Cardiac neural crest ablation inhibits compaction and electrical function of conduction system bundles

Abhijit Gurjarpadhye,1,2 Kenneth W. Hewett,1 Charles Justus,1 Xuejun Wen,1,2 Harriett Stadl,3 Margaret L. Kirby,3 David Sedmera,1,4,5 and Robert G. Gourdie1

1Department of Cell Biology and Anatomy, Medical University of South Carolina; 2Clemson-Medical University of South Carolina Bioengineering Program, Department of Bioengineering, Clemson University, Charleston, South Carolina; 3Neonatal-Perinatal Research Institute-Neonatology, Department of Pediatrics, Duke University Medical Center, Durham, North Carolina; 4Laboratory of Cardiovascular Morphogenesis, Institute of Animal Physiology and Genetics, Videnasa; and 5Institute of Anatomy, First Faculty of Medicine, Charles University, Prague, Czech Republic

Submitted 17 September 2006; accepted in final form 5 December 2006

Gurjarpadhye A, Hewett KW, Justus C, Wen X, Stadt H, Kirby ML, Sedmera D, Gourdie RG. Cardiac neural crest ablation inhibits compaction and electrical function of conduction system bundles. Am J Physiol Heart Circ Physiol 292: H1291–H1300, 2007. First published December 15, 2006; doi:10.1152/ajpheart.01017.2006.—Retroviral and transgenic lineage-tracing studies have shown that neural crest cells associate with the developing bundles of the ventricular conduction system. Whereas this migration of cells does not provide progenitors for the myocardial cells of the conduction system, the question of whether neural crest affects the differentiation and/or function of cardiac specialized tissues continues to be of interest. Using optical mapping of voltage-sensitive dye, we determined that ventricles from chick embryos in which the cardiac neural crest had been laser ablated did not progress to apex-to-base activation by the expected stage [i.e., Hamburger and Hamilton (HH) 35] but instead maintained basal breakthroughs of epicardial activation consistent with immature function of the conduction system. In direct studies of activation, waves of depolarization originating from the His bundle were found to be uncommon in control hearts from HH34 and HH35 embryos. However, activations propagating from septal base, at or near the His bundle, occurred frequently in hearts from HH34 and HH35 neural crest-ablated embryos. Consistent with His bundle cells maintaining electrical connections with adjacent working myocytes, histological analyses of hearts from neural crest-ablated embryos revealed His bundles that had not differentiated a lamellar organization or undergone a process of compaction and separation from surrounding myocardium observed in controls. Furthermore, measurements on histological sections from optically mapped hearts indicated that, whereas His bundle diameter in control embryos thinned by almost one-half between HH30 and HH34, the His bundle in ablated embryos underwent no such compaction in diameter, maintaining a thickness at HH30, HH32, and HH34 similar to that observed in HH30 controls. We conclude that the cardiac neural crest is required in a novel function involving lamellar compaction and electrical isolation of the basally located His bundle from surrounding myocardium.

NEURAL CREST CELLS DELAMINATE from the dorsal neural tube in an anterior to posterior sequence and migrate via stereotypic pathways to various locations in the vertebrate embryo, including the heart (reviewed in Refs. 36 and 41). First identified in the chick during the 19th century, this migratory population has now been characterized in embryos throughout the chondrate lineage (25) as well as in the urochordate ascidians (28).

Neural crest cells have potential to differentiate into diverse cell types (reviewed by Refs. 12, 26, and 35) and, depending in part on the segmental level of delamination, can contribute to craniofacial cartilage, bone, and connective tissue. Additionally, neural crest cells differentiate into nerves and accessory cells involved in the layered myelin insulation of nerves (29, 43), as well as contributing smooth muscle progenitors to the great vessels of the heart (27, 52).

It is well established that neural crest cells originating from posterior rhombencephalic segments of the neural tube contribute to multiple aspects of cardiac development and function (4, 5, 13, 15, 24, 37; reviewed by Refs. 27 and 52). Whereas ongoing focus has been on the outflow pole of the heart, evidence has been provided for an association between neural crest cells and the cardiac conduction system (reviewed by Ref. 17).

The conduction system consists of pacemaking tissues (i.e., the nodes) and the fast-conducting bundles of the ventricular conduction system including the His bundle (reviewed by Ref. 19). In previous lineage-tracing and fate determination studies in chick, we have shown that the cardiac conduction system does not develop by outgrowth from a prespecified pool of conduction cell progenitors. Instead, our data indicated that the conduction system elaborates by progressive commitment to nonproliferation and differentiation by multipotent cardiomyogenic cells (8). Whereas this recruitment process continues in the peripheral network of conduction cells up until hatching, a lamellar pattern of conscription of cells forming the His bundle is completed by Hamburger and Hamilton (HH) stage 36. A similar discrete phase of cellular recruitment to nonproliferation and differentiation was subsequently shown to occur in lamellar patterns around the developing His bundle in rodent embryos (50).

In addition to establishing the cardiomyogenic origin and pattern of differentiation of conduction tissues, our lineage-tracing studies also indicated that direct contributions to the constituent myocardial cells of the conduction system by neural crest was unlikely (8). However, while cell lineage-tracing studies in avian and mouse models have ruled out significant direct contributions, we and others noted that a cohort of neural crest cells showed a preferential migration coinciding with the fascicles of the ventricular conduction system, including the His

Address for reprint requests and other correspondence: R. G. Gourdie, Dept. of Cell Biology and Anatomy, MUSC, 171 Ashley Ave., Charleston, SC 29425-2204 (e-mail: gourdie@musc.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

http://www.ajpheart.org 0363-6135/07 $8.00 Copyright © 2007 the American Physiological Society H1291
bundle (8, 45, 46). In one recent study, two neural crest-specific promoters driving Cre recombinase, Wnt1-Cre and P0-Cre, were used to demonstrate that cells of neural crest origin associate with the conduction systems in both the developing and mature mouse heart (44).

At present, the role of the cardiac neural crest in the embryonic development and adult function of the conduction system remain unknown. In this study, we have used the well-established avian neural crest ablation model (33, 56; reviewed by Ref. 27) to characterize neural crest effects on the cardiac conduction system. Our data indicate that loss of neural crest cells inhibits differentiation of a compact lamellar organization by the His bundle and results in failure of the conduction system to assume its mature function of activating the ventricle in an apex-to-base sequence.

MATERIALS AND METHODS

Embryos. Fertilized Arbor Acre chicken eggs (Goldkist Hatchery, Silver City, NC) were incubated at 37°C and 97% humidity in a forced air incubator. Neural crest-ablated and sham-operated control embryos were prepared as described previously (33, 56) at Duke University Medical Center (Durham, NC). Embryos were staged according to Hamburger and Hamilton (HH) (23). Embryonic hearts between stages HH29 and HH35 were used for these experiments.

Optical mapping. The approaches used for optical mapping in sinus rhythm were similar to those we and others have described for studies of the embryonic chick heart previously (9, 22, 48), with some modifications. The apparatus for data acquisition system is shown in the supplemental figure (the online version of this article contains supplemental data). In the first series of experiments, separate recordings were made of the right ventricular free wall, anterior surface, and posterior surface. In subsequent experiments, the tissue bath was fitted with two s-surface mirrors (Edmond Scientific, Barrington, NJ) at 45° relative to the bottom surface of the bath (supplemental figure, b). The mirrors were positioned a minimum of 3–4 mm apart. The chick embryonic heart was placed between these two mirrors. The right ventricular free wall was positioned upward so that image of the anterior and posterior sides of the heart could be reflected upward by these mirrors. In this way, a single recording could cover three aspects of interest in these hearts. It was estimated that the three perspectives enabled 75–80% imaging coverage of the epicardial surface. The hearts were stained using voltage-sensitive dye (Di-4-ANEPPS) for ~10 min. The hearts were then pinned to a supporting block by the distal outflow tracts before being placed between mirrors in the tissue bath. Hearts were superfused at 35°C with Tyrode solution (pH 7.4) in the tissue bath mounted on a Leica FLZ III dissecting microscope. The spread of depolarization across the myocardium was monitored by changes in the fluorescence signals of Di-4-ANEPPS, using a high-speed, charge-coupled device camera with 80 × 80 pixel array (RedShirt Imaging, Fairfield, CT) at 1,000 or 2,000 frames per second. Tyrode solution was saturated with 100% oxygen, and hearts were maintained in an atmosphere of 100% oxygen. The excitation light was provided by 100-W mercury vapor light source and green filter. An excitation-contraction decoupler (10 mM cytochalasin D) was used to minimize the motion artifacts. Data were spatially and temporally filtered during analysis using Cardioplex software (RedShirt Imaging). The data represent a sequence of the excitatory wave front over the ventricular imaged surface. Isochrone maps indicating site of earliest activation were generated from these data (e.g., Figs. 1 and 2).

For direct assessment of His bundle activation, hearts were isolated from HH34 and HH35 embryos and then immediately cut in an anterior long-axis plane as illustrated in Fig. 3A to expose and cross-section the His bundle. The transected hearts were then stained with Di-4-ANEPPS, and activation of the interventricular septum during pacing (frequency 5 Hz, 1-ms duration, 2 times threshold voltage) from the right atrium was undertaken. Following optical mapping of sequences of voltage transients during induced action potentials (e.g., Fig. 3C), the hearts were stained with eosin, and confocal images were taken of the cut surface of the interventricular septum to identify the location of the cross-sectioned His bundle (e.g., Fig. 3B). The His bundle location for a given preparation was then correlated with the activation map derived from that preparation to determine whether or not activation breakthrough originated at or from the vicinity of the cut His bundle (see Table 1).

Histological techniques and measurements. After the recordings were made, isolated control or ablated hearts were fixed in Dent’s
fixative overnight, rinsed in PBS, and stored in 70% ethanol as described previously (48). The hearts were then processed into paraffin and serially sectioned at 10 μm in frontal long-axis orientation for histological (hematoxylin-eosin) and immunohistochemical analyses. Conduction system was identified on the basis of its histological appearance (2, 54) and HNK-1 (Developmental Studies Hybridoma Bank) antibody staining (55). Immunolabeling for proliferating cell nuclear antigen (PCNA) was carried out using standard immunofluorescence staining protocols detailed at length in previous publications (8, 22, 33, 48). Confocal imaging was carried out on a Leica TCS SP2 AOBS confocal microscope using appropriate filter sets. Measurements of His bundle minimum diameter were made on histological sections four to five optically mapped hearts per stage (i.e., HH30, HH32, and HH34) per experimental group (i.e., sham control and neural crest ablated) using a Leica DMLB microscope (×40 objective) under Normaski illumination. Minimum diameter of the cross section of the bundle was measured because this dimension will be unaffected by variation in bundle orientation between sections. Measurements were made on three to four different sequential sections along the His bundle of each heart.

RESULTS

Development of a measurement of maturity of ventricular activation. As a first step in assessing the effect of neural crest ablation on conduction system development, we devised a quantitative method for indexing the maturity of ventricular activation. Electrode and optical mapping studies of the embryonic chick heart have shown that ventricular activation undergoes a topological change between the 4th and 8th day of development (9, 22, 48). This is characterized by an immature base-to-apex sequence of activation becoming replaced by an apex-to-base sequence, a change diagnostic of a functional His-Purkinje system. Our surveys of isochrone maps of more than 70 hearts at HH stages 29 to 35 ventricles indicated that the site of first activation (i.e., the breakthrough) changed in a progressive trend with developmental stage (9, 22, 48). This is characterized by an immature base-to-apex sequence of activation becoming replaced by an apex-to-base sequence, a change diagnostic of a functional His-Purkinje system. Our surveys of isochrone maps of more than 70 hearts at HH stages 29 to 35 ventricles indicated that the site of first activation (i.e., the breakthrough) changed in a progressive trend with developmental stage (Fig. 1, A–F). In maps of the posterior surface of the ventricle, the initial region of breakthrough (asterisked dark gray sectors in the isochrone...
maps of Fig. 1) tended to change from a first appearance at the left base at more immature stages (Fig. 1, A and B) to later initiations at the right ventricular margin during intermediate stages (Fig. 1, C–E). At the most mature stage, first activation propagated from or near the apex of the heart (Fig. 1F). To obtain an index of this shift in the position of initial activation of the ventricle, we assigned an angular value between $-30^\circ$ and $+180^\circ$ (in $30^\circ$ increments) to the point of first breakthrough (“reference” panel in center of Fig. 1). This estimate was such that the immature breakthroughs at the left base corresponded to a value of $-30^\circ$ (e.g., Fig. 1A), and mature breakthroughs at the tip of the ventricular apex corresponded to values between $140$ and $180^\circ$ (e.g., Fig. 1F). In a pattern consistent with this angular value as an index of the maturity of ventricular activation sequence, the measurement varied in a linear relationship with HH stage ($R^2 = 0.96$) in sham-operated control.
Table 1. Summary of data from 6 sham-operated control and 6 neural crest-ablated hearts optically mapped following bisection along the long axis of the ventricle to expose the His bundle (see Figure 3)

<table>
<thead>
<tr>
<th>Stage/Embryo</th>
<th>Number of Activation Origins</th>
<th>Direction Base-to-Apex?</th>
<th>His Bundle Involvement?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated control hearts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HH34 Embryo 1</td>
<td>2</td>
<td>Bidirectional</td>
<td>No</td>
</tr>
<tr>
<td>HH35 Embryo 1</td>
<td>1</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>HH35 Embryo 2</td>
<td>2</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>HH35 Embryo 3</td>
<td>1</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Neural crest-ablated hearts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HH34 Embryo 1</td>
<td>1</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>HH34 Embryo 2</td>
<td>1</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>HH34 Embryo 3</td>
<td>1</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>HH34 Embryo 4</td>
<td>1</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Six out of six neural crest-ablated hearts demonstrated initiation of depolarization at or around the cut His bundle, whereas only one of the control hearts showed evidence of this. Activation origins in neural crest-ablated hearts tended to occur at a single locus in the interventricular septum and then propagate in a base-to-apex direction. Multiple origins of activation were more common in control hearts. HH, Hamburger-Hamilton stage.

Hearts between HH29 and HH35 (Fig. 1H). It should be noted that the seven breakthrough time points shown in Fig. 1F are each averages based on measurements taken on between 5 and 12 hearts per time point.

Neural crest-ablated hearts do not progress to apex-first activation of the ventricle. The maps shown in Fig. 1 were based on recordings of the posterior aspect of the ventricle. As a further step to ensure the accuracy of the index, we used mirrors of fixed tilt to simultaneously map three ventricular surfaces (anterior, posterior, and right ventricle; see Fig. 2 and supplemental figure). Of ~120 ventricles mapped using this multiple perspective method, we observed three instances in which the timing of the breakthrough sector on the epicardial surface could not be distinguished from another independent activation, and these hearts were excluded from subsequent analysis. The hearts from neural crest-ablated and controls did not appear to differ greatly in size within developmental stages between HH29 and HH35; thus comparison of breakthrough position was probably not complicated by gross differences in myocardial growth between treatment and control groups.

Multiperspective views of ventricular activation sequences from typical HH30 and HH34 control sham-operated and neural crest-ablated embryos are shown in Fig. 2, A–D. Activation breakthrough (asterisked and darkest gray sector) occurs at a basal locus at HH30 in both sham control and neural crest-ablated embryos (Fig. 2, A and B). First breakthrough occurs at the apex in the HH34 control example (Fig. 2C); however, in a HH35 neural crest-ablated embryo (Fig. 2D), breakthrough is seen to retain an immature character, emerging initially from a basal location on the ventricle. The maintenance of an immature pattern of breakthrough in ablated embryos was confirmed in the quantitative analysis summarized in Fig. 2E. In Fig. 2E, the average value of initial breakthrough obtained from multiperspective mapping is plotted in bar graphs against developmental stage between HH29 and HH35 for both sham-operated and neural crest-ablated embryos. Whereas the trend for this HH stage-averaged value for controls (dark gray bars, Fig. 2E) was identical to that shown in Fig. 1H, the average value in neural crest-ablated embryos (light gray bars) showed a distinctive variation with developmental stage. Before HH33, ventricles from neural crest-ablated embryos showed a trend in breakthrough position similar to that of stage-matched controls. However, between HH33 and HH34, there was a significant (P < 0.01) decrease in this average for neural crest-ablated embryos, i.e., average breakthrough position tended to be more basal and less mature. Moreover, HH34 and HH35 neural crest-ablated embryos maintained significant reductions in average breakthrough values relative to control embryos of the same stage (P < 0.001 for HH34 and P < 0.0001 for HH35 neural crest-ablated vs. controls, respectively). The maintenance of basally located activation breakthrough, especially notable at the later developmental stages analyzed (e.g., HH34 and HH35), is consistent with inhibited maturation of conduction system function in the neural crest-ablated embryos.

Reduced electrical isolation of the His bundle from surrounding working myocardium in neural crest-ablated embryos. To further investigate why ventricles from neural crest-ablated hearts did not progress to apex-first activation, we developed a novel protocol to observe activations originating from the His bundle. Our approach was to optically map activation in atrially paced HH34 and HH35 hearts that had been cut in a long-axis plane to expose the basally located His bundle in the dissection (Fig. 3A). Fig. 3B shows a fluorescence...
image of a bisected control heart. The cut His bundle is seen as a circular structure (see arrows on Fig. 3B) near the crest of the interventricular septum, i.e., the septal base. Fig. 3C shows a sequence of voltage maps during an action potential in the same cut interventricular septum as shown in Fig. 3B. As indicated by the transition from blue to yellow to red as depolarization ensues, activation initiates at an ectopic locus of automaticity distal to the His bundle that is situated in the apical half of the interventricular septum (Fig. 3C; the yellow site of activation initiation is also indicated by an asterisk). Activation then propagates from this location, depolarizing the septum in an apex-to-base sequence.

Patterns similar to that shown in Fig. 3C were observed in depolarizations from five out of the six sham control hearts, with action potential origins typically occurring at one, and sometimes more than one, site in the interventricular septum distal from the His bundle (Table 1). These sites of ectopic activation initiation nearly all occurred in subendocardial tissue regions in the apical half of the septum. Activation propagated from multiple locations that included the His bundle in a sixth control heart. Overall, the distribution and multifocality of activation origin sites in control hearts would not be consistent with ventricular activation dominantly originating from the His bundle. Thus, in the controls, there appeared to be an electrical block occurring between the pacing site in the atria and interventricular septum. This block presumably occurred as the basally located His bundle and/or crest of the interventricular septum in the controls were efficiently insulated and did not propagate action potentials initiating from the atria into the ventricular myocardium.

In contrast to the controls, the depolarization sequences of six out of the six neural crest-ablated embryos had action potential origins initiating at a single locus at crest of the interventricular septum, i.e., in or near the cut His bundle (Table 1). In a further distinction from controls, activations in these hearts mostly propagated in a base-to-apex sequence down the septum. An example of this depolarization sequence in neural crest-ablated hearts is provided in Fig. 3D. Here, activation initiates at the septal base at or in the vicinity of the cut His bundle. Depolarization then propagates from this locus in a base-to-apex sequence. The pattern shown in Fig. 3D for neural crest-ablated embryos is what would be expected if the paced activations were being propagated from the atria unimpeded directly into ventricle via the His bundle and/or
other myocardial tissues at the crest of the interventricular septum.

Neural crest ablation inhibits a stage-dependent thinning of the His bundle and its physical segregation from working myocardium. We next sought to probe the structural basis of the activation phenomena observed in neural crest-ablated hearts. Retroviral lineage tracing studies (8, 45) have shown that neural crest cells in chick migrate in tissues surrounding the large fascicles of the developing conduction system, including the His bundle and bundle branches (e.g., Fig. 4a). We undertook histological studies of His bundle in the same hearts that had been used for optical mapping (Fig. 4, b–f). At HH34, the His bundle can be identified as an HNK-1-negative fascicle embedded in a transitional sector of HNK-1-positive tissues at the base of the interventricular septum (21, 55). His bundles in control HH34 hearts were always thinner and appeared to show a greater degree of physical separation from surrounding working myocardial tissues than His bundles in hearts from neural crest-ablated embryos (compare Fig. 4, b and e). Cells in and around the His bundles in control embryos also displayed a concentric lamellar organization that was not as evident in hearts from ablated embryos. As would be expected from previous reports (8), His bundle tissues from control chick embryos also showed low proliferation rates as indicated by decreased levels of nuclear-localized PCNA immunolabeling compared with surrounding working myocardial tissues (Fig. 4c). By contrast, PCNA immunolabeling of His bundle nuclei, in all ablated embryos, was consistently maintained at elevated levels similar to that of surrounding myocardial tissues (Fig. 4f).

The histological analyses indicated that, when compared with controls, His bundles from neural crest-ablated HH34 embryos were thicker in diameter, less concentrically organized, and maintained more tissue contacts with surrounding myocardium than HH34 control bundles. As a next step, we examined control hearts at HH30, -32, and -34 to determine whether or not progressive changes in His bundle organization could be detected over the developmental period of transition to apex-first activation (Fig. 5). At HH30, the His bundle was recognizable as a loosely organized bundle of HNK-1-negative tissues, with relatively little differentiation from surrounding myocardium (Fig. 5a). This organization resembled that observed for the HH34 neural crest-ablated embryo (compare Fig. 5, a and e). Reduced levels of PCNA labeling were nonetheless already evident in the control HH30 bundle (Fig. 5b). As development proceeded from HH30 to HH34, bundles maintained lower levels of proliferation than surrounding working myocardium (Fig. 5, b, d, and f) and progressively compacted, becoming thinner and concentrically organized (Fig. 5, a, c, and e and Fig. 6, inset).

Figure 6 provides a summary of morphological measurements of average His bundle diameter taken on images from optically mapped HH30, -32, and -34 hearts that were sectioned in frontal, long-axis orientation. This bar graph indicates that, whereas average His bundle diameter in sham control

![Fig. 5](http://ajpheart.physiology.org.org/)

**Fig. 5.** Development of His bundle organization between HH30 and 34. Confocal images of the HNK-1-negative His bundle (a, c, and e) and PCNA immunolabeling (b, d, and f) in optically mapped HH30 (a and b), HH32 (c and d), and HH34 (e and f) sham-operated control hearts. Scale bar, 100 μm. Asterisk in e indicates the His bundle.
embryos narrowed by nearly one-half between HH30 and HH34 ($P < 0.001$), the bundle in ablated embryos underwent no such compaction, maintaining a thickness at HH30, HH32, and HH34 similar to that observed in HH30 controls. At HH34, average His bundle thickness in neural crest-ablated hearts was nearly twice that of control hearts ($P < 0.001$).

In conclusion, maintenance of His bundle thickness and a failure of this specialized fascicle to become concentrically organized, compact, and separate from surrounding myocardium are structural phenomena associated with the maintenance of immature patterns of ventricular activation in hearts from neural crest-ablated embryos.

**DISCUSSION**

A function of the His-Purkinje conduction system is to provide for coordinated and rapid propagation of action potential in the ventricles (reviewed by Refs. 2, 19, and 51). Preventing dissipation of excitation into surrounding tissues is key to this function, because the velocity of impulse conduction along a bundle of the ventricular conduction system, or indeed a nerve, is slowed by electrical contacts (i.e., short circuits) with surrounding tissues (30, 31, 49). Here evidence is described for inhibition of concentric remodeling, thinning, and electrical isolation of the developing His bundle from surrounding myocardium in chick embryos subject to neural crest ablation—a treatment causing abnormal and reduced migration of cardiac neural crest cells (reviewed by Ref. 27). These data are consistent with a requirement for the cardiac neural crest in determining patterns of electrical isolation and contact between the conduction system and working ventricular muscle.

The sequence of depolarization of the embryonic chick ventricle has been the subject of a number of pioneering studies (1, 3, 9, 11, 32). The recognition that activation in the chick heart undergoes a change from a base-to-apex to an apex-to-base sequence was made first by Watanabe, Rosenbaum, and coworkers (9). This group reported that the timing of this transition occurred on average at ~HH30, though in individual hearts, earlier and later examples of the topological shift were noted. In the present study, we confirm the plasticity of this shift but show that, as a stage-by-stage average, the differentiation of apex-first activation occurs progressively and is complete by HH35. A numerical index of the average rate of progression to apex-first activation was developed for the purpose of comparing control and neural crest-ablated embryos. Whereas detailed lineage and morphological analyses of ventricular tissues were not undertaken, the developmental progression of mean position of activation breakthrough appears to be consistent with morphological models of chamber formation proposed by Moorman and Christoffels (43a).

In 1906, Tawara (53) described an insulating layer of fibrous tissue around conduction fascicles in adult hearts of all species he examined. Whereas mesenchymal lineages derived from the neural crest contribute to fibrous tissues (reviewed by Refs. 12, 26, and 29), the process of His bundle lamellar compaction described in the present study seems to precede any notable differentiation of the perifascicular layer of fibrous insulation described by Tawara (53). At the stages we examine here, there is as yet little evidence of the deposition of significant amounts of cells or connective tissue around the His bundle (e.g., Fig. 6, inset). Picrosirious red labelings undertaken by us on histological sections from the mapped hearts also provide evidence of negligible differentiation of fibrous tissues around or adjacent to conduction bundles at or before HH35 (data not shown). That fibrous insulation is not a priori necessary, for apex-first activation would also be consistent with our earlier report that apex-to-base spread of impulse in the zebrafish ventricle occurs in the absence of a histologically recognizable conduction...
system, though it does appear to be facilitated by noninsulted tracts of aligned myocytes (50). Further work will be required to determine the origin of the later deposited perifascicular fibrous tissues described by Tawara (53) that differentiate after the His bundle compaction phenomena we report here. We would nonetheless speculate on the basis of the literature that multiple sources of cells may contribute to this specialized layer of interstitial tissue, including progenitors derived from the cardiac neural crest and the proepicardium (reviewed by Refs. 7, 14, 16, and 38).

In addition to development of physical insulation, conduction bundles in the mature heart display patterns of gap junctional connexin (Cx) expression that contribute to electrical connectivity along this specialized network of tissues (42, 51). For example, the His bundle of the adult mouse expresses multiple Cx isoforms including Cx40, Cx45, and Cx30.2 (6, 34, 42, 51). By contrast, surrounding ventricular myocardial cells express only Cx43, a connexin not present in the His bundle of the mouse. Cx40, the predominant isoform of the His Purkinje system in birds and mammals (18), has been shown to form heterotypic gap junction channels with Cx43 (10). Cx40/Cx43 heterotypic channels possess electrophysiological properties that are distinct from homotypic channels comprised of one or the other of the two connexins (10). In vivo, such heterotypic couplings are thought to impede cell-to-cell propagation of action potential, contributing to electrical isolation of conduction cells from surrounding working myocytes. This being said, Cx40 is not detectable in the avian His bundle in distributions characteristic of gap junctions until after hatching, some 12 days subsequent to the HH29–HH35 developmental stages studied here (18, 20). Furthermore, Cx43 is not expressed in avian working myocardial tissues. Thus, although the molecular composition of gap junctional couplings in the His bundle may contribute to its functional isolation from working myocardium in the adult mammal, it seems unlikely that this will be a major factor in the embryonic chick. Purkinje-myocyte electrical junctions are arrayed in complex three-dimensional distributions at the terminal ramifications of the conduction system in the apex of ventricles (47, 53). Our results indicate that there is an abnormal persistence of electrical contact between basal parts of the conduction system and the working myocardium associated with ablation of the cardiac neural crest. An intriguing prospect suggested by this result is that the differentiation of normal points of coupling between the conduction system and working myocardium (i.e., Purkinje-myocyte junctions) occurs preferentially in apical regions of the ventricle, because this is the part of the conduction system that normally receives the lowest level of neural crest cell emigration. In this respect, it is noteworthy that then that Poelmann, Gittenberger-de Groot and coworkers have shown that the density of neural crest cells associated with the conduction system decreases in a base-to-apex gradient in mouse embryos (46). Nakamura and colleagues (44) have also recently demonstrated that a similar relationship between neural crest derivatives and proximal (i.e., basally located) elements of the conduction system is maintained in the adult mouse. This latter study also reported that some of these neural crest-derived cells associated with the conduction system possessed glial markers, i.e., markers expressed in cells functioning in the electrical insulation of nerves. In this respect, it is noteworthy that migratory neural crest cells contribute to myelin layering around nerve fascicles, on the one hand, but are also apparently required for induction of a lamellar organization of cells at and around the His bundle. At the very least, these phenomena suggest that neural crest derivatives make distinct contributions to the lamellar histological organization of both nerves and cardiac conduction fascicles.

GRANTS

This work was supported by National Institutes of Health Grants HL-56728, HD-39946 (to R. G. Gourdie, and RR-16434, MSMT VZ 206100-3, AS CR AVOZ50450515, and the Purkinje Fellowship of the AS CR (to D. Sedmera); and National Heart, Lung, and Blood Institute Grant HL-36059 (to M. L. Kirby).

REFERENCES


