Null Mutation of Connexin43 Causes Slow Propagation of Ventricular Activation in the Late Stages of Mouse Embryonic Development

Dhananjay Vaidya, Houman S. Tamaddon, Cecilia W. Lo, Steven M. Taffet, Mario Delmar, Gregory E. Morley, José Jalife

Abstract—Connexin43 (Cx43) is the principal connexin isoform in the mouse ventricle, where it is thought to provide electrical coupling between cells. Knocking out this gene results in anatomic malformations that nevertheless allow for survival through early neonatal life. We examined electrical wave propagation in the left (LV) and right (RV) ventricles of isolated Cx43 null mutated (Cx43−/−), heterozygous (Cx43+/−), and wild-type (WT) embryos using high-resolution mapping of voltage-sensitive dye fluorescence. Consistent with the compensating presence of the other connexins, no reduction in propagation velocity was seen in Cx43−/− ventricles at postcoital day (dpc) 12.5 compared with WT or Cx43+/− ventricles. A gross reduction in conduction velocity was seen in the RV at 15.5 dpc (in cm/second, mean [1 SE confidence interval], WT 9.9 [8.7 to 11.2], Cx43−/− 9.9 [9.0 to 10.9], and Cx43+/− 2.2 [1.8 to 2.7; P<0.005]) and in both ventricles at 17.5 dpc (in RV, WT 8.4 [7.6 to 9.3], Cx43−/− 8.7 [8.1 to 9.3], and Cx43+/− 1.1 [0.1 to 1.3; P<0.005]; in LV, WT 10.1 [9.4 to 10.7], Cx43−/− 8.3 [7.8 to 8.9], and Cx43+/− 1.7 [1.3 to 2.1; P<0.005]) corresponding with the downregulation of Cx40. Cx40 and Cx45 mRNAs were detectable in ventricular homogenates even at 17.5 dpc, probably accounting for the residual conduction function. Neonatal knockout hearts were arrhythmic in vivo as well as ex vivo. This study demonstrates the contribution of Cx43 to the electrical function of the developing mouse heart and the essential role of this gene in maintaining heart rhythm in postnatal life. (Circ Res. 2001;88:1196-1202.)

Key Words: connexin ■ development ■ arrhythmia ■ electrophysiology ■ knockout

Gap junction channels (connexins) are specialized membrane structures that allow for direct communication between neighboring cells.1 Although previous work documented the spatial and temporal patterns of expression of different connexin isoforms,2–12 the functional correlates of this diversity of connexins are poorly understood. Analysis of mice with null mutations has shed some light on the function of specific connexin genes and their products.13–17 Connexin43 (Cx43) has widespread spatial distribution from the 2-cell stage through postnatal life in the mouse.18 However, the null mutation of the Cx43 gene results in anatomic malformations that are incompatible with postnatal life.19 The cardiac phenotype of the Cx43 null mutation includes delayed looping,19 intertrabecular pouching of the right ventricular cavity, and right ventricular outflow tract obstruction.13 The presence of other connexins, notably Cx40,19 may explain why the heart functions through gestation. However, it is not known at what stage Cx43 becomes the predominant gap junction involved in electrical impulse propagation. Thus, the Cx43−/− embryo presents a unique system in which to study the role of this protein in electrical conduction in the developing heart as it replaces other connexins and becomes the predominant cardiac isoform during late gestation.20 Because of the small size and fragility of the embryonic heart, traditional techniques to study electrical activity such as multiple extracellular electrodes or microelectrode mapping may present great technical difficulties. High-resolution optical mapping of voltage-dependent changes in cardiac tissue21–23 has previously been used in the adult mouse,24,25 the embryonic chick,26,27 and other rodent hearts,26 as well as cellular preparations.28,29 This study uses optical mapping to explore the role of Cx43 in cardiac impulse propagation during the embryonic development and the early postnatal life in the mouse.

Materials and Methods

Heart Preparations

All animal care protocols conformed to institutional and NIH guidelines. The mouse colony was founded by a breeding pair (129Sv/C57BL6/CD1 strain) heterozygous for the Cx43 knockout mutation. Pregnant dams were euthanized at 12.5, 15.5, and 17.5 days postcoitum (dpc) to obtain embryonic hearts. While embryonic preparations were superfused, hearts of 5 days postpartum (dpp) mice were also perfused via aortic cannulation. No pharmacological or mechanical motion-reduction techniques were used during any of
the experimental protocols. Voltage-sensitive dye fluorescence from stained hearts was mapped optically on an upright microscope equipped with a charge-coupled device camera (Dalsa Inc, model CA-D1 128T) as discussed in detail previously.24,30

**Pacing Protocol for Measurement of Conduction Velocity**

Hearts at 12.5 and 15.5 dpc as well as those at 5 dpp were allowed to beat in sinus rhythm, and optical mapping records were obtained to measure the heart rate. A suction glass electrode with an outer diameter of 25 to 100 μm and a fire-polished tip was used to deliver unipolar pacing stimuli to the ventricles. The left (LV) and right (RV) ventricles were separately paced at a basic cycle length (BCL) of 300 ms at 12.5 and 15.5 dpc, 200 ms at 17.5 dpc, and 100 ms at 5 dpp using 4-ms stimuli at 1.5 times diastolic threshold. Activity during pacing and during arrhythmias, if any, was recorded for 4 seconds. Pacing was attempted in arrhythmic Cx43−/− hearts at 5 dpp with unipolar as well as bipolar silver/silver chloride electrodes with diameters ranging from 0.2 to 1 mm. Optical movies of paced activity were signal-averaged as described previously.24,30,33 The conduction velocity in the slowest direction was quantified for the RV and LV for all the time points studied.

**RNase Protection Assay**

Ventricles of hearts at 17.5 dpc used to extract total cellular RNA were dissected in ice-cold Tyrode’s solution and immediately homogenized in Triazol reagent (MRC Inc), and total tissue RNA was extracted according to the manufacturer’s protocol. Antisense probes were designed to recognize regions within the coding sequences of Cx40, Cx43, and Cx45. A probe for the mouse housekeeping gene cyclophilin was used as a control for loading. RNase protection assay (RPA) was carried out using the Riboquant RPA kit (Pharmingen). At 17.5 days, the cyclophilin signal showed no genotype-dependent changes (in million counts per 15 hours, wild type [WT] 3.55 ± 0.87 [n=4], Cx43−/− 3.47 ± 0.38 [n=8], and Cx43+/− 3.62 ± 0.68 [n=3]). Common RPA signals were quantified as a percentage of cyclophilin signal.

**Microelectrode Recordings**

Recordings were obtained using 15- to 30-MΩ microelectrodes filled with 3 mol/L KCl solution. The signals were appropriately conditioned and sampled at 15 to 20 kHz. The resting membrane potential, action potential amplitude, and maximum upstroke velocity (dV/dtmax) were analyzed in 15.5-dpc embryos.

**Electrocardiography in Conscious Mice**

A custom-built chamber with four silver-silver chloride footpads were placed in the chamber such that each foot of the mouse made contact with a separate electrode. No physical restraint is required at this stage. Signals were amplified and low-pass filtered with a differential amplifier (CyberAmp 380, Axon Instruments), digitized (Digidata 1200) at 5 kHz, and stored for offline analysis.

**Statistical Analysis**

All values are reported as confidence intervals of mean±SE. Differences were considered significant at the P<0.05 level using ANOVA.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

**Results**

**Viability of the Preparation**

Heart rates at sinus rhythm were recorded for superfused embryonic hearts at 12.5 and 15.5 dpc, as well as perfused 5-day postnatal hearts. The heart rates were comparable for all genotypes (in bpm, for 12.5 dpc, WT 117.6±13.4 [n=10], Cx43+/− 118.5±8.3 [n=24], and Cx43−/− 99.9±10.7 [n=10]; for 15.5 dpc, WT 172.5±22.1 [n=15], Cx43+/− 154.8±17.6 [n=13], and Cx43−/− 140.7±19.3 [n=14]; and for 5-day postnatal, WT 444.5±16.1 [n=14], Cx43+/− 436.5±12.1 [n=15], and Cx43−/− all arrhythmic [n=4]). The atria of all 17.5-dpc embryonic hearts were crushed to make them asystolic. The above cycle lengths are comparable with those recorded previously for in vivo23,31 and superfused embryonic hearts34 as well as for perfused postnatal hearts.24,31,35

**Optical Mapping of Paced Electrical Activity**

By 12.5 dpc, the muscular septum of the ventricle grows apace and is nearly fully formed.26 An anatomic deformity in the angle of the bending of the heart tube is already apparent in the Cx43−/− heart by this time point.19 At this stage, both Cx43 and Cx40 are strongly expressed throughout the WT ventricles.4 The velocities in the direction of slowest propagation during pacing of the RV and LV at a BCL of 300 ms are tabulated in Table 1. The RV conduction velocities were reduced compared with the LV in all genotypes (P<0.005). However, no significant genotype-related change in velocity was found in either ventricle.

At 16 dpc, the chamber formation in the heart is essentially complete.36 Cx40 is no longer detected in the RV, although it is detected in the LV trabeculated myocardium.19 As seen in Table 1, no LV conduction velocity differences are seen between genotypes during ventricular pacing at the BCL of 300 ms in 15.5-dpc hearts. However, RV conduction is grossly reduced in the Cx43−/− hearts compared with the other genotypes (P<0.005). Thus, it appears that the presence of Cx40 in the trabeculated myocardium of the LV is adequate to maintain propagation within the whole wall. The voltage-dependent fluorescence signal is expected to originate throughout the depth of the ventricular wall17; the optical mapping records may not reflect isolated slow conduction within the compact layers of the LV myocardium, if it occurs.

By day 18, Cx40 is downregulated throughout the left and right ventricular myocardium.19 The effect of this downregulation of all connexins other than Cx43 in the entire ventricular myocardium is seen as an extreme reduction in the conduction velocity in both ventricles of the Cx43−/− heart at this stage (Table 1, P<0.005). Both the LV and the RV show similar conduction times in WT and Cx43+/− hearts. Videos of representative activation sequences in the LV and RV at all embryonic points can be found in an online data supplement available at http://www.circresaha.org.

**Arrhythmias in Cx43−/− Hearts**

No arrhythmias were observed in any of the embryonic hearts at 12.5 or 15.5 dpc. At 17.5 dpc, none of the 20 WT hearts and one of the 35 Cx43+/− hearts showed ventricular arrhythmia at some point during the protocol. Of the 20 Cx43−/−
hearts studied, 10 underwent ventricular arrhythmias throughout the periods of observation and could not be paced. Of the remaining hearts, eight showed arrhythmia at some point during the protocol, often induced by the pacing, whereas two hearts showed no arrhythmias (P < 0.005). An example of the activation pattern during spontaneous arrhythmia showing a transient reentrant circuit initiated by ectopic activity that degenerates into a polymorphic tachycardia has been presented in an online data supplement available at http://www.circresaha.org.

All four of the Cx43−/− hearts mapped at 5 dpp were arrhythmic. One heart showed polymorphic activity, whereas three hearts showed monomorphic activation patterns. The activation map during this polymorphic tachycardia is shown in Figure 1. The reentrant activity lasted for three rotations (thick white arrow), after which the rotor drifted out of the field of view. A complex pattern of breakthrough (asterisk) and block and collision (thin arrows) was observed in the regions adjacent to the reentrant circuit, suggesting a 3-dimensional component to the reentry. The trace below is the pseudo-ECG, which is the summary of the whole movie in time. The polymorphic pattern is apparent in the trace. Two of the monomorphic patterns involved waves entering the field of view from one side, whereas one showed a diffuse breakthrough within the field of view. Although focal activity cannot be excluded, slow conduction in this phenotype makes it probable that anatomic or anchored 3-dimensional reentry may be responsible for these arrhythmias.

**Electrocardiography in 5-Day Postnatal Mice**

We studied the electrocardiographic phenotype of the Cx43−/− mouse to determine whether the arrhythmias observed above were only an artifact of Langendorff perfusion. The ECGs were obtained in the conscious state to avoid the nonspecific and hypothermic effects of anesthesia. In Figure 2, the heart rates recorded in WT mice (heart rate 510 ± 20 bpm and PR interval 44.9 ± 2.1 ms [n = 14], trace A) and Cx43+−/− mice (heart rate 495 ± 16 bpm, PR interval 45.1 ± 1.2 ms [n = 21], trace B) are comparable with those previously reported in conscious neonatal mice. Although none of the WT or Cx43−/− hearts studied showed any arrhythmias, all 5 Cx43−/− mice showed ECG abnormalities. Individual examples are shown in Figure 2, traces C through E. Although the atrial activation rate was not significantly reduced compared with the other genotypes (476 ± 16 bpm [n = 4]), one animal had second-degree heart block (trace C and inset), three had third-degree block (trace D), and one had ventricular arrhythmia (trace E). Thus, Cx43−/− mice have cardiac rhythm abnormalities in the conscious state.

**Microelectrode Recordings in 15.5-dpc Hearts**

The velocity of conduction depends on both the axial resistance for current flow and the excitatory currents available for
depolarization. We excluded genotype-specific reduction in the excitatory currents by recording the upstroke of the action potential in hearts at 15.5 dpc. Figure 3 shows representative action potential upstrokes and dV/dt signals from the RV and LV of the three genotypes. Faster upstroke velocities can be seen in Cx43−/− ventricles compared with WT and Cx43+/− ventricles. Table 2 summarizes the electrophysiologic properties of three WT, 5 Cx43−/−, and three Cx43+/− hearts. Although there is no significant difference between the resting membrane potential or the action potential amplitude between genotypes, the dV/dtmax is higher in the Cx43−/− mouse than in the WT and Cx43+/− hearts. The observation that the dV/dtmax is not reduced in the Cx43−/− embryos suggests that the slow propagation seen at this time point is not a result of a concurrent reduction in excitatory currents. This is consistent with the findings of Johnson et al,40 who studied sodium currents in Cx43−/− animals at term. However, that study found no increase in the upstroke velocity in isolated myocytes.40 The paradoxical increase in the upstroke velocity gives credence to a computational prediction of Rudy and Quan41 that in the presence of unchanged excitatory currents, the higher input resistance of poorly coupled cells must result in higher upstroke velocities. Note that only RV and not LV conduction velocity was found to be reduced in the Cx43−/− heart at 15.5 dpc. However, this conduction function may be sustained by the trabeculated myocardium, which expresses Cx40.19 The microelectrode recordings are superficial and likely to sample only the compact layer of the ventricular myocardium, in which no Cx40 or Cx43 is found in the Cx43−/− heart.19 This may explain the higher dV/dtmax observed in microelectrode recordings from both ventricles at this stage.

### Presence of Other Connexins in the Late Cx43−/− Embryo

By day 18, little or no Cx40 has been demonstrated by immunohistochemistry and in situ hybridization of mRNA, and little or no Cx45 has been demonstrated by in situ hybridization.19 To determine what connexin is responsible for the residual slow conduction, we performed RPAs for Cx40, Cx43, and Cx45 as a sensitive measure of RNA message in 17.5-dpc ventricles. As seen in Figure 4, signals for Cx43, Cx40 and Cx45 are all detected in WT and Cx43+/− hearts in quantities greater than that found in whole Cx43−/− embryonic RNA. Cx43 signal as a percentage of the cyclophilin signal is reduced in the Cx43−/− heart (in percentage of cyclophilin signal, WT 44.8±0.8 [n=4] and Cx43−/− 23.0±0.8 [n=8], P=10−9). Cx40 and Cx45 bands are also observed in microelectrode recordings from both ventricles at this stage.
are probably responsible for the residual conduction function during late gestation in Cx43<sup>−/−</sup> embryos.

**Discussion**

**Connexins and Conduction in Embryonic Life**

This study is the first to demonstrate developmental changes in ventricular conduction velocity in the LV and RV through fetal life and birth in WT and Cx43 knockout mice. The most important result of this study is the demonstration that the lack of Cx43 causes slowing of cardiac propagation late in the embryonic development of the Cx43<sup>−/−</sup> mouse. The important role played by connexins in cell differentiation and development is evidenced by their early and patterned expression in embryonic life. Perturbations in the expression of the cardiac connexins lead to a spectrum of malformations greatly differing in severity. Although the nonexpression of Cx45 causes early embryonic death, no reduction in viability is observed with the nonexpression of Cx40. Mice null mutated for the Cx43 gene show survival until birth, when they die of cyanosis caused by right ventricular outflow tract obstruction. Targeted replacement of the gene for Cx43 by the cardiac connexin Cx40 as well as the noncardiac gene Cx32 results in viable mice that survive into adult life, indicating that some of the vital functions of connexins are shared between these different isoforms. Thus, the developmental pattern of the expression of various connexins may be related to performance of their shared and unique functions during the different stages of embryogenesis. Cx45 has been detected in the embryonic heart at 8.5 dpc. It is downregulated in the ventricular myocardium by 12 dpc, after which very small quantities are detected except in the outflow tract. Between this point and 18 dpc, Cx40 and Cx43 appear in the ventricles at 10.5 dpc and continue to increase until 14.5 dpc. This downregulation first takes place in the RV, whereas the left ventricular trabecular myocardium still expresses Cx40. The results presented above show that the propagation velocity of electrical impulses in Cx43<sup>−/−</sup> embryos is reduced in the same sequence as the downregulation of Cx40. Because optical mapping at this stage involves recording throughout the depth of the ventricular wall, and given that propagation during ventricular pacing involves electrotonic coupling throughout the wall, the rapid propagation in the Cx43<sup>−/−</sup> LV at 15.5 dpc may be explained by the presence of Cx40 coupling deeper myocardial layers.

**Table 2. Microelectrode Measurements in 15.5-dpc Hearts**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Cx43&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Cx43&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Confidence Interval*</td>
<td>Mean</td>
</tr>
<tr>
<td>RMP, mV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV</td>
<td>-71.1</td>
<td>-68.6–73.6</td>
<td>-64.6</td>
</tr>
<tr>
<td>LV</td>
<td>-69.8</td>
<td>-65.1–74.5</td>
<td>-69.1</td>
</tr>
<tr>
<td>APA, mV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV</td>
<td>85.6</td>
<td>77.9–93.3</td>
<td>83.8</td>
</tr>
<tr>
<td>LV</td>
<td>87.6</td>
<td>83.4–89.7</td>
<td>80.9</td>
</tr>
<tr>
<td>dV/dt&lt;sub&gt;max&lt;/sub&gt;,† V/s</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV</td>
<td>56.0</td>
<td>47.7–65.6</td>
<td>54.2</td>
</tr>
<tr>
<td>LV</td>
<td>67.5</td>
<td>47.7–95.7</td>
<td>48.0</td>
</tr>
</tbody>
</table>

RMP indicates resting membrane potential; APA, action potential amplitude. *Confidence interval of 1 SE. †Confidence interval of logarithmic transform reported for dV/dt<sub>max</sub>.

**Figure 4.** RPA demonstrates presence of Cx40 and Cx45 in Cx43<sup>−/−</sup> ventricles at 17.5 dpc. Examples of connexin mRNA protection signals obtained from representative WT, Cx43<sup>−/−</sup>, and Cx43<sup>−/−</sup> hearts. For each connexin tested (Cx43, Cx40, and Cx45), lanes with the undigested probe and in vitro–transcribed positive control RNA show the location of the unprotected and protected lengths of the probe. Connexin signals were normalized with cyclophilin mRNA as internal control. Cx43 signal from WT and Cx43<sup>−/−</sup> hearts and the Cx40 and Cx45 signal from all genotypes are stronger than the signal seen from whole embryonic RNA from a Cx43<sup>−/−</sup> embryo. The Cx43 signal as a percentage of cyclophilin signal is seen to be reduced in the Cx43<sup>−/−</sup> heart compared with WT, whereas it is absent in the Cx43<sup>−/−</sup> heart. Similar Cx40 and Cx45 signals are seen in all hearts.
finding argues that conduction of electrical activation waves in the ventricle is a shared function of Cx43 and Cx40. The gross reduction in conduction velocity at 17.5 dpc in the Cx43−/− mouse also highlights the fact that Cx43 is largely responsible for the conductance of electrical impulses in the ventricles during late embryonic life. We may only speculate that the 2.5- to 3-fold increase in conduction velocity seen in WT and Cx43+/− mice between embryonic life and 5 dpp may occur as a result of an increase in Cx43 expression, or other ionic channels, or mechanical changes associated with circulation that occur during this period.

Conduction in the Cx43+/− Heart

There have been conflicting reports about reductions in conduction velocity in the Cx43+/− mouse compared with WT. In this study no significant difference was found in the conduction velocity in either ventricle. Indeed, previously reported differences in conduction velocities between Cx43+/− and WT were less dramatic at birth than during adult life.

Residual Conduction in the Cx43+/− Embryo

Cx40 has not been identified either by in situ hybridization of mRNA or by immunohistochemistry in 18-dpc mice, whereas little if any Cx45 has been detected by in situ hybridization at this stage either in WT, Cx43−/−, or Cx43+/− mice. Our results in pooled whole-ventricular RNA at this stage suggest that small quantities of Cx40 and Cx45 persist in the ventricles and may account for the residual conduction function seen in Cx43+/− embryos at this stage.

Relationship Between Excitatory Currents and Coupling

The excitatory current that travels from cell to cell is provided by inward ionic flow through sodium and calcium channels. It has been shown that reduction in inward currents can lead to a slowing in propagation velocity before conduction block ensues. However, this slowing of propagation is not as profound as that which can be achieved by a reduction of coupling. A key difference between the two mechanisms is that the dV/dt max is reduced with a primary deficit in excitatory currents, whereas it has a biphasic relationship with the degree of coupling. The first phase of reduction in coupling is associated with an increase in the dV/dt max caused by the higher-input resistance of the cell, whereas extreme degrees of uncoupling close to the onset of block are associated with a decrease in the dV/dt max. Our measurements in the 15.5-dpc embryo show that the knockout heart has a high dV/dt max, indicating that a reduction in excitatory currents is not the primary cause of slow propagation. These data are in agreement with previously published results showing that the sodium current in isolated ventricular knockout myocytes is not reduced compared with WT. Indeed, the higher dV/dt max suggests that reduced coupling is the primary cause of the reduction in conduction velocity. It is possible that the higher dV/dt max observed in the LV at this stage is due to the poor coupling of epicardial cells, although well-coupled subendocardial cells maintain conduction function as discussed above.

Arrhythmias in Cx43+/− Mice

Arrhythmias were commonly observed in Cx43−/− mice at 17.5 dpc, and as a rule after birth. The arrhythmias in 5-dpp mice were observed in the conscious state as well as ex vivo. Although the triggers for spontaneous arrhythmias may be focal, slow conduction likely makes sustained reentrant arrhythmias possible. It is possible that the hypoxia suffered by the mouse during postnatal life may be responsible for the arrhythmias. However, the embryonic circulation largely bypasses the pulmonary bed and receives oxygenation at the placenta. It is not expected that the late embryonic heart is hypoxic. The observation of spontaneous as well as pacing-induced arrhythmias at 17.5 dpc suggests that reduction in conduction velocity makes the heart an arrhythmogenic substrate. This strengthens the conjecture that slow propagation due to the absence of Cx43 and the hypoxia caused by the outflow tract malformation likely makes probable causes of death in the Cx43+/− heart.

Acknowledgments

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