II. THE IMMUNOHISTOCHEMICAL STUDY: MORPHOLOGY OF THE CARDIAC NERVE PLEXUS ON THE RABBIT HEART BASE

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Abstract. The purpose of this study was to investigate the neurochemistry of the epicardiac ganglionated nerve plexus in the whole-mount preparations of the rabbit atria, because the intrinsic cardiac nervous system of the rabbit has not been investigated so far. The cardiac nerve structures were revealed by the immunofluorescence labelling the choline acetyltransferase (ChAT), the tyrosine hydroxylase (TH) and the neuronal nitric oxide synthase (nNOS) in whole-mount atrial preparations derived from 10 young rabbits. The somata of the intrinsic cardiac neurons (ICNs) displayed the immunoreactivity for ChAT and nNOS. The TH-IR was not observed in the somata of the ICNs. The majority of the ICNs were immunoreactive for ChAT (52±11 %), the small population of the ICNs (19±16 %) exhibited the immunoreactivity for nNOS and 30±5 % of the ICNs were biphenotypic for ChAT and nNOS. The mean number of the ChAT-IR neurons was statistically significantly bigger than the mean number of both the nNOS-IR and the biphenotypic neurons ($P<0.05$). The ChAT-IR neurons were surrounded by the baskets of the ChAT-IR varicose nerve terminals. The nNOS-IR nerve fibres did not form pericellular complexes with nNOS-IR or ChAT-IR neurons. The singular varicose TH-IR nerve terminals were observed within ganglia but did not form pericellular complexes with ChAT-IR neurons. The extrinsic cardiac nerves entering the heart base were predominantly composed of the TH-IR nerve fibres that spread in the left atrium. The sparse ChAT-IR nerve fibres proceeded on the heart base toward the large intrinsic ganglia and on the epicardium towards the root of the right cranial vein (RCV). We concluded that the lack of the TH-IR ICNs within ganglia and lack of the mesh-work of the extrinsic nNOS-IR nerve fibres within the rabbit epicardiac ganglionated nerve plexus may support the hypothetic increase of reactivity to the rabbit coronary vessels to the effects of the catecholamines released by the adrenal medulla during the stress.

Keywords: intrinsic cardiac neurons, rabbit, tyrosine hydroxylase, choline acetyltransferase, neuronal nitric oxide synthase

Introduction. In contrast to the humans, the rabbit heart attack is always caused by a spasm of a coronary artery and frequently leads to a sudden death of the domestic rabbits. The spasm of a coronary artery brought on by scare leads to reduction of the blood supply to the rabbit heart muscle. The heart diseases have occurred in the rabbits and were treated by veterinarians with some success (Smith, 2003). The intrinsic cardiac nervous system is a crucial regulator of the coronary blood flow (Zong et al., 2004). Catecholamines released by the adrenal medulla and norepinephrine released from sympathetic nerve terminals activate both α and β adrenoreceptors, and activation of α-adrenoreceptors of vascular smooth muscle causes vasoconstriction of the coronary vessels (Zong et al., 2004). Significant sympathetic vasoconstriction has been previously demonstrated in the coronary circulation during exercise (Gorman et al., 2000), and the sympathetic coronary vasoconstriction limits rabbit ventricular function and cardiac output during exercise and stress. Physiological studies have shown that the coronary blood flow as well as cardiac functions, i.e. chronotropic, dromotropic and inotropic, are influated by intrinsic cardiac ganglia (ICG) working as an integrative nerve centres modulating extrinsic autonomic inputs from the spinal cord, brain stem, and insular cortex as well as mediates local cardio-cardiac reflexes (Armour, 2008). Classically, the ICG consist of parasympathetic efferent neurons, however, evidence that augmentation of cardiac function can be evoked by focal activation of the somata of some ICNs has been suggested that the somata of non-cholinergic neurons may be present in the heart (Armour, 2008).

According to the recent data, the ICG were mainly distributed within atria of the rabbit heart (unpublished data). We hypothesize that the rabbit heart contains the population of ICNs potentially involved in the sympathetically mediated coronary vasoconstriction. The ICNs involved in the coronary pressor reflexes might be expected to display the adrenergic phenotype, including expression of tyrosine hydroxylase (TH, the rate-limiting enzyme in norepinephrine synthesis). The present study aimed to identify the pattern of distribution of TH-IR nerve structures within the whole-mount preparations of the rabbit atria. Inhibition of the neuronal nitric oxide synthase (nNOS) in cardiac postganglionic sympathetic neurons leads to enhanced cardiac sympathetic responsiveness (Choate and Paterson, 1999). Recent data have demonstrated that nNOS is localized in both intrinsic cardiac vagal and stellate sympathetic neurons, and evidence has established neuronal nitric oxide (NO) as an important modulator (Herring and Paterson, 2008). In cholinergic neurons, NO acts to increase the release of acetylcholine (Herring and Paterson, 2008), while, conversely, NO generated in sympathetic neurons reduces the release of norepinephrine (Choate and Paterson, 1999). The possibility that NO might be involved in the sympathetically mediated coronary vasoconstriction has never been investigated. This study aimed to establish the distribution of the nNOS-IR nerve structures within the whole-mount preparations of the rabbit atria. In contrast

to the hearts of other mammalian species, the neuroanatomy of the rabbit heart has been poorly examined. This is the first immunohistochemical investigation of the rabbit intrinsic cardiac ganglia performed in whole mount preparations of the rabbit atria using immunofluorescence labelling for ChAT, TH and nNOS.

Materials and Methods. Ten White New Zealand rabbits of both sexes (4–6 weeks old and weighing 0.658 ± 0.093 kg) were used in the following study. The animals were anesthetized with a lethal dose of sodium thiopental (30 mg/kg i.v) in accordance with local and state guidelines for the care and use of laboratory animals (Permission No. 0206).

Whole mount preparation. To examine the intrinsic cardiac neural plexus using immunofluorescence, hearts perfused with 0.01 M phosphate-buffered saline (PBS; 0.9% NaCl, pH 7.4) were removed from the chest and placed into a dissecting dish containing cold 0.01 M PBS as described previously (Rysevaite et al., 2011). For optimal visualization of neural structures, the atrial walls were separated from the ventricles and then pinned on a silicone pad in a dissecting dish, in which tissues were prefixed for 45 min at 4 °C in a 4% paraformaldehyde solution in 0.01 M phosphate buffer (pH 7.4). To decrease the background light during fluorescence mocroscopy, the tissues were cleared using a dimethylsulfoxide and hydrogen peroxide solution and dehydrated as reported previously (Rysevaite et al., 2011). After tissue clearing, the whole-mount preparations were rehydrated by successive 15-minute washes through a graded ethanol series, then washed, and permeabilized in 3 x 10-minute changes of 0.01 M PBS containing 0.5% Triton X-100 (Carl Roth, Karlsrube, Germany). Nonspecific binding was blocked for 2 h in PBS containing 5% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and 0.5% Triton X-100. Next, preparations were washed three times for 10 min in 0.01 M PBS and incubated in a mixture of double primary antibodies for 48 h in a dark humid chamber at +4 °C (table 1). After three 10 min-washes in 0.01 M PBS, the whole-mount heart preparations were incubated in an appropriate combination of secondary antibodies for 4 h in a dark humid chamber on a shaker stage at room temperature (Table 1). All antibodies were diluted in 0.01 M PBS. Thereafter, the specimens were again washed in 0.01 M PBS, incubated for 2 h in a dimethylsulfoxide and PBS mixture, 1:4 (v:v) ratio, and mounted in Vectashield Mounting Medium (Vector Laboratories, California, USA). A cover slip was placed on the tissue and then sealed with clear nail polish. Both positive and negative controls were used.

Table 1. **Primary and secondary antisera used in the study**

Antigen	Host	Dilution	Supplier	Catalogue number
Primary				
TH	Mouse	1:500	Invitrogen[a]	32-2100
CHAT	Goat	1:100	Chemicon[b]	AB 144P
NOS	Mouse	1:100	SantaCruz[c]	SC-5302
PGP 9.5	Mouse	1:200	Nordic BioSite[d]	7863-1004
Secondary				
Mouse^{FITC}	Donkey	1:100	ImmunoStar[e]	715-095-151
Goat^{Cy3}	Donkey	1:300	Chemicon[b]	AP 180C
Ginea pig^{FITC}	Donkey	1:500	Sigma-Aldrich[f]	CF^{TM} 488A

[a] Invitrogen Corporation, Flynn Rd, Camarillo, CA; [b] Chemicon International, Temecula, California, USA; [c] Santa Cruz Biotechnology, Inc., Dallas, Texas, USA; [d] Nordic BioSite By Your Side^{TM} In Life Science Research, Täby, Sweden; [e] ImmunoStar Incorporation, Wisconsin, USA; [f] Sigma-Aldrich, St. Louis, MO, USA.

Microscopic examination and quantitative analysis. Whole-mount preparations stained immunohistochemically were analyzed utilizing an AxioImager Z1 fluorescence microscope (Zeiss, Gottingen, Germany) equipped with a set of filters to observe the fluorescein isothiocyanate (FITC) and cyanine (Cy3) fluorescence, an Apotome (Zeiss, Gottingen, Germany), and a digital monochrome camera AxioCam MRm (Zeiss, Gottingen, Germany). When necessary, the same whole-mount preparations were additionally analyzed and photographed employing a laser scanning microscope LSM 700 (Zeiss, Jena, Germany) with its ZEN 2010 software (Zeiss, Jena, Germany). The number of neurons inside intrinsic cardiac ganglia was estimated in 0.3–0.5 μm optical sections of the whole-mount preparations by counting exclusively the nNOS-IR, ChAT-IR and ChAT+nNOS-IR immuno-reactive nerve cells that contained well-visible nuclei. The adjustments of final images and measurements of cardiac neural structures were performed using AxioVision 4.8.1 software (Zeiss, Jena, Germany).

Statistical analysis. Data were processed using the Origin Lab software version 6.1 (OriginLab Corporation, Northampton, MA, USA). Data shown both in the text and graphs are expressed as mean ± standard error of mean (SEM). Statistical significance of the difference between the means was performed with Student's paired and independent tests. Differences were considered statistically significant at $P<0.05$.

Results. The distribution of the cholinergic nerve structures was determined in ten whole-mount

preparations of the rabbit atria using the antibodies to ChAT. The majority of neuronal somata demonstrated the immunoreactivity for ChAT, while the small population of the ICNs were not labelled for ChAT and it was later demonstrated that somata of these neurons were immunoreactive for the nNOS (Fig. 1). The immunoreactivity for ChAT was identified in the perinuclear region of soma, and the intensity of the fluorescence varied from neuron to neuron (Fig. 1). These ChAT-IR neuronal somata were surrounded by the baskets of the varicose nerve terminals that expressed the ChAT more intensively than the perinuclear region (Figs 2b,c,d). The abundant ChAT-IR nerve fibres were identified only in the thin nerves that interconnected ganglia or distributed towards the root of the RCV, i.e. the SAN region as well as in the nerve bundles that proceeded within ganglia (Fig. 2a).

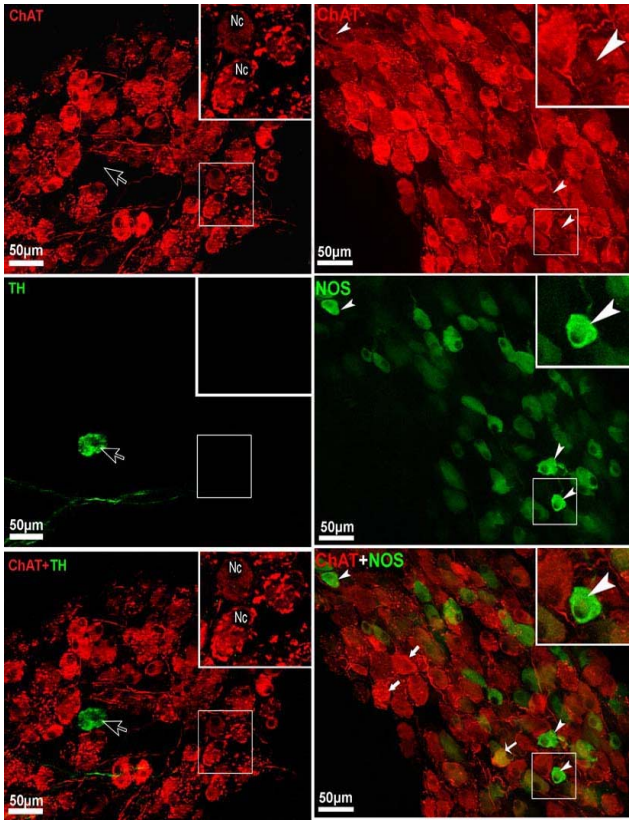

Fig 1. **The microphotographs of the whole-mount preparations of atria demonstrate the cholinergic neurons, nerve fibres and bundles labelled in red, the adrenergic nerve fibres and small intensely fluorescent cells** (SIF) **and the nitrergic neurons and fibres labelled in green.** The boxed areas and panels are enlarged in the right upper corners. The majority of the ICNs were ChAT-IR. The adrenergic marker TH is localised in the SIF cells and varicose nerve fibres. The nitrergic marker nNOS is identified in a population of the cholinergic ICNs but some of neurons were immunoreactive for the nNOS only. Open white arrow – cluster of SIF cells; white arrowheads – nNOR-IR neurons; thin arrow – biphenotypic neuron; thick arrows – ChAT-IR neurons. Abbreviation: Nc – nucleus.

The adrenergic nerve structures were examined in five whole-mount preparations of the rabbit atria using the antibodies to the TH. The immunoreactivity for TH was not observed in the somata of the ICNs and in the baskets of varicose nerve terminals that surrounded the neuronal somata (Figs 2b,c,d). Yet singular varicose TH-IR terminals were observed within ganglia (Fig. 2c). Only the compact clusters of small cells located within ICG demonstrated the strong immunoreactivity only for TH (Fig. 1). These cells were smaller than neuronal somata (10–15 μm diameter), elongated and did not possess pericellular basket of the varicose terminals (Fig. 1). Therefore, the small TH-IR cells were considered in the present study as the small intensely fluorescent cells. The double labelling for TH and ChAT showed that the extrinsic cardiac nerves that entered the nerve plexus on the heart base were predominantly composed of the TH-IR nerve fibres (Fig. 2a). These nerves branched on rabbit heart base, and part of the TH-IR brunches: (1) extended directly through the root of the RCV to the ventral surface of atria; (2) passed the RC of the ICG, accepted some ChAT-IR fibres and reached the SAN region; (3) entered the ganglia of the LC, joined ChAT-IR nerve fibres and reached the dorsal surface of the atria (Fig. 2a).

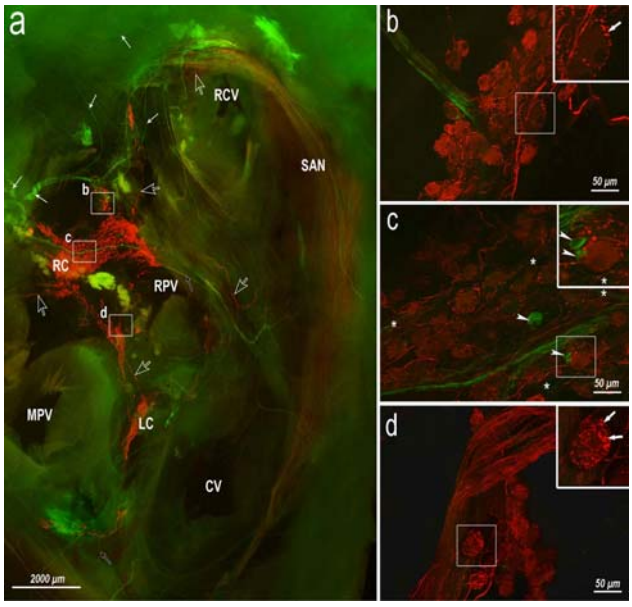

Fig 2. **The whole-mount preparation of the rabbit atria** (a) **shows the morphologic pattern of the nerve structures labelled immunohistochemically for tyrosine hydroxylase** (in green) **and choline acetyltransferase** (in red). The left cranial cava vein and left pulmonary vein were extirpated in order to flatten the atria. The boxed areas in panel a are enlarged as b-d to illustrate the ChAT-IR neurons (b-d), the SIF cells (c) and the baskets of varicose nerve terminals that expressed the ChAT more intensively than the perinuclear region (b, d). Boxed areas in b-d panels are enlarged in the right upper corners. The asterisks point the TH-IR varicose nerve fibres; white arrowheads – the SIF cells; white thick arrows - the baskets of varicose nerve terminals; white thin arrows - the extrinsic cardiac nerves entering the

ganglionated nerve plexus and their brunches (adrenergic); black thin arrows – mixed nerves consisted of TH + ChAT-IR fibres; white open arrows – the cholinergic nerves. Abbreviations: CV – caudal cava vein; LC – left cluster; MPV – middle pulmonary vein; RC – right cluster; RCV – right cranial vein; RPV – right pulmonary vein; SAN – sinoatrial nodal region.

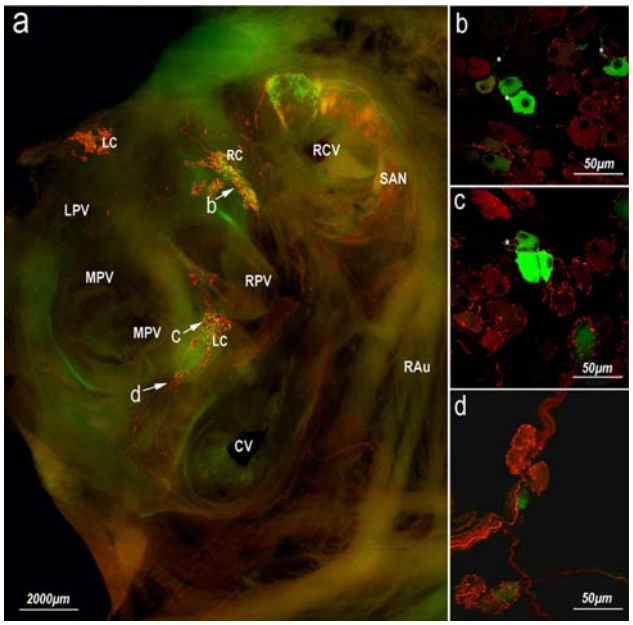

Fig 3. **The whole-mount preparation of the rabbit atria** (a) **shows the morphologic pattern of the nerve structures labelled immunohistochemically for neuronal nitric oxide synthase** (in green) **and choline acetyltransferase** (in red). The left cranial cava vein was extirpated in order to flatten the atria. The white arrows point ganglia in a panel that are enlarged as b-d to illustrate the nNOS-IR neuronal somata and processes; asterisk – neuronal processes. Abbreviations: CV – caudal cava vein; LC – left cluster; LPV – left pulmonary vein; MPV – middle pulmonary vein; RAu – right auricle; RC – right cluster; RCV – right cranial vein; RPV – right pulmonary vein; SAN – sinoatrial nodal region.

The nitrergic nerve structures examined in five whole-mount preparations of the rabbit atria using the antibodies to nNOS (Fig. 3). The immunoreactivity for nNOS was observed in the neuronal somata and processes, i.e. dendrites and nerve preterminals (Figs 3b,c). The double labelling for the nNOS and the ChAT demonstrated colocalization of both markers within somata of the moderate neuronal population (Fig. 3) that involved, on average, up to 30±5 % of all examined neurons (Fig. 4). The double labelling for both markers revealed some neurons which somata were immunoreactive for nNOS-IR only (Fig. 1c), and these nNOS-IR neurons comprised, on average, up to 19±16 % of total neuronal number (Fig. 4). The mean number of the ChAT-IR neurons was statistically significantly bigger than the mean number of both the nNOS-IR and the biphenotypic neurons ($P<0.05$) (Fig. 4). Both the nNOS-IR and the biphenotypic neurons were unevenly distributed within ganglia. Fig. 5 shows that a majority of the nNOS-IR and the biphenotypic neurons were concentrated within the large ganglia containing up to several hundred ICNs, while the small ganglia ranging from 3 to 90 neurons were predominantly composed of the ChAT-IR. There were no identified the nNOS-IR fibres within large nerve bundles and small interganglionic connective running between ganglia (Fig. 6).

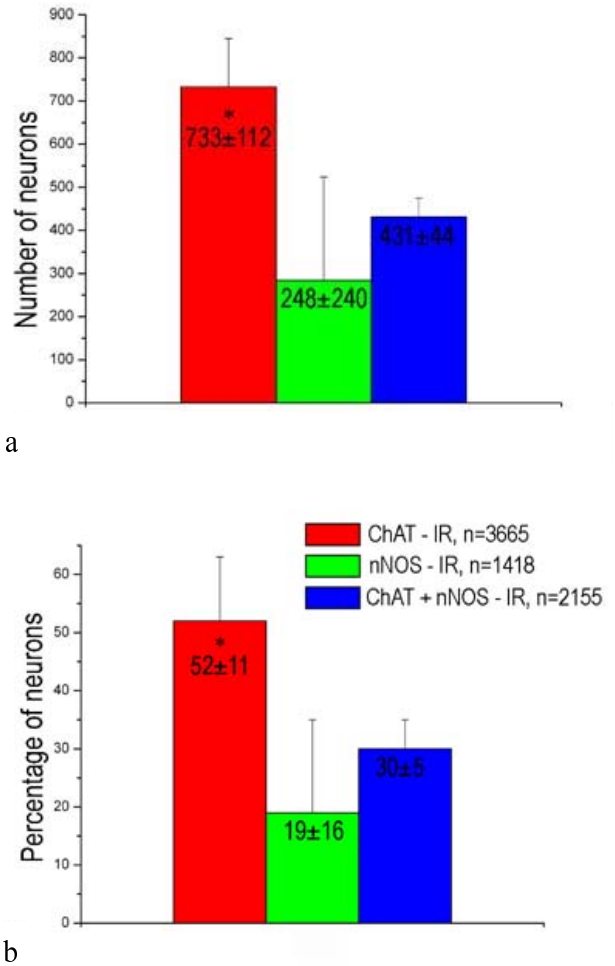

Fig 4. **The graphs demonstrate the number** (a) **and the percentage** (b) **of ChAT-IR** (red), **nNOS-IR (green) and ChAT + nNOS-IR (blue) neurons visualized in the double labelled ganglia** (Figs. 2, 4). N – number of examined neurons; asterisk – the mean number and the mean percentage of the ChAT-IR neurons that are statistically significantly bigger than the mean numbers of both the nNOS-IR and the biphenotypic neurons ($P < 0.05$).

Discussion. In this study, the majority of neuronal somata demonstrated the immunoreactivity for ChAT. The immunoreactivity for ChAT was identified in the perinuclear region of soma surrounded by the baskets of varicose nerve terminals expressing the ChAT-IR more intensely than perinuclear region. Our results confirm the

findings of the previous studies showed that the majority of neurons in the human, guinea pig, rat and mouse hearts display the cholinergic phenotype (Leger et al., 1999; Richardson et al., 2003; Hoover et al., 2009; Rysevaite et al., 2011). Although the majority of the ICNs of the rabbit heart are cholinergic, but they are neurochemically very complex, expressing a number of neuropeptides in addition to acetylcholine (Richardson et al., 2003; Hoover et al., 2009).

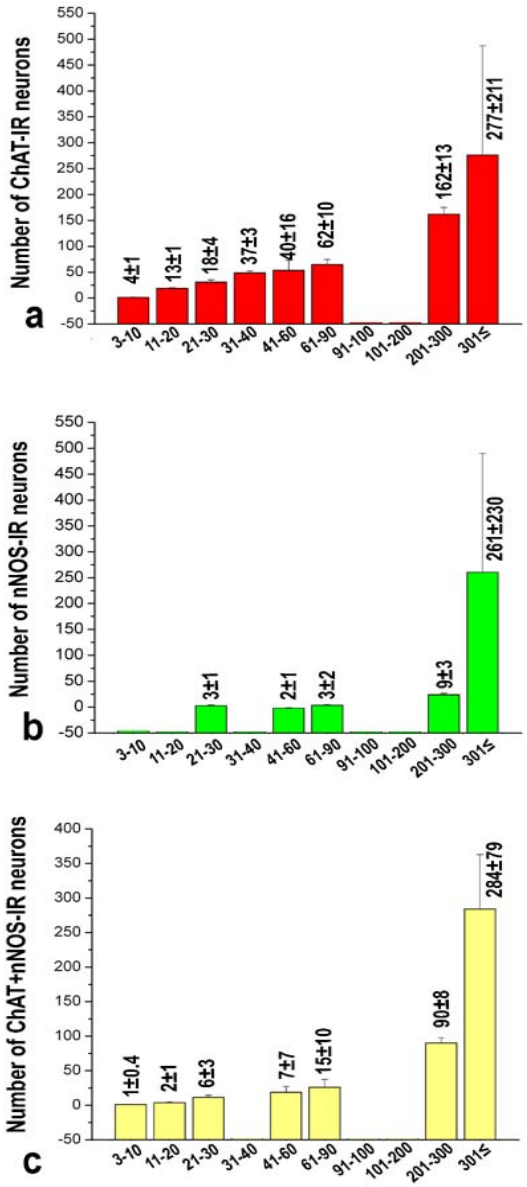

Fig 5. **The graphs demonstrate the distribution of the ChAT-IR neurons** (a), **the nNOS-IR neurons** (b) **and the ChAT + nNOS-IR neurons** (c) **within the double labelled rabbit cardiac ganglia** (Figs 2, 4). The numerals at the horizontal axis point the number of neurons within ganglia, the value labels at the columns show the mean numbers of neurons expressed as mean ± SEM.

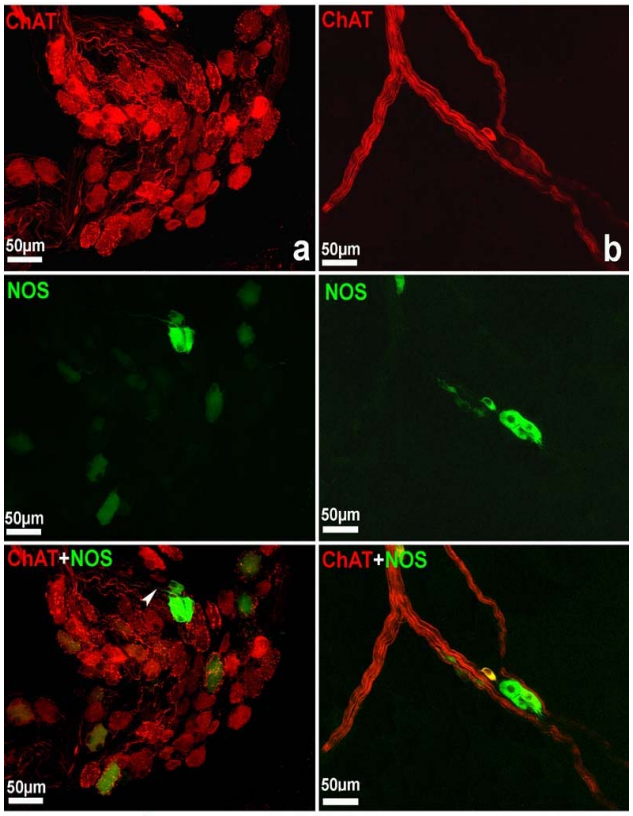

Fig 6. **The microphotographs of the whole-mount rabbit atrial preparation double labelled for the ChAT (red) and the nNOS (green) demonstrating the NOS-IR nerve fibres identified within the nerve bundles. White arrowhead demonstrates the NOS-IR fibre within nerve bundle.**

In the present study, the most intrinsic cardiac neuronal somata and nerve preterminals in the rabbit atria showed the immunoreactivity for the nNOS. Also the present study shows that rabbit cardiac ganglia contain numerous biphenotype nNOS-IR + ChAT-IR, small population of nNOS-IR neurons and single immunoreactive for nNOS fibres, that do not form complexes with neurons within ganglia. Contrary to the present findings in the rabbit heart, previous studies identified the numerous nNOS-IR neurons in many cardiac ganglia and the nNOS-IR fibres within large nerve bundles and small interganglionic connectives running between cardiac ganglia in the human and the guinea-pig hearts (Calupca et al., 2000; Hoover et al., 2009). The comparison of control versus 72-hours explant culture preparations indicated that most of the nitrergic fibres within cardiac nerve plexus and ganglia were extrinsic (Calupca et al., 2000). The extrinsic cardiac nNOS-IR nerve fibres were not immunoreactive for ChAT, TH or CGRP and represented an afferent fibre input that was separate from the SP/CGRP-containing population of sensory fibres (Calupca et al., 2000). Furthermore, many extrinsic nNOS-IR nerve fibres probably derived from the neurons of the nodose ganglion and the dorsal root ganglia (Calupca et al., 2000; Hoover et al., 2008). These neurons might have the dual afferent and efferent

functions (Hoover et al., 2008), because a selective inhibitor of the nNOS attenuates the pressor reflex evoked by the application of bradykinin to the left ventricular epicardium of anesthetized cats (Tjen et al., 2001).

In contrast to the observations in the human, rat and mouse hearts (Richardson et al., 2003; Hoover et al., 2009; Rysevaite et al., 2011), TH-IR neuronal somata were not observed in this study. Although, the singular varicose TH-IR nerve terminals were observed within ganglia. These findings are consistent with the previous reports that did not show the immunoreactivity for TH in the INCs in the guinea-pig heart and the primary culture of the guinea pig atrial neurons (Leger et al., 1999). The release of the catecholamines from the SIF cells of the heart may be an alternative explanation for intrinsically mediated cardioaugmentation (Leger et al., 1999). In our study, the morphology of the rabbit atrial TH-IR cells were similar to the SIF cells in the ICG and the ganglia of the sympathetic chain of the other animal species (Heym et al., 1994; Cheng et al., 1997; Leger et al., 1999; Rysevaite et al., 2011). The SIF cells are unevenly distributed within rabbit atria. Most of them are clustered within ganglia. The SIF cells have the tendency to group together, and occurr in relatively small clusters. The four or five SIF cells grouped together may have the appearance of the TH-IR neuronal somata.

The goal of the present study was to prove that the extrinsic rabbit cardiac nerves entering heart base were composed of the TH-IR nerve fibres Previous studies have reported that the numerous ChAT and TH immunoreactive nerve fibres were within the accessing nerves of the mouse heart base (Rysevaite et al., 2011). However, in accordance with the present data, the epicardiac TH-IR nerve fibres were predominant in the left atrium, whereas the ChAT-IR nerve fibres proceeded on the heart base toward the large intrinsic ganglia and on the epicardium of the root of the RCV (Rysevaite et al., 2011).

Previously it has been reported that the adrenergic nerve plexus of the mammalian heart play the crucial role in the regulation of the coronary blood flow as well as in the processes of the neural inactivation of the catecholamines (Shvaliov et al., 1982; Zong et al., 2004). In contrast to the other mammalian species, the rabbits often suffer from the heart attack that it caused by a spasm of a coronary artery evoked by scare. The deficit of adrenergic cardiac innervation and the attenuation of the processes of the neural inactivation of catecholamines are accompanied by an increase in the sensitivity of myocardium and coronary arteries to the epinephrine (Shvalev et al., 1982). It is known that the sensitivity of the myocardium and the smooth-muscle cells of the coronary vessels to catecholamines released by the adrenal medulla after experimental cardiac desympathization increases (Shvalev et al., 1982). In the present study, we did not estimate the density of the adrenergic nerve plexus and did not compare with other species. In contrast to the observations in the human, rat and mouse hearts (Richardson et al., 2003; Hoover et al., 2009; Rysevaite et al., 2011), the immunoreactivity for TH was not observed in the somata of the ICNs and in the baskets of varicose nerve terminals that surrounded the neuronal somata. Although, the singular varicose TH-IR nerve terminals were observed within ganglia.

This study provides detailed morphologic data on the neurochemical phenotype of the epicardiac nerve ganglionated plexus of the rabbit heart. Our data indicate that (1) the majority of the neurons found in the rabbit atria were immunoreactive for ChAT and these neurons are cholinergic; (2) cardiac ganglia contain numerous biphenotype nNOS-IR + ChAT-IR, small population of nNOS-IR neurons and single immunoreactive for nNOS fibres, that did not form complexes with neurons within ganglia; (3) the singular varicose TH-IR nerve terminals were observed within ganglia but the TH-IR was not observed in the somata of the ICNs; (4) the extrinsic cardiac nerves entering the rabbit heart base were predominantly composed of the TH-IR nerve fibres; (5) the sparse ChAT-IR nerve fibres proceeded on the heart base, spread toward the large intrinsic ganglia and on the epicardium of the root of the RCV. We concluded that the lack of the TH-IR ICNs within ganglia and lack of the mesh-work of the extrinsic nNOS-IR nerve fibres within the rabbit epicardiac ganglionated nerve plexus supports the hypothetic increase of reactivity to the rabbit coronary vessels to the effects of the catecholamines released by the adrenal medulla during the stress.

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