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

Candice Baker [a,1], David G. Taylor [a,1,2], Kingsley Osuala [a,3], Anupama Natarajan [b], Peter J. Molnar [b,4], James Hickman [b], Sabikha Alam [a], Brittany Moscato [a], David Weinshenker [c], Steven N. Ebert [a,*]

[a] Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, 6900 Lake Nona Blvd, Orlando, FL 32827, USA
[b] Nanoscience Technology Center, University of Central Florida, 12424 Research Parkway, Suite 400, Orlando, FL 32826, USA
[c] Department of Human Genetics, Emory University School of Medicine, Whitehead 301, 615 Michael Street, Atlanta, GA 30322, USA

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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.

* Corresponding author. Fax: +1 407 266 7002.
E-mail address: Steven.Ebert@ucf.edu (S.N. Ebert).
[1] These two authors contributed equally to this work.
[2] Department of Biology, Seminole State College of Florida, 100 Weldon Blvd., Sanford, FL 32773.
[3] Wayne State University, Department of Pharmacology, 540 E. Canfield, Detroit, MI 48201.
[4] Institute of Biology, Faculty of Natural Sciences, University of West Hungary, Károlyi Gáspár tér 4, Szombathely, H-9700, Hungary.

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"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,