Biochemical and Biophysical Research Communications 423 (2012) 536–541

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Adrenergic deficiency leads to impaired electrical conduction and increased arrhythmic potential in the embryonic mouse heart

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ARTICLE INFO

Article history:
Received 21 May 2012
Available online 7 June 2012

Keywords:
Adrenergic regulation
Arrhythmia
Cardiac development
Cardiac gap junctions
Conduction velocity

ABSTRACT

To determine if adrenergic hormones play a critical role in the functional development of the cardiac pacemaking and conduction system, we employed a mouse model where adrenergic hormone production was blocked due to targeted disruption of the *dopamine β-hydroxylase* (*Dbh*) gene. Immunofluorescent histochemical evaluation of the major gap junction protein, connexin 43, revealed that its expression was substantially decreased in adrenergic-deficient ($Dbh^{-/-}$) relative to adrenergic-competent ($Dbh^{+/+}$ and $Dbh^{+/-}$) mouse hearts at embryonic day 10.5 (E10.5), whereas pacemaker and structural protein staining appeared similar. To evaluate cardiac electrical conduction in these hearts, we cultured them on microelectrode arrays (8 × 8, 200 μm apart). Our results show a significant slowing of atrioventricular conduction in adrenergic-deficient hearts compared to controls (31.4 ± 6.4 vs. 15.4 ± 1.7 ms, respectively, $p < 0.05$). To determine if the absence of adrenergic hormones affected heart rate and rhythm, mouse hearts from adrenergic-competent and deficient embryos were cultured *ex vivo* at E10.5, and heart rates were measured before and after challenge with the β-adrenergic receptor agonist, isoproterenol (0.5 μM). On average, all hearts showed increased heart rate responses following isoproterenol challenge, but a significant ($p < 0.05$) 225% increase in the arrhythmic index (AI) was observed only in adrenergic-deficient hearts. These results show that adrenergic hormones may influence heart development by stimulating connexin 43 expression, facilitating atrioventricular conduction, and helping to maintain cardiac rhythm during a critical phase of embryonic development.

1. Introduction

Mice that lack the ability to produce the adrenergic hormones, norepinephrine (NE) and epinephrine (EPI), due to targeted disruption of the *dopamine β-hydroxylase* (*Dbh*) gene die at mid-gestation from apparent heart failure [25]. Structural formation of the heart was not markedly perturbed in the adrenergic-deficient embryos, though subtle abnormalities such as dilated atria and disorganized ventricular myofibrils were observed in the deficient group. In addition, blood pooling in major organs and slower *in vivo* heart rates led to the conclusion that heart failure was the likely cause of death in adrenergic-deficient embryos. The mechanism of action appears to be primarily through β-adrenergic receptor activation because isoproterenol (β-agonist) but not L-phenylephrine (α-agonist) could rescue the adrenergic-deficient ($Dbh^{-/-}$) mouse embryos when supplied via the maternal drinking water [26]. Despite a relatively wide body of data on adrenergic mechanisms in the adult heart, only rudimentary information exists regarding adrenergic actions in the embryonic heart. A major gap in our current knowledge is how embryonic activation of β-adrenergic signaling specifically affects cardiac function and embryonic survival at these critical formative stages of development.

Independent studies have shown that the heart itself is a source of adrenergic hormones during early development [4,8,13,14].

Abbreviations: APX, Apex of ventricle; A-V, Atrioventricular; AVJ, Atrioventricular junction; Cx43, Connexin 43; Dbh, Dopamine β-hydroxylase; EPI, Epinephrine (adrenaline); Hcn4, Hyperpolarization-activated cyclic nucleotide-modulated channel isoform 4; ICA, Intrinsic Cardiac Adrenergic (cells); MEA, Microelectrode array; NE, Norepinephrine (noradrenaline); OT, Outflow tract; SAN, Sinoatrial node; SAR, Sinoatrial region.

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http://dx.doi.org/10.1016/j.bbrc.2012.05.163

"Intrinsic Cardiac Adrenergic" (ICA) cells appear in the heart at about the time that it first starts to beat [4,13]. There is a transient clustering of ICA cells in regions of the heart progressively associated with development of the cardiac pacemaking and conduction system, including the pacemaker cells in the sinoatrial node, the atrioventricular node, bundle of His, and Purkinje fibers [8]. Some of these transient ICA cells appear to differentiate into cardiac myocytes, including the specialized myocytes that serve as pacemaker cells in the sinoatrial and atrioventricular nodes as well as extensive labeling of myocytes throughout the ventricular conduction system [5]. These observations have led us to hypothesize that NE and/or EPI play a critical role in the embryonic development of the cardiac pacemaking and conduction system [6].

In the present study, we utilized the *Dbh* knockout mouse model to test the hypothesis that NE and EPI play a critical role in the development of the cardiac pacemaking and conduction systems. Our initial experiments evaluated the *in situ* expression of a key pacemaker channel protein, the hyperpolarization-activated cyclic nucleotide-modulated channel isoform 4 (Hcn4) [24], and a major gap junction protein responsible for fast ventricular conduction, connexin 43 (Cx43) [28], in adrenergic-competent and deficient embryos. We then used microelectrode arrays (MEAs) to evaluate electrical conduction, and videomicroscopy to examine heart rate and rhythm.

2. Materials and methods

2.1. Animals

The *Dbh* mouse strain and collection of embryos used in this study has been described previously [25]. All animal procedures were performed in accordance with NIH guidelines and were approved by the University of Central Florida Animal Care and Use Committee. Most of our analyses were performed using embryonic day 10.5 (E10.5) mouse embryos because E10.5 is the latest stage of development when adrenergic-deficient embryos are still largely asymptomatic [25].

2.2. Immunofluorescence histochemistry

Dual immunofluorescent histochemical staining of hearts was performed essentially as described previously [5,8].

2.3. Ex vivo embryonic mouse heart cultures

E10.5 mouse hearts were isolated under aseptic conditions and cultured in Dubelcco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Hyclone Labs, Logan, UT) that had been charcoal-stripped to remove catecholamine and steroid hormones [17]. The media was additionally supplemented with the following (final concentrations given): d-glucose (25 mM), sodium pyruvate (1 mM), penicillin G (100,000 U/L), streptomycin (100 mg/L), β-mercaptoethanol (55 μM), l-glutamine (2 mM), and 1% 100x α-minimum nonessential amino acids [7]. Hearts were cultured for 20–24 h prior to any measurements of beating activity or conduction properties.

2.4. Microelectrode arrays (MEAs)

General MEA procedures were similar to those described previously [19], with the exceptions described here. Freshly isolated E10.5 hearts were placed in the center of gelatin-coated 8 × 8 MEAs (#200/10iR-Ti, Multichannel Systems, Reutlingen, Germany) with the flat ventral surface in contact with electrodes (i.e., outflow tract projecting upward). A representative video of an E10.5 spontaneously beating mouse heart on a MEA is shown in Supplemental Video 1. Electrodes were 10 μm in diameter and 200 μm apart. MEA analysis was performed using Clampfit v10.0.0.61 (Molecular Devices, Sunnyvale, CA). The first depolarizing atrial electrode was considered the sinoatrial region (SAR). The electrode with the largest depolarization in the atrioventricular region was considered the atrioventricular junction (AVJ), and the ventricular apex (APX) was identified both by location and bifurcation of impulse propagation to the distal Purkinje fibers. Within the AVJ and APX regions, adjacent electrodes depolarized usually near-simultaneously (<0.5 ms of each other). Conduction time was measured between field potential minimums (FP_{min}) [12,22].

2.5. Beating rate and rhythmicity measurements

Beating rate and rhythmicity measurements were performed as described previously [9,17]. Arrhythmic index (AI) was calculated as the median cycle length divided by the standard deviation [9].

2.6. Statistics

Data are expressed as mean ± S.E.M. Student *t*-tests were performed to compare means, with $p < 0.05$ required to reject the null hypothesis. No significant differences were observed between wild-type ($Dbh^{+/+}$) and heterozygous ($Dbh^{+/-}$) hearts at E10.5 in any examined parameter. Since there was no significant difference between $Dbh^{+/+}$ and $Dbh^{+/-}$ embryos [25], these two genotypes were combined into a single group referred to as "adrenergic-competent." Homozygous knockout ($Dbh^{-/-}$) mice were designated as "adrenergic-deficient" due to their inability to produce NE or EPI [25].

3. Results

Since ICA cells have previously been identified in regions of the developing heart associated with conduction and pacemaking function [5,8], we employed immunofluorescent histochemical staining to evaluate a key pacemaking protein (Hcn4) and a major gap junction protein (Cx43) important for the generation and propagation, respectively, of electrical signaling in adrenergic-competent and deficient embryonic hearts. As shown in Fig. 1, our results indicate that Cx43 immunofluorescent staining intensity in adrenergic-deficient hearts was substantially less than that observed in adrenergic-competent hearts (compare red fluorescence in panels a and b). In this example, co-immunofluorescent staining with Hcn4 showed similar distribution and intensity in both groups. Higher magnification of the dual immunofluorescent staining in the SAN region is shown in panels c and d. The arrowhead points to red Cx43-expressing cells while the arrow indicates the Hcn4-expressing cells shown in green. Cx43-expressing cells showed only marginal overlap with Hcn4-positive cells in the SAN region, but were found in the adjacent atrial myocardial cells and extending into the myocardium of the ventricle and outflow tract regions as well. This was true for both adrenergic-competent and deficient E10.5 hearts, though the intensity of the Cx43 staining in the deficient hearts was much less than that seen in the competent hearts (compare panels c and d). This effect appeared to be specific for Cx43 since anti-sarcomeric α-actinin staining in adjacent sections showed similar intensity and distribution for both adrenergic-competent and deficient hearts (compare red staining in panels e and f). Further, since the same secondary antibody source and concentration were used for both sarcomeric α-actinin and Cx43, the relative decrease in Cx43 staining intensity was probably not due to differential effects of the secondary antibody,

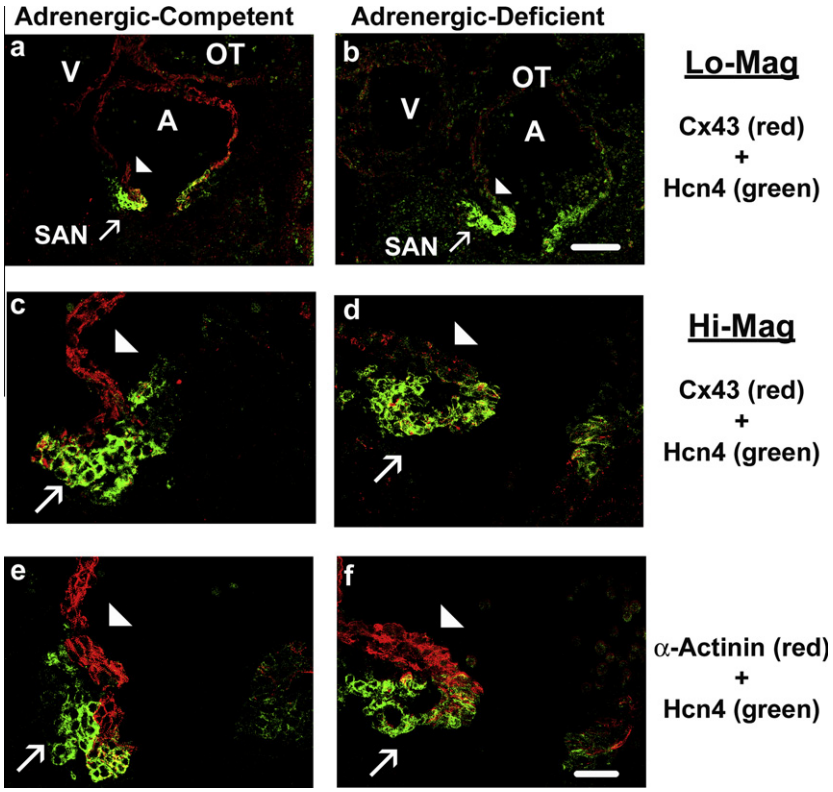

Fig. 1. Immunofluorescent histochemical evaluation of Cx43 relative to Hcn4 and sarcomeric α-actinin expression in adrenergic-competent and deficient E10.5 mouse hearts. (a,b) Low-magnification (Lo-Mag, 20X objective) views of adrenergic-competent and deficient mouse hearts, respectively, for Cx43 (red) and Hcn4 (green). Co-localized areas appear yellow. Scale bar for panels a-b, 0.1 mm. (c,d) High-magnification (Hi-Mag, 60X objective) views of the corresponding SAN regions (arrows) depicted in panels a and b, respectively, for adrenergic-competent and deficient hearts stained for Cx43 and Hcn4. (e,f) Hi-Mag view of an adjacent section co-stained for sarcomeric α-actinin (red) and Hcn4 (green) in adrenergic-competent and deficient hearts. Arrow indicates Hcn4 staining (a-f), and arrowhead indicates either Cx43 (a-d) or α-actinin (e,f) in approximately equivalent regions of each heart. Scale bar for panels c-f, 25 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

but instead likely reflects changes in Cx43 expression resulting from lack of adrenergic stimulation.

We similarly analyzed other regions of the heart, and found that Cx43 immunofluorescent histochemical staining was also decreased in the atrioventricular (A-V) area of adrenergic-deficient compared to adrenergic-competent E10.5 embryos (Supplemental Fig. 1). Low-magnification views of sarcomeric α-actinin immunofluorescent staining in adrenergic-competent and deficient hearts (panels a and b) are provided to aid in orientation of the higher-magnification views of Cx43 staining in these same sections, as shown in panels c-f. When camera exposure times and image processing were optimized for viewing of Cx43 in adrenergic-competent E10.5 mouse hearts, virtually no Cx43 staining was observed in the A-V junctional region of adrenergic-deficient hearts using equivalent image acquisition and processing settings (compare panels c and d, "dim"). Cx43 was not entirely absent from the A-V region of the adrenergic-deficient hearts, however, as it can be seen when image processing was adjusted to increase sensitivity. Equivalent processing of the images from adrenergic-competent hearts led to overexposure of Cx43 immunofluorescent staining, though the comparison with the deficient example under the same conditions ("bright") still clearly demonstrate the disparity of staining intensities between adrenergic-competent and deficient E10.5 in the A-V regions (compare panels e and f). These results further demonstrate that Cx43 expression is selectively diminished in E10.5 adrenergic-deficient myocardium relative to that found in adrenergic-competent hearts at this stage of development.

When examined one day earlier in development (E9.5), however, Cx43 staining intensity and distribution appeared similar in adrenergic-competent and deficient mouse hearts (Fig. 2). Hcn4 immunostaining was also similar between these groups at E9.5 (compare panels a and b). Comparative views of Cx43 are shown at low (panels c and d) and high (panels e and f) magnification. These results demonstrate that the Cx43 immunofluorescent staining is not inherently diminished in adrenergic-deficient hearts throughout development, suggesting that decreased Cx43 expression occurs after E9.5.

To determine if the electrical properties of the developing embryonic heart were altered as a result of adrenergic-deficiency, we cultured isolated E10.5 adrenergic-competent and deficient hearts on MEAs and measured extracellular field potentials from different regions of the heart. These included the sinoatrial region (SAR) near the sinoatrial node (SAN), the interchamber atrioventricular junction region (AVJ), and ventricular apex (APX), as illustrated in Fig. 3. Representative field potential traces from adrenergic-competent and deficient hearts are shown from simultaneous MEA recordings at the SAR and APX electrodes, indicating a general lengthening of the conduction time from SAR to APX in deficient hearts. MEA measurements were thus obtained for a series of these *ex vivo* embryonic heart preparations, and the combined results are summarized in Table 1, where it can be seen that the atrioventricular conduction time (AVJ-APX), which includes transmission time through the AVJ, was roughly twice the duration in adrenergic-deficient hearts compared with competent control hearts (31 ± 6 vs. 15 ± 2 ms, respectively, $n = 5/6$, $p < 0.05$). In contrast, atrial conduction times (SAR-AVJ) were not significantly altered between the groups. The adrenergic-deficient hearts displayed slightly slower intrinsic heart rates and longer

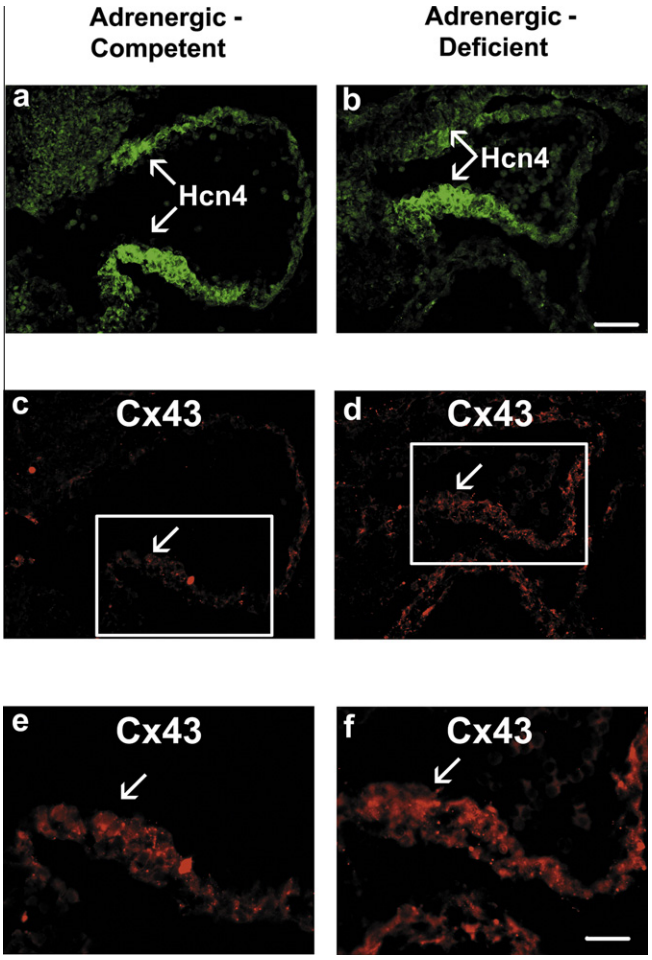

Fig. 2. Immunofluorescent histochemical evaluation of Cx43 and Hcn4 expression in adrenergic-competent and deficient E9.5 mouse hearts. (a,b) Hcn4 (green) staining (arrows) in adrenergic-competent and deficient hearts, respectively. (c,d) Cx43 (red) staining (arrow) in adrenergic-competent and deficient hearts, respectively. Scale bar for a-d, 50 μm. (e,f) Expanded view of the Cx43 (boxed insets from panels c and d) in adrenergic-competent and deficient hearts, respectively. Scale bar for e-f, 25 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

SAR-APX conduction times than adrenergic-competent controls on average, but these differences were not significant. These data suggest that there was a selective slowing of atrioventricular conduction velocity in adrenergic-deficient E10.5 hearts whereas atrial conduction and average beating rates were not significantly altered under these conditions.

To determine if adrenergic-deficient embryonic mouse hearts show increased susceptibility to arrhythmia, we cultured adrenergic-deficient and adrenergic-competent hearts *ex vivo* following isolation at E9.5 and E10.5. Heart rate and rhythmicity were measured at baseline and after challenge with the β-adrenergic receptor agonist, isoproterenol (0.5 μM). This challenge resulted in consistent 30–40% increases in heart rates for all groups within 2 min after drug application (Fig. 4a). Baseline and isoproterenol-induced heart rates were not significantly different between adrenergic-deficient and competent hearts at either E9.5 or E10.5 (Supplemental Table). Arrhythmic index (AI) was used to measure the rhythmicity of these isolated hearts before and after isoproterenol treatment as previously reported and described in the Materials and Methods section [9]. A significant ($p < 0.05$) 225% increase in AI from the adrenergic-deficient E10.5 mouse hearts was observed following the isoproterenol challenge (Fig. 4b). Representative examples of adrenergic-competent (rhythmic beating) and adrenergic-deficient (arrhythmic beating) hearts are provided in Supplemental Videos 2 and 3, respectively. Relatively small non-significant ($p > 0.05$) increases in AI (< 50%) were observed for adrenergic-competent hearts at E10.5, and no significant differences were observed in AI for either group at E9.5 (Fig. 4b). These results demonstrate that by E10.5, adrenergic-deficient mouse hearts were significantly more arrhythmic compared with adrenergic-competent controls following acute challenge with isoproterenol.

4. Discussion

Initially, we aimed to examine protein staining intensity and distribution patterns for Cx43 and Hcn4 to determine if these were altered in adrenergic-deficient embryonic hearts around the time of their demise. Cx43 staining was less intense relative to that for sarcomeric α-actinin and Hcn4 in adrenergic-deficient hearts compared with competent controls at E10.5. Our co-immunofluorescent histochemical staining data show that was not true one day earlier at E9.5, where Cx43 expression was not diminished in adrenergic-deficient hearts relative to controls or to sarcomeric α-actinin and Hcn4 staining. These results indicate that Cx43 appears to develop normally through E9.5 in adrenergic-deficient hearts, but that it either becomes down-regulated or fails to be up-regulated due to the lack of NE and EPI, such that by E10.5, Cx43 expression was substantially lower in $Dbh^{-/-}$ hearts than in controls. The pattern of Cx43 distribution, which remained diffuse and spotty as has been seen in other studies at these early developmental stages [2,3,10,18], did not appear to be altered in adrenergic-deficient mouse hearts, but the intensity of the staining for Cx43 was substantially reduced compared with equivalent staining in the control group.

Disruption of both Cx43 alleles ($Cx43^{-/-}$) results in lethality at birth due to cardiac malformation resulting from swelling and blockage of the right outflow tract [20]. Surprisingly, cardiac conduction speeds were not altered much at E12.5, the earliest developmental timepoint measured in $Cx43^{-/-}$ hearts, though significant slowing became apparent by E15.5 and continued to lengthen thereafter [27]. Cardiac-restricted Cx43 knockouts survive through birth, but die within the first two months of life from sudden cardiac death due to severe arrhythmias [11]. Ventricular conduction velocity was also markedly slowed in these animals. Additionally, cultured embryonic myocytes derived from $Cx43^{-/-}$ hearts show dramatic reductions in conduction speeds relative to $Cx43^{+/-}$ or $Cx43^{+/+}$ controls [1]. Thus, it is well-established that Cx43 plays a critical role in mediating ventricular conduction through the fetal and early postnatal periods. At earlier stages of embryonic development, it may be less critical as compensation by Cx40 and Cx45 appear to help ameliorate conduction slowing due to the absence of Cx43 [27], though there is evidence suggesting that their expression and activity may also be compromised in $Cx43^{-/-}$ myocytes [1,15,16,29]. These caveats notwithstanding, lowered Cx43 expression in adrenergic-deficient hearts could contribute to the observed slowing of atrioventricular conduction. We expect to find additional targets of embryonic adrenergic hormone action in the developing heart that will also likely contribute to the slowed atrioventricular conduction and arrhythmogenic phenotype of $Dbh^{-/-}$ mice.

Microelectrode arrays provided *ex vivo* heart rate and conduction analysis of cultured adrenergic-competent and deficient embryonic hearts. One of the limitations of the MEA analysis, however, was the relatively large electrode-electrode distance (200 μm) compared to the small heart (~600 × ~800 μm). In addition, the 2-dimensional MEA surface could only examine the ventral plane of the 3-dimensional heart. Despite these limitations,

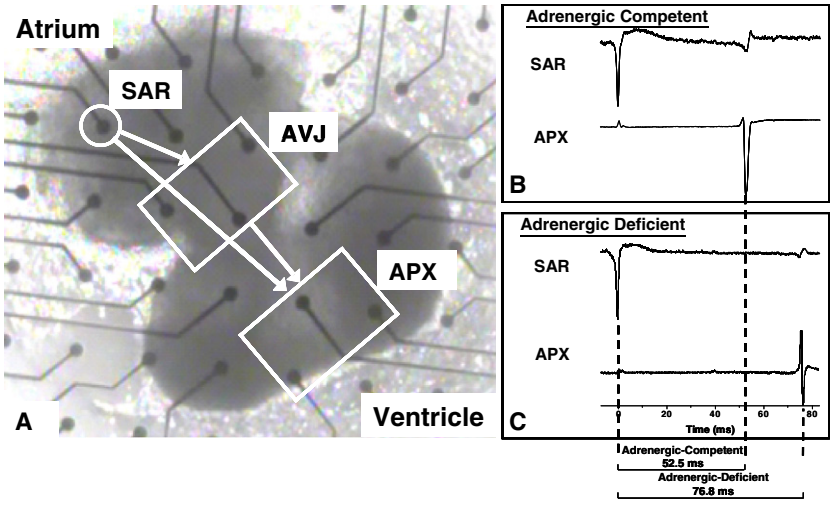

Fig. 3. Illustrative conduction paths and representative field potential traces from adrenergic-competent and deficient E10.5 mouse hearts cultured *ex vivo* on MEAs. (a) Representative heart shown without anchoring grid for visual clarity. Circle indicates first electrode to depolarize in the sinoatrial region (SAR). Boxes indicate electrodes depolarizing near-simultaneously (within 0.5 ms of each other) in the atrioventricular junction region (AVJ) and ventricular apex (APX). Arrows display overall evaluated conduction paths. (b and c) Representative field potential recordings were aligned and compared from the SAR and APX regions of adrenergic-competent and deficient hearts, respectively. Vertical dashed lines were drawn to show how conduction times were measured from the FP_{min} (see Materials and Methods for more details on how these measurements were performed) [9]. Cumulative data from several experiments is shown in Table 1.

Table 1
Summary of MEA data from E10.5 mouse hearts.

	Adrenergic Competent	Adrenergic-Deficient
Heart Rate (bpm)	148.6 ± 10.7 (11)	120.7 ± 6.7 (6)
Conduction Time: SAR-APX (ms)	52.1 ± 4.0 (11)	64.8 ± 9.1 (6)
Conduction Time: SAR-AVJ (ms)	33.8 ± 3.0 (6)	29.8 ± 4.8 (5)
Conduction Time: AVJ-APX (ms)	15.4 ± 1.7 (6)	31.4 ± 6.4*(5)

* $p < 0.05$.

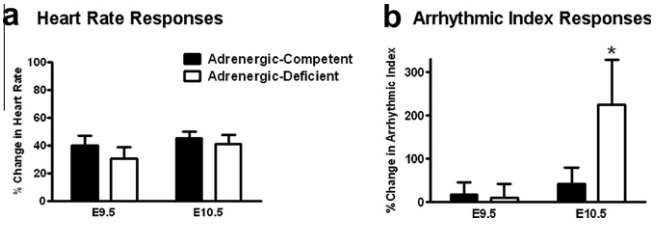

Fig. 4. Heart rate and arrhythmic index responses to isoproterenol challenge in adrenergic-competent (black columns) and adrenergic-deficient (white columns) embryonic mouse hearts isolated at E9.5 and E10.5. (a) Percent change in heart rates following acute (2-min) challenge with 0.5 μm isoproterenol. (b) Percent change in arrhythmic index (AI) in response to acute isoproterenol (0.5 μm) challenge in adrenergic-competent and deficient embryonic mouse hearts. *, $p < 0.05$ relative to control adrenergic-competent E10.5 hearts.

the data from the MEA experiments demonstrated that atrioventricular conduction was slower in adrenergic-deficient hearts. Of note, it has been reported that E10.5 is approximately the stage of development in the mouse when ventricular conduction transitions from a base-to-apex to an apex-to-base pattern of excitation [21]. It is therefore conceivable that adrenergic hormones could play a role in mediating this transition in the developing heart. Future experiments are needed to further explore the underlying molecular mechanisms that mediate adrenergic influence on the development of atrioventricular conduction.

Failure to complete this transition in a coordinated and timely manner could have critical consequences for heart development and embryonic survival if the myocardium becomes more susceptible to arrhythmogenesis. Adrenergic-competent and deficient embryonic hearts did not show a difference in basal AI; however, isoproterenol challenge did induce significantly increased AI in adrenergic-deficient E10.5 hearts compared to controls. This indicates that in *ex vivo* cultured conditions adrenergic hormones help maintain a balance of rhythmic beating even after increased stimulation. Other mechanisms of β-adrenergic regulation, such as synchronization of intracellular Ca^{2+} oscillations [23], may also impact arrhythmia susceptibility in adrenergic-deficient hearts. Consequently, we cannot say for certain if the altered Cx43 expression and slowed atrioventricular conduction observed in adrenergic-deficient hearts were contributory to the increased propensity for arrhythmias. If such arrhythmias are triggered *in vivo*, they could certainly contribute to the observed heart failure and fetal lethality in adrenergic-deficient mice, but further study is required to make that determination.

In summary, we have shown that adrenergic deficiency led to decreased Cx43 in E10.5 but not E9.5 mouse hearts. We have also for the first time shown that atrioventricular conduction is significantly slower in adrenergic-deficient hearts, and that arrhythmic activity was significantly induced in adrenergic-deficient hearts compared with adrenergic-competent controls. In contrast, pacemaking cell development and gross cardiac structural development of the early muscle chambers appear relatively normal in adrenergic-deficient embryos through E10.5. Thus, our data do not support the hypothesis that adrenergic hormones are critical for development of cardiac pacemaking, but they are supportive of a role for adrenergic hormones in stimulating Cx43 expression, facilitating atrioventricular conduction, and helping to maintain cardiac rhythm during a critical early period of embryonic heart development.

Acknowledgments

This work was supported by NIH Grants to SNE (HL078716) and JH (5RO1EB005459), and a postdoctoral fellowship (DGT) from the American Heart Association (0825395E).

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