in the SHR; (iii) the increased innervation of the right ventricle and atria in SH rats and an opposite effect on the left ventricle; (iv) ultrastructural alterations of intracardiac ganglia and nerve glial cells indicative of an early onset on neuropathic processes in the SH rat hearts.

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The authors thank Tomas Ragauskas and Dmitrij Kvitka for technical assistance. We are extremely grateful to Audrys G. Pauza from the Laboratories for Integrative Neuroscience and Endocrinology, University of Bristol (United Kingdom), for his careful reading of the manuscript, constructive criticisms, and generous editorial assistance. This study was supported by the Grant SMIP-19-23/PRM19-113 from the Research Council of Lithuania and Grants No. V-786, V-789 from the Lithuanian University of Health Sciences Research Foundation.

A conflict of interest statement

Authors declare no conflicts of interest.

REFERENCES:


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Table 1. The mean parameters (± standard error) of the analyzed neural structures in the SHR and WKY rats. Neural structures were immunohistochemically labeled for PGP9.5.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WKY</th>
<th>SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Juvenile</td>
<td>Adult</td>
</tr>
<tr>
<td></td>
<td>Juvenile</td>
<td>Adult</td>
</tr>
<tr>
<td>Gagal number</td>
<td>121</td>
<td>148</td>
</tr>
<tr>
<td>Mean (± SE)</td>
<td>29.5 (13.2)</td>
<td>23.3 (4.8)</td>
</tr>
<tr>
<td>Ganglion area (µm²)</td>
<td>121</td>
<td>148</td>
</tr>
<tr>
<td>Mean (± SE)</td>
<td>52348.9 (6565.5)</td>
<td>45097.0 (4402.7)</td>
</tr>
<tr>
<td>Neuron area (µm²)</td>
<td>697</td>
<td>996</td>
</tr>
<tr>
<td>Mean (± SE)</td>
<td>643.6 (10.8)</td>
<td>532.2 (7.1)**</td>
</tr>
<tr>
<td>Neuronal number in ganglion</td>
<td>5876</td>
<td>6811</td>
</tr>
<tr>
<td>Mean (± SE)</td>
<td>52.4 (10.1)</td>
<td>33.0 (2.8)</td>
</tr>
<tr>
<td>Epicardiac nerves</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atrial nerve width (µm)</td>
<td>544</td>
<td>368</td>
</tr>
<tr>
<td>Mean (± SE)</td>
<td>28.6 (29.8)</td>
<td>32.2 (28.3)**</td>
</tr>
<tr>
<td>Ventricular nerve width (µm)</td>
<td>295</td>
<td>673</td>
</tr>
<tr>
<td>Mean (± SE)</td>
<td>22.2 (1.1)</td>
<td>27.1 (0.7)</td>
</tr>
<tr>
<td>PGP 9.5 in epicardiac nerve cross-sections (%)</td>
<td>151</td>
<td>149</td>
</tr>
<tr>
<td>Mean (± SE)</td>
<td>72.5 (0.9)</td>
<td>81.6 (1.6)**</td>
</tr>
<tr>
<td>Density of myocardial nerve fibers (%)</td>
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<td></td>
</tr>
<tr>
<td>Atrial</td>
<td>144</td>
<td>70</td>
</tr>
<tr>
<td>Mean (± SE)</td>
<td>0.96 (0.02)</td>
<td>1.0 (0.03)</td>
</tr>
<tr>
<td>Ventricular</td>
<td>195</td>
<td>235</td>
</tr>
<tr>
<td>Mean (± SE)</td>
<td>0.65 (0.4)</td>
<td>1.1 (0.6)**</td>
</tr>
<tr>
<td>Right ventricular</td>
<td>78</td>
<td>69</td>
</tr>
<tr>
<td>Mean (± SE)</td>
<td>1.04 (0.1)</td>
<td>1.1 (0.1)</td>
</tr>
<tr>
<td>Left ventricular</td>
<td>60</td>
<td>103</td>
</tr>
<tr>
<td>Mean (± SE)</td>
<td>0.6 (0.1)</td>
<td>0.7 (0.04)**</td>
</tr>
<tr>
<td>Interventrical septal</td>
<td>57</td>
<td>63</td>
</tr>
<tr>
<td>Mean (± SE)</td>
<td>0.3 (0.03)</td>
<td>0.4 (0.03)**</td>
</tr>
</tbody>
</table>

* - statistically significant difference between SHR and WKY groups of the same age, p<0.05.

** - statistically significant difference between juvenile and adult groups in WKY or SHR groups, p<0.05.
Table 2. The mean parameters (± standard error) of neural structures in WKY rats versus SHR were analyzed with the aid of a transmission electron microscope.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WKY</th>
<th>SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Atrial</td>
<td>Ventricular</td>
</tr>
<tr>
<td>Unmyelinated fibers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of axons measured (n)</td>
<td>1381</td>
<td>978</td>
</tr>
<tr>
<td>Axon area (µm²)</td>
<td>1.5 (0.03)</td>
<td>1.2 (0.04)**</td>
</tr>
<tr>
<td>Axon diameter (µm)</td>
<td>1.3 (0.01)</td>
<td>1.07 (0.02)</td>
</tr>
<tr>
<td>Mean axonal number in nerve fiber</td>
<td>2.5 (0.1)</td>
<td>4.4 (0.6)**</td>
</tr>
<tr>
<td>Axon area in nerve (%)</td>
<td>48.9 (1.2)</td>
<td>54.4 (1.4)**</td>
</tr>
<tr>
<td>Myelinated fibers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of axons measured (n)</td>
<td>92</td>
<td>28</td>
</tr>
<tr>
<td>Axon area (µm²)</td>
<td>4.1 (0.1)</td>
<td>5.5 (0.3)</td>
</tr>
<tr>
<td>Axon diameter (µm)</td>
<td>2.3 (0.04)</td>
<td>2.6 (0.1)**</td>
</tr>
<tr>
<td>Nerve fiber area (µm²)</td>
<td>7.2 (0.3)</td>
<td>8.8 (0.6)**</td>
</tr>
<tr>
<td>Nerve fiber diameter (µm)</td>
<td>3.0 (0.1)</td>
<td>3.3 (0.1)**</td>
</tr>
<tr>
<td>G-ratio</td>
<td>0.77 (0.01)</td>
<td>0.8 (0.01)**</td>
</tr>
</tbody>
</table>

*- statistically significant difference between WKY and SHR groups, p<0.05.

**- statistically significant difference between atrial and ventricular fibers in WKY or SHR groups, p<0.05.
Legends for illustrations (MS_Ranceviene et al.)

Fig. 1. Laser-scanning (confocal) microphotographs to illustrate a general view of morphological patterns of intracardiac ganglionated nerve plexus of the SHR atria of 8 (A) and 14 week (B) old animals. The general views of whole mounts of the rat atria were assembled with the aid of software ZEN 2010B SP1 from 108 images displaying neural structures immunochemically labeled for the antigen PGP 9.5. Note, there are decreased ganglionic clusters in the aged (panel B) compared to the young rats (panel A). White arrows indicate some epicardiac nerves, arrowheads point to some ganglia, and thick arrows point to preganglionated nerves, which enter the heart through the hilum at the left cranial vein. Abbreviations: LPV – left pulmonary vein, MPV – middle pulmonary vein, RPV – right pulmonary vein.

Fig. 2. Electron micrographs to demonstrate an ultrastructure of intrinsic cardiac ganglia and unmyelinated nerve fibers in WKY (A, C) and SH (B, D) rats. A – A fragment of a WKY neuronal soma with a satellite cell covering it. The top of the electronogram is occupied by a neuronal body (N) from which the dendrite (D) extends downwardly. At this location, the neuron body is surrounded by a thick layer of neuroglia (G) which is multi-layered (arrowheads). In some sites, deep invaginations intervene in the neuroglia, and endoneurial collagen fibers (arrows) interpose between the satellite cell processes. Fragments of several axons and dendrites are seen around the neuronal body. B - Fragment of an SHR neuronal body. The satellite cell that covers the neuron looks porous with deep invaginations containing endoneurial collagen fibers (arrows). A few dendrites (D) and one axon (ax) with vesicles are visible at a distance from the neuronal body. C – Unmyelinated nerve fiber from the WKY rat nerve, in which the axons are arranged compactly, and there are no Schwann cell invaginations. Some axons are completely covered by the glia cell; others are opened and covered only with the basal membrane (arrowheads). Some axons are placed in one throat of a Schwann cell and adjoin each other (*). D – Unmyelinated nerve fibers of the SH rat. A dotted line marks the basal membrane of each fiber, indicating the boundaries of the fiber. Schwann cells in these fibers are fractioned with specific invaginations of the endoneurium that separate a single nerve fiber into discernible parts interconnected by narrow glial bridges. Numerous very thin axons with a diameter of less than 0.2 µm (white arrowheads) are common in these fibers. Some axons adjoin each other (*). Abbreviations: D – dendrite, ax – axon, Max – myelinated axon, En – endoneurium, P – perineurium. Scale bar – 1 µm.
**Fig. 3.** Immunofluorescent images from whole-mount preparations labeled for PGP 9.5 of 14 week old WKY (panel A) and SHR (panel B) cardiac ventricles. Whole-mount preparations were made, cutting off the bases of cardiac ventricles and flattening them under a stereoscopic microscope. The displayed general views were assembled from 500 images with the aid of the software AxioVision (Rel. 4.8.2). Panel C demonstrates the nerve meshwork in the atrial myocardium of 14 week old SHR, while panel (D) is a transverse section of cardiac ventricles demonstrating profiles of myocardial nerve fibers immunohistochemically stained for PGP 9.5. Panel E shows one of the thick epicardiac nerves in 16 week old SHR ventricles that is full of PGP 9.5 positive nerve fibers. *Abbreviations:* LLV - lateral left ventricle; ALRV - anterolateral right ventricle.

**Fig. 4.** Abundance in percentages of the different axonal diameters in adult SH and WKY rats. Vertical lines on the bars indicate limits of standard errors (Mann-Whitney U test, asterisks marks p<0.05).

**Fig. 5.** Electron micrographs of intracardiac nerves in WKY (A) and SH (B-F) rats. A – Normal myelinated (ax) and unmyelinated (*) nerve fibers from the WKY rat nerve. Myelin sheath is attached to the axon and corresponds to its diameter and shape. Compact and thin unmyelinated nerve fibers are scattered between the myelinated ones. In the right upper part of the image, a fragment of thick myelinated fiber (axon diameter is 4.9 µm, that is unusual for intracardiac nerves) is seen. B – Typical electron microscopic image of intracardiac nerve in SH rats. Some myelinated nerve fibers (ax) are of normal ultrastructure. In other ones, there are typical visible alterations: ax1 - depicts the collapsed/empty myelin sheath encasing an axon with its outer surface (enlarged in panel C), ax2 - endoneurium intervenes between the myelin and the axon covered by a layer of glial cells (enlarged in panel D), ax3 - endoneurium is between the myelin and a myelinated axon (enlarged in panel E), ax4 and ax5 - cytoplasm is interposed between the myelin layers which formed specific protrusions that varied in size, yet remain inside the Schwann cell always (white arrowheads). Note the same protrusion which penetrates the myelin of ax3. ax5 (1.18 µm in diameter) is noticeably thinner than the adjacent ones and encloses a very thin myelin sheath. The unmyelinated nerve fibers are marked by asterisks (*). F - Intra-axon myelin ring (double arrowhead) of the myelinated nerve fiber in the SH rat.
**Fig. 6.** Typical alterations of myelinated fibers within intracardiac nerves of SH rats. **A** - Nerve with abundant myelinated fibers at low magnification. Some myelinated fibers are of a typical structure when the myelin is attached to the axon and replicates its shape. The myelin sheath of the other fibers (*) is significantly larger than the axon diameter, resulting in the formation of a larger or smaller myelin fold (arrowheads). Among myelinated fibers are scattered the unmyelinated ones. **B** - Three axons (ax1-ax3) are enveloped in the same myelin sheath. Around it are seen tiny unmyelinated fibers containing very few (1-2) axons (diamonds). **C** - Two axons (ax1-ax2) enclosed in the same myelin sheath. Adjacent unmyelinated fibers are cuddled up together and are covered by a common basal membrane (arrow). The myelin of the upper axon (*) forms the fold (arrowhead). **D** - Two axons (*) enclosed by the myelin sheaths forming folds (arrowheads). Note the tiny unmyelinated nerve fiber with two axons (diamonds) specific to intrinsic cardiac nerves in SH rats. **E** - Four myelinated axons (ax1-ax4) in one Schwann cell. The myelin sheath covering the main axon (ax1) forms the three folds (arrowheads). **F** - The myelinated nerve fibers of different diameter with a typical thickness of myelin. One of the axons (ax1) exposes an inclusion specific to the SHR nerves, where many vesicles are surrounded by dual membranes (double arrowheads). The other two very small-diameter myelinated axons (ax2-ax3) are in the same Schwann cell. Scale bar: A – 2 µm, B-F – 1 µm.

**Fig. 7.** Density in percentages of myocardial nerve fibers in adult SH and WKY rats. Vertical lines on the bars indicate limits of standard errors (Mann-Whitney U test, asterisks marks p<0.05).
The bar chart shows the distribution of axons by diameter in two groups, SHR and WKY, with the number of axons expressed as a percentage. The axons are categorized into the following diameter ranges: <0.5, 0.5 - <1, 1 - <1.5, 1.5 - <2, and >2 µm. The chart indicates a significant difference (*) between the two groups in the 0.5 - <1 and 1 - <1.5 µm diameter ranges.