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DEVELOPMENT OF CARDIAC PARASYMPATHETIC NEURONS, GLIAL CELLS, AND REGIONAL CHOLINERGIC INNERVATION OF THE MOUSE HEART

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Abstract—Very little is known about the development of cardiac parasympathetic ganglia and cholinergic innervation of the mouse heart. Accordingly, we evaluated the growth of cholinergic neurons and nerve fibers in mouse hearts from embryonic day 18.5 (E18.5) through postnatal day 21(P21). Cholinergic perikarya and varicose nerve fibers were identified in paraffin sections immunostained for the vesicular acetylcholine transporter (VAChT). Satellite cells and Schwann cells in adjacent sections were identified by immunostaining for S100β calcium binding protein (S100) and brain-fatty acid binding protein (B-FABP). We found that cardiac ganglia had formed in close association to the atria and cholinergic innervation of the atrioventricular junction had already begun by E18.5. However, most cholinergic innervation of the heart, including the sinoatrial node, developed postnatally (P0.5–P21) along with a doubling of the cross-sectional area of cholinergic perikarya. Satellite cells were present throughout neonatal cardiac ganglia and expressed primarily B-FABP. As they became more mature at P21, satellite cells stained strongly for both B-FABP and S100. Satellite cells appeared to surround most cardiac parasympathetic neurons, even in neonatal hearts. Mature Schwann cells, identified by morphology and strong staining for S100, were already present at E18.5 in atrial regions that receive cholinergic innervation at later developmental times. The abundance and distribution of S100-positive Schwann cells increased postnatally along with nerve density. While S100 staining of cardiac Schwann cells was maintained in P21 and older mice, Schwann cells did not show B-FABP staining at these times. Parallel development of satellite cells and cholinergic perikarya in the cardiac ganglia and the increase in abundance of Schwann cells and varicose cholinergic nerve fibers in the atria suggest that neuronal-glial interactions could be important for development of the parasympathetic nervous system in the heart. © **2012 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: cardiac ganglia, cholinergic innervation, heart, parasympathetic, satellite cells, Schwann cells.

INTRODUCTION

Neural control of the heart is mediated through parasympathetic and sympathetic branches of the autonomic nervous system (Ardell, 2001). These branches differ in their neuroanatomy, neurotransmitters, and their influence on cardiac function. Cardiac sympathetic neurons are located in paravertebral ganglia, have long axonal projections to the heart, and produce cardioexcitatory effects mediated by the noradrenergic transmitter norepinephrine. Conversely, cardiac parasympathetic neurons are located in cardiac ganglia on the surface of the heart, have shorter axonal projections, and produce cardioinhibitory effects mediated by the cholinergic transmitter acetylcholine (ACh). Substantial knowledge about the development of sympathetic ganglia, growth of cardiac sympathetic nerves, and the regional distribution and abundance of sympathetic nerves in the adult heart has been gained through the use of catecholamine histofluorescence, immunohistochemistry for specific noradrenergic markers, and transgenic mouse models (Ernsberger and Rohrer, 2009; Ernsberger, 2009). Artemin, neurotrophin-3, and nerve growth factor (NGF) are major neurotrophic factors, which act at different stages to control sympathetic development. NGF is further required for the maintenance of sympathetic nerves in adults (Ruit et al., 1990). In marked contrast, there are major gaps in our knowledge about the development of cardiac parasympathetic neurons and cholinergic innervation of the mammalian heart.

Parasympathetic ganglia differ from their sympathetic counterparts in being smaller, more diffuse in distribution, and located at the target tissue. The cardiac parasympathetic system is an extreme example with numerous ganglia of various sizes forming a network distributed primarily or exclusively over the dorsal atrial surface (Leger et al., 1999; Pauza et al., 2000; Ai et al., 2007). These ganglia contain cholinergic neurons that innervate the myocardium. Studies of adult hearts from various species have shown that cholinergic nerve density is much higher in atrial compared to ventricular myocardium, and in small mammals like the mouse and guinea pig, cholinergic nerve fibers are very sparse in working ventricular

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Abbreviations: ACh, acetylcholine; AVN, atrioventricular node; B-FABP, brain-fatty acid binding protein; BSA, bovine serum albumin; E, embryonic day; HCN4, hyperpolarization-activated cyclic nucleotide-gated cation channel 4; NGF, nerve growth factor; P, postnatal day; PBS, phosphate-buffered saline; SAN, sinoatrial node; S100, S100β calcium binding protein; VAChT, vesicular ACh transporter.

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myocardium (Crick et al., 1999a,b; Hoover et al., 2004; Mabe et al., 2006).

Recent studies of transgenic mouse models have implicated neurturin as a neurotrophic factor for some cardiac parasympathetic neurons. Mice with deletion mutations for neurturin or its receptor have prominent deficits in cholinergic nerve density at specific sites in the myocardium and a decreased number of cholinergic perikarya in the cardiac ganglia compared to wild-type controls (Hiltunen et al., 2000; Mabe and Hoover, 2009). Neurturin knockout mice also exhibit defects in the negative chronotropic response to vagal nerve stimulation. However, the precise role of neurturin in the development of cardiac parasympathetic innervation remains unknown, as do many details regarding the development of the cardiac parasympathetic nervous system.

Recent studies of transgenic mouse embryos with tagged neural crest cells have shown that the cardiac ganglia begin to form by embryonic day 10.5 (E10.5) in this species and grow substantially over the next week of embryonic life (Hildreth et al., 2008). These developing ganglia contained neurons and non-neuronal neural crest-derived cells presumed to be satellite cells, and they received vagal input. Preganglionic vagal efferent nerve fibers and neurons within the cardiac ganglia began showing the cholinergic phenotype by E12.5, based on staining for the vesicular ACh transporter (VAChT), and some cholinergic nerves were detected in the dorsal mesocardium. However, subsequent development of the cardiac ganglia and regional cholinergic innervation of the heart were not evaluated.

Our goal in this study was to characterize the development of cardiac ganglia and the growth of cholinergic nerve fibers in the mouse heart from E18.5 through the third week of postnatal life. Immunohistochemistry was performed on paraffin sections of mouse hearts to localize the VAChT, a specific marker for cholinergic perikarya and nerve fibers. Cardiac nodes and other specialized regions of the developing hearts were identified on the basis of anatomical location and immunostaining for hyperpolarization-activated cyclic nucleotide-gated cation channel 4 (HCN4) (Garcia-Frigola et al., 2003; Yamamoto et al., 2006). Additionally, the development of satellite glial cells in the cardiac ganglia and Schwann cells in cardiac tissue were studied by performing immunohistochemistry for glial cell markers.

EXPERIMENTAL PROCEDURES

Animals and tissue collection

Hearts were collected from C57BL/6 mice bred from stock purchased from Harlan Laboratories (Indianapolis, IN, USA). Procedures conformed to protocols approved by the East Tennessee State University Animal Care and Use Committee and to the guidelines of the US National Institutes of Health published in the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85–23, revised 1996). Hearts were collected on E18.5, E19.5, postnatal day 0.5 (P0.5), P3.5, P7.5, and P21 (n = 3–6/age). Two additional hearts were obtained from P28 and adult mice. Animals were deeply anesthetized with isoflurane and euthanized by cervical dislocation or decapitation. Hearts were excised quickly, washed briefly in phosphate-buffered saline (PBS, pH 7.4), and fixed in 10% neutral buffered formalin for 5–7 days at 4 °C. Fixed tissue was embedded in paraffin and sectioned at 5-μm thickness using a Microm HM 310 microtome. Sections were mounted on charged slides and deparaffinized for 1 h at 60 °C prior to immunostaining.

Immunohistochemistry

Slide-mounted sections were immunostained at room temperature using the ABC technique (Vector Laboratories, Burlingame, CA, USA). Prior to immunostaining, tissue sections were rehydrated and treated with *Citra Plus* antigen retrieval solution per instructions from the manufacturer (Biogenex, San Ramon, CA, USA). Following several washes in PBS (pH 7.3), tissue sections were permeabilized with 0.4% Triton X-100 in PBS containing 0.5% bovine serum albumin (BSA) and treated with 1% H_2O_2 in PBS to quench endogenous peroxidase activity. Tissues were incubated in primary antibody diluted with 0.4% Triton X-100 in PBS containing 1% BSA for 16–18 h, washed in PBS, and incubated for 2 h with biotinylated secondary antibody to IgG of the same species as the primary antibody source. Localization of the antigen was visualized using the ImmPACT VIP Peroxidase Substrate Kit (Vector). Primary antibodies used included goat anti-VAChT (1:1000; Immunostar, 24286), rabbit anti-HCN4 (1:200; Alomone Labs, APC-052), rabbit anti-S100β calcium binding protein (S100; 1:2000; DakoCytomation, Z0311), and rabbit anti-brain-fatty acid binding protein (B-FABP; 1:5000; gift from Dr. Thomas Müller, Max-Delbrück-Center for Molecular Medicine).

Collection and analysis of digital images

Slides were viewed and photographed using an Olympus BX41 microscope equipped with a MagnaFire SP digital camera. The cross-sectional areas of cardiac neurons were determined by analysis of digital images using Stereo Investigator 7 software (MicroBrightField, Inc.). Briefly, the isotropic nucleator probe with six rays was applied to neurons with visible nuclei, and the intersection of each ray with the cell perimeter was marked for the determination of area. Nerve fiber densities in the sinoatrial node (SAN) and atrioventricular node (AVN) terminal field regions were evaluated by using ImageJ 1.36b software (NIH, USA). Briefly, digital images were converted to 8-bit, and the threshold was adjusted until only the VAChT-positive varicosities and nerves fibers were highlighted. Nerve fiber densities are reported as a percentage of the total area evaluated. Digital images of sections stained for S100 were collected and used to quantify Schwann cells in specific regions of the heart at different postnatal ages.

Statistics

Statistical analyses were performed using GraphPad Prism 4 (GraphPad Software, Inc.). Differences were considered significant at $P < 0.05$.

RESULTS

Cardiac parasympathetic ganglia are formed prenatally and mature postnatally

Immunostaining for VAChT showed that several small ganglia were already present in the mouse heart on E18.5 and associated exclusively with the atria. VAChT immunoreactivity was localized to the cytoplasm of ganglionic neurons and to varicose nerve processes that were found around most of the cholinergic perikarya (Fig. 1). The cross-sectional area of the neurons increased significantly over the next 3 weeks and doubled by P21 (Fig. 2).