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IMMUNOHISTOCHEMICAL CHARACTERIZATION OF THE INTRINSIC CARDIAC NEURAL PLEXUS IN WHOLE-MOUNT MOUSE HEART PREPARATIONS

Kristina Rysevaite¹, Inga Saburkina¹, Neringa Pauziene¹, Raimundas Vaitkevicius¹, Sami F. Noujaim², José Jalife², and Dainius H. Pauza^{1,*}

¹Institute of Anatomy, Lithuanian University of Health Sciences, Lithuania

²Center for Arrhythmia Research, University of Michigan, USA

Summary

BACKGROUND—The intrinsic neural plexus of the mouse heart has not been adequately investigated despite the extensive use of this species in experimental cardiology.

OBJECTIVE—We determined the distribution of cholinergic, adrenergic and sensory neural components in whole-mount mouse heart preparations using double immunohistochemical labeling.

METHODS AND RESULTS—Intrinsic neurons were concentrated within 19 ± 3 ganglia ($n = 20$ mice) of varying size, scattered on the medial side of the inferior caval (caudal) vein on the right atrium and close to the pulmonary veins on the left atrium. Of a total of 1082 ± 160 neurons, most somata (83%) were choline acetyltransferase (ChAT)-immunoreactive, while 4% were tyrosine hydroxylase (TH)-immunoreactive; 14% of ganglionic cells were biphenotypic for ChAT and TH. The most intense ChAT staining was observed in axonal varicosities. ChAT was evident in nerve fibers interconnecting intrinsic ganglia. Both ChAT and TH immunoreactivity were abundant within the nerves accessing the heart. However, epicardial TH-immunoreactive nerve fibers were predominant on the dorsal and ventral left atrium, whereas most ChAT-positive axons proceeded on the heart base toward the large intrinsic ganglia and on the epicardium of the root of the right cranial vein. Substance P-positive and calcitonin gene-related peptide-immunoreactive nerve fibers were abundant on the epicardium and within ganglia adjacent to the heart hilum. Small intensely fluorescent cells were grouped into clusters of 3–8 and dispersed within large ganglia or separately on the atrial and ventricular walls.

CONCLUSIONS—While some nerves and neuronal bundles of the mouse epicardial plexus are mixed, most express either adrenergic or cholinergic markers. Therefore, selective stimulation and/or ablation of the functionally distinct intrinsic neural pathways should allow the study of specific effects on cardiac function.

Keywords

Heart; intrinsic cardiac neural plexus; autonomic ganglia and nerves; sinuatrial and atrioventricular nodes; tyrosine hydroxylase; choline acetyltransferase; substance P; calcitonin gene related peptide; mouse

*Correspondence to: Prof. Dainius H. Pauza, Institute of Anatomy, Lithuanian University of Health Sciences, A. Mickevieciaus Street 9, Kaunas LT-44307, Lithuania, dainius.pauza@kmu.lt, Phone: + 370 682 39366.

No Conflicts

Introduction

The intrinsic ganglionated cardiac plexus integrates input from multiple sources including vagal efferent and afferent neurons, extrinsic adrenergic and spinal sensory neurons. A balance between the stimulatory sympathetic and inhibitory parasympathetic inputs is crucial for the control of cardiac function^{1–3}. Intrinsic cholinergic neurons are primarily located within the atrial epicardial ganglia. They contain choline acetyltransferase (ChAT)⁴, an enzyme required for the synthesis of acetylcholine. Acetylcholine released by postganglionic cholinergic axons directly affects the membrane potential of atrial, sinuatrial, AV nodal, and His-Purkinje cells by activating the acetylcholine sensitive inward rectifier potassium current ($I_{K,ACH}$) through the G protein coupled receptors. In addition, acetylcholine has prejunctional inhibitory effects on adrenergic axons⁵. In contrast, adrenergic neurons, whose axons innervate the heart, co-reside with non-cardiac adrenergic neurons in the superior and middle cervical, mediastinal, and stellate ganglia within the chest cavity⁶. These adrenergic neurons contain tyrosine hydroxylase (TH), which is required for the synthesis of norepinephrine⁷. TH has been identified within intrinsic cardiac neurons (ICNs) of adult rats, guinea pigs and humans^{8,9,12–14,27}.

To define the sensory neuronal subpopulations, recent anatomical investigations relied on immunohistochemical assessment of the calcitonin gene-related peptide (CGRP) and substance P (SP). These neuropeptides have been employed to identify the peptidergic class of nociceptors, although CGRP is clearly expressed in some non-nociceptive neurons as well^{15–17}. Even though SP is considered to be a pain transmitter, receptors for this neuropeptide are expressed not only in neurons, but also on the surface of cardiomyocytes and endothelial cells, as well as lymphocytes and macrophages^{18–21}. Furthermore, it has been demonstrated that SP depolarizes cardiac neurons, triggers action potentials of intrinsic cardiac neurons, contributes to dilated cardiomyopathy and is essential for the pathogenesis of encephalomyocarditis and viral myocarditis^{22,23,47}. Further, CGRP and SP play counter-regulatory roles in hypertension and coronary flow²³.

The mouse heart is widely used as an experimental model to investigate the effects of modifying specific genes involved in cardiac development and function²⁴. Yet the consequences of such genetic modifications on neural control of heart function have not been adequately studied. It has been reported that the intrinsic cardiac neurons of adult mice and humans are comparable. They both exhibit a similar neurochemical phenotype manifested predominantly by ChAT and TH⁴. However, in contrast to the human heart, the neuroanatomy of the mouse heart has been poorly examined to date. Here we identified the course of cholinergic, adrenergic and peptidergic nerve fibers in the mouse heart using correspondent immunofluorescence labeling for ChAT, TH, SP and CGRP in whole-mount heart preparations that allow both transmural and expanded examinations of the intact intrinsic cardiac nerve plexus.

Methods

Animals

Twenty young mice of either gender were used to examine the intrinsic cardiac neural plexus in whole-mount preparations of the atria and ventricles. We used immunofluorescence labeling for ChAT, TH, SP and CGRP (see Online Supplement, OLS, Table 1 for antibodies used and microscopic examination as described in the OLS).

Statistical analyses

Data are expressed as mean $\pm$ standard error. The letter “n” represents the number of samples. Linear regression was used to quantify the relationship between the number of

neurons in a given ganglion and the ganglion area (see Fig. 2). Statistical analysis of neuron soma size was performed with both the paired and independent Student's tests using Origin software (v. 6.1, OriginLab Corporation, Northampton, MA, USA). Significance was considered at $p < 0.05$.

Results

Distribution of immunochemically distinct intrinsic cardiac ganglia and neurons

The location of ganglionic cells positive for ChAT and/or TH was reproducible from animal to animal. Intrinsic cardiac ganglia (ICG) varied in shape and size, and were mostly located at the limits of the venous part of the heart hilum, consistently near the vicinity of pulmonary vein roots (Fig. 1). Very few solitary ganglia were distributed epicardially on the dorsal side of the left azygos vein root and close to the roots of the aorta and pulmonary trunk. The ICG situated around the roots of three pulmonary veins were regularly interconnected by thin nerves that formed a ring-like ganglionated neural chain thread. Some ganglia in this chain were connected with commissural nerves that extended across the chain (Fig. 1). Since most of the ICG had clear borders, an assessment of the ganglion number was simple and reliable. The whole-mount mouse heart preparations had 11 to 30 ICG that were very variable in size. The smallest ICG could involve only 3 neuronal somata, whereas the largest ICG enclosed up to 700 neurons. On average, a mouse cardiac ganglion contained 87 ± 9 neurons (Table 1). We observed individual intrinsic neurons as well (Table 1). They were usually attached to epicardial nerves extending along the root of the left azygos vein and/or on the ventral side of the right cranial vein.

Based on the direct interdependence of the ganglion area and the number of neurons (Fig. 2), the total number of intrinsic cardiac neurons in the mouse was estimated to be 1082 ± 160 (Table 1).

ChAT-IR neurons

ChAT immunoreactivity (IR) was characteristic of 83% of the neurons identified within the intrinsic cardiac ganglia (Table 2). These cholinergic neurons were surrounded by baskets of varicose neural terminals which expressed ChAT more intensely than the perinuclear regions of cholinergic ganglionic cells (Fig. 3b, c, e, f). The ChAT immunoreactive nerve fibers were evident in the nerves which accessed the heart and those which interconnected the large ganglia located at the root of the pulmonary veins (Fig. 1). The ChAT positive neuronal somata varied in size and shape (Figs. 1, 3). Their long axis was $20 \pm 0.3 \mu\text{m}$, but occasionally, they could be larger ($31.9 \mu\text{m}$) or smaller ($12.9 \mu\text{m}$) (Table 2).

TH-IR neurons and small intensively fluorescent (SIF) cells

We detected neurons that were immunoreactive for TH only. We also found ChAT positive neurons that were immunoreactive for TH (Fig. 3a–f). The biphenotypic neurons were ubiquitously present and amounted to about 14% of all ganglionic cells (Table 2, Figs. 1, 3). All the TH-IR neurons usually had neuronal processes and were positive for PGP 9.5, regardless of size (Fig. 3g–i). Compared with the purely ChAT-IR neurons, the TH immunoreactivity was evenly distributed throughout the neuronal body. However, TH reactivity varied among individual neurons and was not dependent on the neuron location inside the ganglion. Commonly, weakly positive TH neurons were significantly larger than cholinergic neurons (Table 2). The purely positive TH neurons were relatively rare compared to other intrinsic cardiac neuron types (Table 2). However, the purely TH positive neurons were considerably larger than the cholinergic and biphenotypic counterparts (Table 2).

We also observed small intensely fluorescent cells (SIF; Fig. 3a–c). These cells displayed very strong TH-IR and were smaller than other TH-IR nerve cells. These cells were grouped into small clusters of 3–8 cells and were dispersed within large ganglia or separately on the atrial and ventricular walls. Commonly, SIF cells were more frequent on the left atrium close to the roots of the pulmonary veins and at the trunk of the left coronary artery.

Distribution of ChAT-IR and TH-IR nerve fibers

Extrinsic nerves access the mouse heart on the arterial and venous sections of the heart hilum (HH, Fig. 1). In the venous part, the accessing nerves were mainly concentrated in two locations, i.e., at the medial side of the right cranial vein root and on the anterior side of the left cranial (azygos) vein root (Fig. 1). At the boundary of the HH, the accessing nerves formed a chain-like ganglionated neural plexus with nerve extensions that passed epicardially to the atria and ventricles (Fig. 1). The heart accessing nerves were predominantly TH-IR, but some ChAT-IR fibers were present as well. In the arterial part of the HH, the TH-IR and ChAT-IR axons inside the accessing nerves entered the heart on the left side between the aorta and the pulmonary trunk (Fig. 1) and extended epicardially to the left ventricular wall. In the epicardial neural plexus, the nerves were mixtures of ChAT-IR and TH-IR axons. However, the TH-IR axons were more abundant than ChAT-IR axons in the epicardial nerves traveling along the left cranial (azygos) vein, coronary sinus and on the anterior sides of the atria and ventricles.

These heart preparations showed substantial regional differences in the density of ChAT-IR nerve fibers. Both the sinuatrial and atrioventricular node regions contained meshworks of ChAT-IR nerve fibers that were particularly dense and extended beyond the typical sites of these nodes (Figs 1 and 4). The sinuatrial nodal neural meshwork extended to the anterior, posterior and even medial side of the right cranial vein root, while the atrioventricular nodal meshwork occupied the entire lower part of the interatrial septum and proceeded along the possible location of the His bundle and its right and left branches within the interventricular septum (Figs 1 and 4). The density of the ChAT-IR neural meshwork close to the sinuatrial node region was not equal everywhere on the right cranial vein root. However it was noticeably greater than in the surrounding atrial walls. It was evident, moreover, that ChAT-IR nerve fibers dominated the nerves interconnecting the chain of intrinsic cardiac ganglia (ICG) on the heart base (Fig. 1). The wall of the left atrium was distinguished by a lower density of ChAT-IR nerves compared to the right atrial wall (Fig. 1). In the myocardial layer, there were numerous varicosities and spine-like processes in the preterminal and terminal parts of both ChAT-IR and TH-IR axons (Fig. 5a–c). Nevertheless, it was clear that some axonal terminals contained a mixture of neurotransmitters; i.e., they were immunoreactive for both TH and ChAT (Fig. 5a–c). Interestingly, such biphenotypic axons were not identified within the epicardial or myocardial nerves and neural bundles, in which TH-IR axons proceeded in parallel with, but separately from ChAT-IR axons (Fig. 5a–c).

Distribution of SP-IR and CGRP-IR nerve fibers

SP-IR and CGRP-IR nerve fibers were the most abundant in the mouse epicardium and in ganglia adjacent to the HH. The SP-IR and CGRP-IR nerve fibers were thin and generally identified within the mixed nerves and nerve bundles together with ChAT- and TH-IR nerve fibers (Fig. 5d–f). Frequently, both the SP- and CGRP-IR nerve fibers were situated near blood vessels of varying sizes. We failed to observe neuronal somata that were even dimly immunoreactive to SP and/or CGRP. Inside the ganglia, SP- and CGRP-IR nerve fibers contained numerous varicosities and appeared as if in contact with ganglionic cells (Fig. 5g–i). On the other hand, many SP- and CGRP-IR nerve fibers simply passed through the ganglia without having any specialized ending. SP- and CGRP-IR nerve fibers were also identified within the heart accessing nerves, intrinsic nerve bundles and interganglionic

nerves that passed between intrinsic cardiac ganglia adjacent to the pulmonary vein roots. Within the myocardial neural network, the SP- and CGRP-IR nerve fibers were considerably rarer than cholinergic and adrenergic fibers, but they appeared to proceed jointly within the common myocardial nerve bundles (Fig. 5d–f).

Discussion

This study demonstrates the distribution of cholinergic, adrenergic and peptidergic (putative sensory) nerve fibers and neurons in whole-mount preparation of the mouse heart. The technique of whole-mount preparation allowed us to precisely identify and map all the intrinsic cardiac ganglia in this species. We also classified their immunohistochemical properties and the interconnections of their neurons in the atria and the interventricular septum.

As expected, in the mouse heart most of the intrinsic neurons are cholinergic. This confirms the classical point of view that the intrinsic cardiac neurons are mainly cholinergic and, therefore, they play an inhibitory role in cardiac regulation. Prior immunohistochemical studies have indicated that all intrinsic cardiac neurons are exclusively immunoreactive to ChAT in the guinea pig^{10,13}, rat¹¹ and mouse²⁶. Despite the fact that most neurons observed in our study showed immunoreactivity for a cholinergic marker, 13% of neurons were nevertheless positive for both TH and ChAT; i.e. they were biphenotypic. The TH patterns of fluorescence in the somata of these neurons did not overlap with those of ChAT. Therefore, the distribution of these particular neurons was reliably assessed within the intrinsic ganglia. Our finding of biphenotypic neurons in the mouse heart is consistent with studies on tissue sections and cultured intrinsic neurons derived from mouse and human hearts^{5,26,27}. In addition, we report for the first time in the mouse heart the presence of a population of intrinsic neurons that are exclusively positive for TH. It should be noted that isolated rat intracardiac neurons were found to be responsive to noradrenaline application in vitro^{28,29}. Therefore, the adrenergic input on some cholinergic neurons in the heart needs to be addressed in the future.

We have identified biphenotypic TH- and ChAT-IR axons in the mouse atrial myocardium. Interestingly, immunochemically distinct axons contained numerous varicosities and were closely juxtaposed with each other up to their terminals. At the terminals, TH- and ChAT-IR axons usually split and petered out on the target cells with button and spine like endings. Based on the present observations, it is evident that TH is colocalized with ChAT in neuronal somata, as well as in most terminal parts of the axons. This is in sharp contradiction with the conclusion reached by^{26,27}, who proposed that TH is synthesized by a certain population of intrinsic cardiac neurons but is not transported to their processes.

In contrast to histological sections, the whole-mount heart preparation enables transmural examination of the entire intrinsic nerve plexus. Using this technique, we show that the density of the intrinsic nerves and fibers is different in distinct heart regions. ChAT-IR fibers are especially densely distributed in the sinuatrial node region, the right cranial vein root, and the interatrial septum, at the site of the atrioventricular node. The dense innervation of the sinuatrial node was demonstrated earlier by histochemical staining of acetylcholinesterase in mouse, rat, rabbit, dog, pig, human^{30–33} as well as by immunohistochemical staining of ChAT^{34–35} and choline transporters in mouse and human^{4,34,35}. These morphological findings correlate well with physiological studies showing that vagal nerve stimulation activates the cholinergic input to the sinuatrial node, causing a decrease in the heart rate³⁶.

TH and ChAT staining in rabbit atrioventricular node sections revealed a heterogeneous distribution³⁷. However, no morphological data have been published to date about the innervation of the intact mouse atrioventricular node. Here we found an extremely dense and wide neural meshwork in the vicinity of the atrioventricular node, which extended beyond its typical anatomical location. This is in line with recent reports in laboratory animals, including mouse^{38–41}. Presumably, all conductive cardiomyocytes distributed widely on the lower interatrial septum are under extensive control of efferent nerve fibers that make up the meshwork we have described here.

Yasuhara and colleagues³⁵ demonstrated that the rat ventricular myocardium contains a small number of ChAT-IR fibers. Coronary blood vessels in the ventricular myocardium are also supplied by ChAT-IR fibers. Our observations confirm that the innervation of the mouse ventricles is by ChAT-IR nerve fibers that are always accompanied by TH-IR fibers. Interestingly, vagal stimulation decreases ventricular contractility in dogs⁴², pigs and humans⁴³, but does not affect ventricular contractility in rats⁴⁴ or guinea pigs³⁴. It is possible that the cholinergic innervation in ventricular myocardium varies significantly between species.

The SP- and CGRP-IR nerve fibers observed in the mouse cardiac ganglia exhibited varicosities and free endings, but never showed specific pericellular baskets on or next to other cardiac cells, including intrinsic neurons. We were unable to perform double labeling of these antigens. However, we noticed the predominance of CGRP-IR compared to SP nerve fibers. In the rat heart, SP and CGRP colocalized in nerve fibers distributed inside intrinsic ganglia^{8,11,12,45,46}.

We have described and characterized the cholinergic, adrenergic and biphenotypic intrinsic neurons of the mouse heart. Adrenergic, cholinergic and peptidergic (SP- and CGRP-IR) nerve fibers extend to their targets in mixed nerves and nerve bundles, but the mouse intrinsic cardiac neural plexus contains numerous nerves that contain either adrenergic or cholinergic axons. Studies such as ours, which map in detail the distribution of the intrinsic cardiac nerve fibers, may help in guiding attempts to selectively stimulate and/or ablate functionally distinct intrinsic neural pathways in the arrhythmic heart.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Ardell JL, Butler CK, Smith FM, Hopkins DA, Armour JA. Activity of in vivo atrial and ventricular neurons in chronically decentralized canine hearts. *Am J Physiol.* 1991; 260:H713–H721. [PubMed: 2000967]
2. Pauza DH, Skripkiene G, Skripka V, Pauziene N, Stropus R. Morphological study of neurons in the nerve plexus on heart base of rats and guinea pigs. *J Auton Nerv Syst.* 1997; 62:1–12. [PubMed: 9021644]
3. Smith FM. Extrinsic inputs to intrinsic neurons in the porcine heart in vitro. *Am J Physiol.* 1999; 276:R455–R467. [PubMed: 9950925]

4. Mabe AM, Hoard JL, Duffourc MM, Hoover DB. Localization of cholinergic innervation and neurturin receptors in adult mouse heart and expression of the neurturin gene. *Cell Tissue Res.* 2006; 326:57–67. [PubMed: 16708241]
5. Weihe E, Schutz B, Hartschuh W, Anlauf M, Schafer MK, Eiden LE. Coexpression of cholinergic and noradrenergic phenotypes in human and nonhuman autonomic nervous system. *J Comp Neurol.* 2005; 492:370–379. [PubMed: 16217790]
6. Richardson RJ, Grkovic I, Allen AM, Anderson CR. Separate neurochemical classes of sympathetic postganglionic neurons project to the left ventricle of the rat heart. *Cell Tissue Res.* 2006; 324:9–16. [PubMed: 16418838]
7. Maslyukov PM, Shilkin VV, Timmermans JP. Immunocytochemical characteristics of neurons in the stellate ganglion of the sympathetic trunk in mice during postnatal ontogenesis. *Neurosci Behav Physiol.* 2006; 36:851–855. [PubMed: 16964463]
8. Forsgren S, Moravec M, Moravec J. Catecholamine-synthesizing enzymes and neuropeptides in rat heart epicardial ganglia; an immunohistochemical study. *Histochem J.* 1990; 22:667–676. [PubMed: 1706694]
9. Steele PA, Gibbins IL, Morris JL, Mayer B. Multiple populations of neuropeptide-containing intrinsic neurons in the guinea-pig heart. *Neuroscience.* 1994; 62:241–250. [PubMed: 7816202]
10. Leger J, Croll RP, Smith FM. Regional distribution and extrinsic innervation of intrinsic cardiac neurons in the guinea pig. *J Comp Neurol.* 1999; 407:303–317. [PubMed: 10320213]
11. Richardson RJ, Grkovic I, Anderson CR. Immunohistochemical analysis of intracardiac ganglia of the rat heart. *Cell Tissue Res.* 2003; 314:337–350. [PubMed: 14523644]
12. Moravec M, Moravec J, Forsgren S. Catecholaminergic and peptidergic nerve components of intramural ganglia in the rat heart. An immunohistochemical study. *Cell Tissue Res.* 1990; 262:315–327. [PubMed: 1706221]
13. Horackova M, Armour JA, Byczko Z. Distribution of intrinsic cardiac neurons in whole-mount guinea pig atria identified by multiple neurochemical coding. A confocal microscope study. *Cell Tissue Res.* 1999; 297:409–421. [PubMed: 10460488]
14. Horackova M, Slavikova J, Byczko Z. Postnatal development of the rat intrinsic cardiac nervous system: a confocal laser scanning microscopy study in whole-mount atria. *Tissue Cell.* 2000; 32:377–388. [PubMed: 11201277]
15. Lawson SN, Perry MJ, Prabhakar E, McCarthy PW. Primary sensory neurones: neurofilament, neuropeptides, and conduction velocity. *Brain Res Bull.* 1993; 30:239–243. [PubMed: 7681350]
16. Lawson SN, McCarthy PW, Prabhakar E. Electrophysiological properties of neurones with CGRP-like immunoreactivity in rat dorsal root ganglia. *J Comp Neurol.* 1996; 365:355–366. [PubMed: 8822175]
17. Lawson SN, Crepps B, Perl ER. Calcitonin gene-related peptide immunoreactivity and afferent receptive properties of dorsal root ganglion neurones in guinea-pigs. *J Physiol.* 2002; 540:989–1002. [PubMed: 11986384]
18. Cook GA, Elliott D, Metwali A, et al. Molecular evidence that granuloma T lymphocytes in murine schistosomiasis *mansoni* express an authentic substance P (NK-1) receptor. *J Immunol.* 1994; 152:1830–1835. [PubMed: 8120392]
19. Church DJ, Arkinstall SJ, Vallotton MB, Chollet A, Kawashima E, Lang U. Stimulation of atrial natriuretic peptide release by neurokinins in neonatal rat ventricular cardiomyocytes. *Am J Physiol.* 1996; 270:H935–H944. [PubMed: 8780188]
20. Ho WZ, Lai JP, Zhu XH, Uvaydova M, Douglas SD. Human monocytes and macrophages express substance P and neurokinin-1 receptor. *J Immunol.* 1997; 159:5654–5660. [PubMed: 9548509]
21. Goode T, O'Connell J, Sternini C, et al. Substance P (neurokinin-1) receptor is a marker of human mucosal but not peripheral mononuclear cells: molecular quantitation and localization. *J Immunol.* 1998; 161:2232–2240. [PubMed: 9725216]
22. D'Souza M, Garza MA, Xie M, Weinstock J, Xiang Q, Robinson P. Substance P is associated with heart enlargement and apoptosis in murine dilated cardiomyopathy induced by *Taenia crassiceps* infection. *J Parasitol.* 2007; 93:1121–1127. [PubMed: 18163347]
23. Robinson P, Garza A, Moore J, et al. Substance P is required for the pathogenesis of EMCV infection in mice. *Int J Clin Exp Med.* 2009; 2:76–86. [PubMed: 19436834]

24. Feintuch A, Zhu Y, Bishop J, et al. 4D cardiac MRI in the mouse. *NMR Biomed.* 2007; 20:360–365. [PubMed: 17451168]
25. Dent JA, Polson AG, Klymkowsky MW. A whole-mount immunocytochemical analysis of the expression of the intermediate filament protein Vimentin in *Xenopus*. *Development.* 1989; 105:61–74. [PubMed: 2806118]
26. Hoard JL, Hoover DB, Wondergem R. Phenotypic properties of adult mouse intrinsic cardiac neurons maintained in culture. *Am J Physiol Cell Physiol.* 2007; 293:C1875–C1883. [PubMed: 17913847]
27. Hoard JL, Hoover DB, Mabe AM, Blakely RD, Feng N, Paolucci N. Cholinergic neurons of mouse intrinsic cardiac ganglia contain noradrenergic enzymes, norepinephrine transporters, and the neurotrophin receptors tropomyosin-related kinase A and p75. *Neuroscience.* 2008; 156:129–142. [PubMed: 18674600]
28. Xu ZJ, Adams DJ. Alpha-adrenergic modulation of ionic currents in cultured parasympathetic neurons from rat intracardiac ganglia. *J Neurophysiol.* 1993; 69:1060–1070. [PubMed: 8098358]
29. Ishibashi H, Umezumi M, Jang IS, Ito Y, Akaike N. Alpha 1-adrenoceptor-activated cation currents in neurones acutely isolated from rat cardiac parasympathetic ganglia. *J Physiol.* 2003; 548:111–120. [PubMed: 12598585]
30. Kent KM, Epstein SE, Cooper T, Jacobowitz DM. Cholinergic innervation of the canine and human ventricular conducting system. Anatomic and electrophysiologic correlations. *Circulation.* 1974; 50:948–955. [PubMed: 4430098]
31. Crick SJ, Wharton J, Sheppard MN, et al. Innervation of the human cardiac conduction system. A quantitative immunohistochemical and histochemical study. *Circulation.* 1994; 89:1697–1708. [PubMed: 7908612]
32. Crick SJ, Sheppard MN, Anderson RH, Polak JM, Wharton J. A quantitative study of nerve distribution in the conduction system of the guinea pig heart. *J Anat.* 1996; 188 (Pt 2):403–416. [PubMed: 8621340]
33. Petrecca K, Shrier A. Spatial distribution of nerve processes and beta-adrenoreceptors in the rat atrioventricular node. *J Anat.* 1998; 192 (Pt 4):517–528. [PubMed: 9723979]
34. Hoover DB, Ganote CE, Ferguson SM, Blakely RD, Parsons RL. Localization of cholinergic innervation in guinea pig heart by immunohistochemistry for high-affinity choline transporters. *Cardiovasc Res.* 2004; 62:112–121. [PubMed: 15023558]
35. Yasuhara O, Matsuo A, Bellier JP, Aimi Y. Demonstration of choline acetyltransferase of a peripheral type in the rat heart. *J Histochem Cytochem.* 2007; 55:287–299. [PubMed: 17142806]
36. Randall WC, Ardell JL, Becker DM. Differential responses accompanying sequential stimulation and ablation of vagal branches to dog heart. *Am J Physiol.* 1985; 249:H133–H140. [PubMed: 4014479]
37. Hucker WJ, Nikolski VP, Efimov IR. Autonomic control and innervation of the atrioventricular junctional pacemaker. *Heart Rhythm.* 2007; 4:1326–1335. [PubMed: 17905339]
38. Efimov IR, Nikolski VP, Rothenberg F, et al. Structure-function relationship in the AV junction. *Anat Rec A Discov Mol Cell Evol Biol.* 2004; 280:952–965. [PubMed: 15368340]
39. Hou Y, Scherlag BJ, Lin J, et al. Ganglionated plexi modulate extrinsic cardiac autonomic nerve input: effects on sinus rate, atrioventricular conduction, refractoriness, and inducibility of atrial fibrillation. *J Am Coll Cardiol.* 2007; 50:61–68. [PubMed: 17601547]
40. Anderson RH, Yanni J, Boyett MR, Chandler NJ, Dobrzynski H. The anatomy of the cardiac conduction system. *Clin Anat.* 2009; 22:99–113. [PubMed: 18773472]
41. Yanni J, Boyett MR, Anderson RH, Dobrzynski H. The extent of the specialized atrioventricular ring tissues. *Heart Rhythm.* 2009; 6:672–680. [PubMed: 19328044]
42. Levy MN.; Warner, MR. Parasympathetic effects on cardiac function. In: Armour, JA.; Ardell, JL., editors. *Neurocardiology*. New York: Oxford University Press; 1994. p. 53-76.
43. Lewis ME, Al-Khalidi AH, Bonser RS, et al. Vagus nerve stimulation decreases left ventricular contractility in vivo in the human and pig heart. *J Physiol.* 2001; 534:547–552. [PubMed: 11454971]

44. Takahashi H, Maehara K, Onuki N, Saito T, Maruyama Y. Decreased contractility of the left ventricle is induced by the neurotransmitter acetylcholine, but not by vagal stimulation in rats. *Jpn Heart J.* 2003; 44:257–270. [PubMed: 12718487]
45. Onuoha GN, Alpar EK, Chukwulobelu R, Nicholls DP. Distributions of VIP, substance P, neurokinin A and neurotensin in rat heart: an immunocytochemical study. *Neuropeptides.* 1999; 33:19–25. [PubMed: 10657467]
46. Onuoha GN, Alpar EK, Nicholls DP, Buchanan KD. Calcitonin gene-related peptide, neuropeptide Y and atrial natriuretic peptide distribution in guinea pig heart from paraffin wax-embedded and formalin-cryoprotected tissues. *Histochem J.* 1999; 31:617–621. [PubMed: 10579631]
47. Hoover DB, Tompkins JD, Parsons RL. Differential activation of guinea pig intrinsic cardiac neurons by the PAC1 agonists maxadilan and pituitary adenylate cyclase-activating polypeptide 27 (PACAP27). *J Pharmacol Exp Ther.* 2009; 33:197–203. [PubMed: 19602551]

Fig. 1.

Whole-mount preparation. Both atrial appendages, pulmonary vein roots, and the major part of the ventricles were dissected in order to flatten the atria. ChAT-IR or cholinergic neural structures are labeled red, TH-IR or sympathetic structures are green. Arrows indicate the extrinsic nerves accessing the mouse heart, whereas arrowheads point to intrinsic ganglia. The boxed areas in panel **a** are enlarged in the insets to illustrate: (b) the predominance of ChAT-IR ganglionic cells; (c) the small ganglia, and single neurons; (d) the nerves accessing the heart on the anterior side of the left cranial vein root in which the TH-IR nerve fibers predominate compared to neighboring solitary ChAT-IR nerve fibers; (e) the nerves accessing the heart on the medial side of the right cranial vein root with absolute predominance of TH-IR nerve fibers; (f) the considerable difference in density of the cholinergic neural meshwork on the right cranial vein root (RCV) compared with the rest of right atrial wall; (g) the occurrence of ChAT-IR fibers together with TH-IR fibers in epicardial nerves. *Abbreviations:* **PT** - orifice of pulmonary trunk, **AO** – orifice of aorta, **RCV** – orifice of right cranial vein, **CV** – orifice of caudal vein, **LCV** - orifice of left cranial vein, **PVs** – orifice of left ant middle pulmonary veins, **RPV** – orifice of right pulmonary vein.

Fig. 2. The number of neurons plotted against the area of 132 ganglia from 15 mouse hearts. The straight line indicates the linear regression of the plotted data. Each point corresponds to one of the analyzed ganglia (some points overlap in the graph). R – correlation coefficient at $P < 0.0001$.

Fig. 3.

a–c: Microphotographs illustrating the predominance of ChAT-IR ganglionic cells inside a ganglion that contains also the biphenotypic (arrowheads) and small intensively fluorescent (SIF) (arrows) cells immunoreactive to TH. Note that immunostaining for ChAT (**b**) does not exhibit the SIF cells (arrows), while only the double-channel fluorescence of simultaneous immunostainings for TH and ChAT (**c**) reveals reliably the biphenotypic neurons (arrowheads) that were dimly seen in mono-channel fluorescence applied to display immunostaining for TH (**a**); **d–f:** Microphotographs demonstrating three types of neurons in small ganglion of the mouse heart identified applying mono-channel (**d** and **e**) and double-channel (**f**) fluorescence. Note the baskets of ChAT-IR neural terminals that surround the neurons and contain numerous varicosities. White crosses indicate the neuron that is exclusively positive to TH, asterisks - neurons positive only to ChAT, and diamonds - two biphenotypic nerve cells; **g–i:** Microphotographs confirming the neuronal origin of all ganglionic cells and showing the diversity of sizes and staining intensities of TH-IR neurons located within one ganglion. Note the intensely (diamonds) and weakly (crosses) stained TH-IR neurons, which are positive for the general neuronal marker PGP 9.5.

Fig. 4.

Whole-mount preparation of the interatrial (**a–c**) and interventricular (**d–f**) septa illustrating the pathways of intrinsic nerves proceeding toward the atrioventricular node region (**a**) as well as along the His bundle and its right branch (**d**). Note the predominance of ChAT-IR fibers in the region of the atrioventricular node, at His bundle and its right branch. The nerves (arrowheads) from epicardial ganglia (solid white arrow) extend toward the atrioventricular node and form the dense neural meshwork therein. The boxed areas in panel **a** are enlarged as the insets in **b** and **c**, while ones in panel **d** – as the insets in **e** and **f**. Double arrowheads in **a** and **b** point out the solitary ChAT-IR neuron located beneath the endocardium of the interatrial septum. Asterisks in **d** track the blood vessels in the interventricular septum. *Abbreviations:* **FO** – fossa ovalis, **AVN** - atrioventricular node region, **CS** – orifice of coronary sinus.

Fig. 5.
a–c: Microphotographs showing the myocardial preterminal and terminal aspects of the ChAT-IR and TH-IR axons that contain numerous varicosities and spine-like processes. Arrowheads indicate the ChAT-IR axons, while arrows - the axons with a mixture of TH and ChAT; **d–i:** Microphotographs illustrating SP-IR nerve fibers in close vicinity to atrial myocytes (**d–f**) and CGRP-IR nerve fibers between the ChAT positive ganglionic cells (**g–i**). Note the numerous varicosities and button-like terminal endings of the CGRP-IR nerve fibers at ChAT-IR nerve cells. In **d–f** panels, asterisks point to two atrial myocytes, in which myofibril striations are seen, while in **g–i** panels, double arrowheads – the varicosities of CGRP-IR nerve fiber.

Table 1

The mean number and range of neurons located inside an intrinsic ganglion, solitary intrinsic neurons and the total number of intrinsic cardiac neurons identified in 13 whole-mount mouse heart preparations

Parameter	On average	Range
Neuronal number per ganglion	87 ± 9	3 – 678
Number of solitary neurons per heart	19 ± 3	8 – 30
The approximate neuronal number per heart*	102 ± 160	600 – 1938

* Approximations were based on a strong correlation between ganglion area and neuronal number shown on Fig. 3.

The number, percentage and size of the immunohistochemically distinct intrinsic cardiac neurons identified in whole-mount preparations

Table 2

	ChAT – IR			TH – IR			Biphenotypic (ChAT +TH –IR)		
	<i>n</i>	Mean $\pm$ SD	Range	<i>n</i>	Mean $\pm$ SD	Range	<i>n</i>	Mean $\pm$ SD	Range
<i>Number of neurons per one heart</i>	13	583 $\pm$ 99	292 – 969	13	25 $\pm$ 5	10 – 45	13	97 $\pm$ 36	47 – 279
<i>Percentage of neurons per one heart</i>	13	83 $\pm$ 2	76 – 88	13	3 $\pm$ 1	2 – 6	13	14 $\pm$ 2	7 – 22
<i>Long axis of neurons (μm)</i>	140	20 $\pm$ 0.3**	13 – 32	110	22 $\pm$ 0.7*	13 – 30	90	21 $\pm$ 0.4***	13 – 30
<i>Short axis of neurons (μm)</i>	140	14 $\pm$ 0.2**	7 – 23	110	15 $\pm$ 0.3*	9 – 23	90	14 $\pm$ 0.4***	6 – 24
<i>Area of neurons (μm²)</i>	140	223 $\pm$ 6	94 – 560	110	260 $\pm$ 9*	99 – 647	90	240 $\pm$ 9	75 – 566

* Differences between the axes and area of ChAT-IR and TH-IR neuronal somata were statistically significant at $P < 0.05$.

** Differences between the axes of ChAT IR and biphenotypic neuronal somata were statistically significant at $P < 0.05$.

*** Differences between the axes of TH IR and biphenotypic neuronal somata were statistically significant at $P < 0.05$.