Replacement of Connexin40 by Connexin45 in the Mouse
Impact on Cardiac Electrical Conduction

Sébastien Alcoléa, Thérèse Jarry-Guichard, Jacques de Bakker, Daniel González, Wouter Lamers, Steven Coppen, Luis Barrio, Habo Jongsma, Daniel Gros, Harold van Rijen

Abstract—Gap junction channels, required for the propagation of cardiac impulse, are intercellular structures composed of connexins (Cx). Cx43, Cx40, and Cx45 are synthesized in the cardiomyocytes, and each of them has a unique cardiac expression pattern. Cx40 knock-in Cx45 mice were generated to explore the ability of Cx45 to replace Cx40, and to assess the functional equivalence of these two Cxs that are both expressed in the conduction system. ECGs revealed that the consequences resulting from the biallelic replacement of Cx40 by Cx45 were an increased duration of the P wave, and a prolonged and fractionated QRS complex. Epicardial mapping indicated that the conduction velocities (CV) in the right atrium and the ventricular myocardium, as well as conduction through the AV node, were unaffected. The significant reduction of the CV in the left atrium would be the most likely cause of the P-wave lengthening. In the right ventricle, a changed and prolonged activation in sinus rhythm was found in homozygous mutant mice, which may explain the prolongation and splitting of the QRS complex. Electrical mapping of the His bundle branches revealed that this was due to slow conduction measured in the right branch. The CV in the left branch was unchanged. Therefore, in the absence of Cx40, the upregulation of Cx45 in the heart results in a normal impulse propagation in the right atrium, the AV node, and the left His bundle branch only. (Circ Res. 2004;94:100-109.)

Key Words: gap junction channels ■ connexins ■ cardiac conduction

In the mammalian heart the electrical activity, spontaneously generated in the sinoatrial (SA) node, is propagated through the atria, then to the ventricular walls via specialized tissues that constitute the conduction system (CS). The CS includes the atrioventricular (AV) node, the His bundle, and the two bundle branches (BBs), which ramify into peripheral Purkinje fibers. Gap junction channels are responsible for the electrical coupling between the various types of myocytes, and they are required for the propagation of cardiac impulse.1

Gap junction channels are intercellular structures constituted by transmembrane proteins belonging to the connexin (Cx) family.2,3 Nineteen Cx genes have been identified in the mouse genome,4 but so far only four Cxs, Cx43, Cx40, Cx45, and Cx46, have been shown to be expressed in mammalian cardiomyocytes with each having a unique expression pattern in the adult heart.5 Hence, in the mouse heart, Cx43 is abundant in both the atrial and ventricular working myocytes, and the distal part of the CS; Cx40 is strongly expressed in the atrial working myocytes and the CS,6,7 whereas Cx45 is detected mainly in the SA node and the CS.8-10 Expression of Cx45 has been reported in the working ventricular myocardium,11 but this finding has not been corroborated by other investigations.9,12 Lastly, Cx46 was detected in the rabbit SA node associated with Cx40 and Cx45.13,14

Analysis of Cx-deficient mice has provided evidence that Cx43, Cx40, and Cx45 are involved in both heart function and development. The Cx45 null mutation is lethal with the embryos dying in utero around embryonic day 10. 2 days after cardiac contractions are normally initiated.15,16 Several anomalies including defects in the endocardial cushions are thought to contribute to the lethal phenotype. Conditional knock-out (KO) mice in which Cx43 gene expression in the cardiomyocytes is reduced by about 90% have ventricular conduction velocity (CV) slowed by 50%. All these mice undergo sudden cardiac death from spontaneous ventricular arrhythmias by 2 months of age.17 Cx40KO mice are viable and fertile,18,19 and several studies have focused on impulse propagation in the hearts of these mice.20-25 Findings from these studies include impaired conduction at various levels of the CS, and increased incidence of inducible atrial tachyarrhythmias, indicating that Cx40 is an important determinant of impulse propagation in the atria and the AV conduction system. Recent investigations also suggest that Cx40 may play a role in cardiac morphogenesis.26

Original received May 12, 2003; resubmission received October 2, 2003; revised resubmission received November 6, 2003; accepted November 11, 2003.

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DOI: 10.1161/01.RES.0000108261.67979.2A
Figure 1. Expression of the transgene. A, Diagram indicating the positions of the primers (arrows) used in RT-PCR experiments to amplify fragments from wild-type (WT) Cx40, transgenic Cx45, and endogenous WT Cx45 transcripts. Transcribed exons 1 and 2 (E1 and E2) of the Cx40 gene are represented as white boxes. Transcribed exons 1, 2, and 3 of the Cx45 gene are represented as dotted boxes but only E3 is indicated. The expected sizes of amplicons are indicated. B, Representative results of RT-PCR experiments performed with RNA extracted from the atria of Cx40/H11001/H11001, Cx40/H11001/KICx45 (H11001/KI), and Cx40KICx45/KICx45 (KI/KI) 129Sv/CD1 mice. Similar results were obtained with 129Sv/C57Bl/6 mice. Amplicons of the expected sizes were detected in all samples investigated. The intensity of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signals indicated that equivalent amounts of synthesized cDNAs were analyzed. C, Representative results of Northern blot experiments performed with RNA extracted from atria of Cx40/H11001/H11001, Cx40/H11001/KICx45 (H11001/KI), and Cx40KICx45/KICx45 (K1/KI) 129Sv/CD1 mice. Similar results were obtained with 129Sv/C57Bl/6 mice. Intensity of signals obtained with the hypoxanthine phosphoribosyl-transferase (HPRT) probe indicated that equal amounts of cDNA were analyzed. Exposure times: 3 days, 5 days, 1 day, and 12 hours after hybridization with Cx40, Cx45, Cx43, and HPRT probes, respectively. D, Representative results of Western blot experiments performed with samples of atrial tissue collected from Cx40/H11001/H11001, Cx40/H11001/KICx45 (H11001/KI), and Cx40KICx45/KICx45 (K1/KI) 129Sv/CD1 mice. Similar results were obtained with 129Sv/C57Bl/6 mice. Intensity of the signals obtained after treatment with anti-desmin antibody (Des.) indicated that similar amounts of proteins were probed with each of the anti-Cx antibodies. Molecular mass of the detected proteins is indicated on the right.
The analysis of KO mice has therefore provided important information on the in vivo functions of Cxs in heart. Another approach for analyzing the function of Cxs is to generate knock-in (KI) mice to assess the functional equivalence of two Cxs. Cx40 and Cx45 are both expressed in the mouse CS, albeit with very different levels of expression. Channels composed of Cx40 or Cx45 have quite different properties. Cx40 channels have a large unitary conductance (≈160 pS), in contrast with Cx45 channels, which have a small conductance (≈30 pS).27,28 Macroscopic conductance of Cx40 channels is not very sensitive to the transjunctional voltage (Vj) as compared with that of Cx45 channels, which is very sensitive to Vj.29–32 Furthermore, both types of channels are differently regulated by phosphorylation.28,33 To assess the functional equivalence of Cx40 and Cx45, we have generated Cx40KICx45 mouse lines in which Cx45 is expressed in lieu and place of Cx40.

Materials and Methods

The strategy used to generate the transgenic mice and the techniques used for genotyping the ES cells and mice and for identifying expression of the transgene in the KI mice are described in detail in the expanded Materials and Methods in the online data supplement (available at http://www.circresaha.org). The expression of the transgene was assessed on three hearts from adult mice (8 to 12 weeks old), from three independent litters, for each type of experiment performed (RT-PCR, Northern and Western blots, immunofluorescence), and for each genotype investigated. In addition, all experiments were performed with samples from both Sv129/CD1 and 129Sv/C57Bl/6 mice.

ECG recordings were performed on anesthetized mice as described previously.25,34 The hearts were dissected, connected to a Langendorff setup, and extracellular epicardial electrograms were recorded during sinus (SR) or paced rhythm.25,34 Protocols for measuring epicardial activation and the conduction velocity (CV) in the various cardiac tissues including the BBs are described in the online data supplement. Multiple group comparisons were performed using ANOVA with LSD post hoc analysis using SPSS 10 for Macintosh. Values are mean±SEM. Values of P≤0.05 were considered statistically significant.

Results

Generation of the KI Mice

KI mice were generated on two genetic backgrounds:129129/CD1 (50%/50%) and 129Sv/C57Bl/6 (50%/50%). No abnormal embryonic lethality was detected. The mice grew normally, were fertile, and had no obvious anatomical cardiac defects.

Transcriptional and Translational Expressions of the Transgene

The results revealed no significant difference dependent on the genetic background and were reproducible for each type of experiment performed. The expression of Cx40, Cx43, and transgenic Cx45 mRNAs in the atria of wild-type (wt, Cx40+/+), heterozygous (Cx40+/KICx45) and homozygous (Cx40KICx45/KICx45) mice was determined by semiquantitative RT-PCR (Figure 1B) and Northern blot experiments (Figure 1C). No endogenous Cx45 expression was detected (Figures 1B and 1C), but a gene-dependent gain of Cx45 mRNA, accompanied by a gene-dependent loss of Cx40 transcript (Figures 1B and 1C) was seen in Cx40+/KICx45 mice. The expression level of Cx45 mRNA in Cx40+/KICx45 mice was comparable to that of Cx40 mRNA in Cx40+/+ mouse (Figures 1B and 1C).
The levels of expression of the transcript of the Cx43 gene in the three types of mice investigated were similar (Figure 1C).

Cx45 protein was correctly translated in the transgenic mice, as shown by Western blot experiments performed with atrial samples (Figure 1D). The reduction of the level of expression of Cx40 protein in the atria of Cx40<sup>H11001/H11001</sup> mice (Figure 1D) was associated with the detection of Cx45 protein in the same tissue. Cx40 protein was not detected in the atria samples of Cx40<sup>KICx45/KICx45</sup> mice. In contrast, Cx45 protein was shown to be expressed in these same samples with a level of expression higher than that observed in the heterozygotes. The level of expression of Cx43 protein was unaffected by the expression of the transgene (Figure 1D).

The pattern of expression of Cx40, Cx45, and Cx43 was further investigated by immunofluorescence on sections of Cx40<sup>H11001/H11001</sup> and Cx40<sup>KICx45/KICx45</sup> mouse heart. No signal was detected in the atria of Cx40<sup>KICx45/KICx45</sup> mice treated with anti-Cx40 antibodies (Figure 2B'), or in the atria of Cx40<sup>H11001/H11001</sup> mice treated with anti-Cx45 antibodies (Figure 2C). In contrast, analysis of sections from atria of Cx40<sup>H11001/H11001</sup> and Cx40<sup>KICx45/KICx45</sup> mice showed that Cx45 protein was detected in the His bundle branches (white arrow in H), and in the first layer cells of the septal working myocytes (black arrows in H). It was undetectable in the major portion of the septum (asterisks in H). Similar results were obtained with 129Sv/C57Bl/6 mutant mice. Bar in A=50 μm for A and B; bar in D=50 μm for D, E, and F; and bar in G=20 μm for G and H.
mice indicated that Cx45 was strongly expressed in this tissue with a pattern of expression similar to that of Cx40 in the samples of Cx40+/−/− mice (compare Figures 2A and 2D). The pattern of expression of Cx43, and the intensity of signals, were similar in the atria of both Cx40+/−/− and Cx40KICx45/KICx45 mice (Figures 2E and 2F). Cx40 and Cx45 proteins were detected in the cardiac CS of Cx40+/−/− mice (Figures 3E and 3F), as reported previously.5 The area of Cx45 labeling was larger than that of Cx40, corresponding to the so-called "extended conduction system" and the intensity of signal was weaker than that detected for Cx40 protein (Figures 3E and 3F). In the Cx40KICx45/KICx45 mouse the pattern of expression of Cx45 delineated an extended CS similar to that seen in the Cx40+/−/− mice (Figure 3H). Cx40 was detected between the endothelial cells of the coronaries irrigating the cardiac walls of the Cx40+/−/− mice (Figure 3A). In the coronaries of Cx40KICx45/KICx45 mice, Cx45 had replaced Cx40, as expected (Figure 3B).

Analysis of the Impulse Conduction in the Heart

Surface ECGs

Figure 4 illustrates the ECG recordings of the 129Sv/CD1 mice. The values of the parameters of ECGs and their statistical analysis are summarized in the Table. No difference was found between the three genotypes investigated for the RR or the PQ intervals. However, the P wave duration was significantly increased from 9.1±0.3 ms in the Cx40+/−/− mice to 10.7±0.5 ms in the Cx40KICx45/KICx45 mice, suggesting slower atrial activation or other mechanisms such as a delayed activation in the interatrial pathways or ectopic activation. The latter hypothesis is unlikely because the P-wave morphology was similar for all three genotypes. The QRS complexes were fractionated and their duration was increased from 10.8±0.4 ms in the Cx40+/−/− mice to 14.0±0.6 ms in the Cx40KICx45/KICx45 mice, pointing to a delayed activation of the ventricles. The profiles of the ECGs of 129Sv/C57Bl/6 mice were similar to those of the 129Sv/CD1 mice (not shown). However the absolute values of the measured parameters were different from those of 129Sv/CD1 mice (Table), indicating a strain dependency, as expected.35 The P wave and the QRS complex durations were significantly increased in the Cx40KICx45/KICx45 129Sv/C57Bl/6 mice, as compared with the Cx40+/−/− mice. Because the modifications identified were similar in the two genetic backgrounds, the experiments that followed were performed on one background only, 129Sv/CD1.

Ventricular Activation

Epicardial mapping was performed in SR on Langendorff-perfused hearts to investigate the delayed activation of the ventricles in Cx40KICx45/KICx45 mice. Figure 5 shows representative activation maps of the right (Figure 5A) and left ventricles (Figure 5B) in SR. In the Cx40+/−/− and Cx40+/−/− mice, the sites of first activation were found as breakthrough, apical, or basolateral sites (see Table for the frequencies of events). In the Cx40KICx45/KICx45 mice no midventricular breakthrough activation was found, and the earliest activations were predominantly found at apical sites (Table), indicating a different mode of ventricular activation in these animals.

The total activation time was calculated from all RV and LV activation maps recorded in SR by subtracting the latest activation times from the earliest ones. The activation time of the RV was significantly increased from 5.4±0.4 ms in Cx40+/−/− mice to 9±0.7 ms (67%) in Cx40+/−/− mice (Table). The activation times of the LV of the mice of the three genotypes investigated were not significantly different (Table).

Atrioventricular Conduction

Figure 5C shows examples of the AV delay as a function of the coupling interval of the premature stimulus (conduction curves). No significant differences were detected between the three groups of mice for neither the AV effective refractory period (ERP), the minimum AV delay at infinitely long coupling intervals (D0), nor the time constant (τ) (Table).

Atrial Conduction

Activation maps of the paced right (RA) and left (LA) atria of the three groups of mice investigated are shown in Figures 6A and 6B, respectively. In the RA, the CV was 30.8±1.8 cm/s for the Cx40+/−/− mice and was not significantly changed in the Cx40+/−/−/− mice and Cx40+/−/− mice (32.9±2.3 and 30.0±2.3 cm/s, respectively) (Table). In the LA, there was a significant reduction of the CV, from 31.5±1.6 cm/s in Cx40+/−/− mice to 24.3±2.2 cm/s in Cx40+/−/− mice.

Ventricular Conduction

Examples of activation maps of the paced RV and LV are shown in Figures 6C and 6D, respectively. RV and LV activation maps showed anisotropic conduction independent of the genotype. Both longitudinal and transversal CVs in the ventricles were similar in all three genotypes (Table).

Bundle Branch Conduction

Examples of RBB and LBB activation maps are shown in Figures 6E and 6F, respectively. RBB activation was always...
Electrophysiological Parameters of Wild-Type and Mutant Mice

<table>
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<tr>
<th>ECG parameters, ms (129Sv/CD1)</th>
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<th>Cx40−/−</th>
<th>Cx40−/−Cx45/−Cx45</th>
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<td>126.5±1.1 (n=6)</td>
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<td>QRS duration</td>
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<td>10.5±0.3 (n=12)</td>
<td>12.5±0.5 (n=13)†</td>
<td>0.001</td>
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Ventricular activation (129Sv/CD1)

| Breakthrough RV (LV)             | 33% (33%) | 11% (25%) | 0% (0%) |      |
| Apical/lateral RV (LV)           | 50% (50%) | 89% (37.5%) | 64% (92%) |      |
| Basal/lateral RV (LV)            | 17% (17%) | 0% (37.5%) | 36% (8%) |      |
| RV activation time, ms           | 5.4±0.4 (n=6) | 6.4±0.8 (n=9) | 9±0.7 (n=11)† | 0.04  |
| LV activation time, ms           | 6.1±0.8 (n=6) | 5.7±0.7 (n=8) | 6.7±0.5 (n=12) | 0.51  |

Conduction velocities, cm/s (129Sv/CD1)

| RA CV                            | 30.8±1.8 (n=6) | 32.9±2.3 (n=11) | 30.0±2.3 (n=12) | 0.63  |
| LA CV                            | 31.5±1.6 (n=6) | 35.6±3.1 (n=11) | 24.3±2.2 (n=11)† | 0.01  |
| RV CV longitudinal               | 37.5±2.7 (n=6) | 33.1±6.7 (n=10) | 33.8±2.2 (n=10) | 0.45  |
| RV CV transversal                | 21.0±3.4 (n=6) | 21.2±2.0 (n=10) | 19.6±0.6 (n=10) | 0.80  |
| RV anisotropic ratio             | 2.0±0.3        | 1.7±0.2         | 1.7±0.1         | 0.52  |
| LV CV longitudinal               | 46.3±4.2 (n=6) | 39.1±3.2 (n=9)  | 38.8±3.2 (n=12) | 0.33  |
| LV CV transversal                | 20.5±1.1 (n=6) | 18.6±1.1 (n=9)  | 16.1±1.0 (n=12) | 0.11  |
| LV anisotropic ratio             | 2.3±0.3        | 2.2±0.2         | 2.3±0.2         | 0.8   |
| RBB CV                           | 36.5±5.4 (n=5) | 44.6±5.6 (n=9)  | 19.2±2.9 (n=10)† | 0.001 |
| LBB CV                           | 47.8±7 (n=5)   | 49.2±3.5 (n=6)  | 48.5±8.6 (n=6)  | 0.98  |

AV node function (129Sv/CD1)

| AV ERP, ms                       | 68±3.4 (n=6)  | 68±5.5 (n=10)  | 74.2±5.1 (n=12) | 0.63  |
| AV delay t=∞, ms                 | 55.7±2.5 (n=6) | 64.2±3.5 (n=10) | 64.9±2.4 (n=12) | 0.12  |
| AVτ                              | 31.5±5.7 (n=6) | 22.1±3.8 (n=10) | 24.4±2.4 (n=12) | 0.14  |

Values are given as mean±SEM. *P≤0.05 compared to Cx40+/+; †P≤0.05 compared to Cx40−/−. n indicates number of independent experiments.

recorded in a one-electrode wide strand, whereas LBB activation was electrically much wider and was always recorded in several electrode strands parallel to each other. In the Cx40+/+ mice, the CV in the RBB was 36.5±5.4 cm/s (Table), and it was not significantly different in the Cx40−/−Cx45/− mice. Interestingly, in the Cx40−/−Cx45/−Cx45 mice, the CV in the RBB was significantly reduced by 46% to 19.2±2.9 ms. The CVs in the LBB were not significantly different between the three genotypes investigated (Table).

Discussion

The targeted replacement of one Cx gene by another Cx gene has been performed for several Cxs.36–38 Analysis of the resulting transgenic mice has indicated that Cxs could have either unique or redundant functions, and has provided important clues regarding the role of Cxs in vivo. In this study, we have generated Cx40KICx45 mice, and assessed the involvement of Cx45 in the propagation of cardiac impulse in the absence of Cx40.

For both genetic backgrounds investigated, the major phenotypic effects of the biallelic replacement of Cx40 by Cx45 were an increased duration of the P wave, and a prolonged and fractionated QRS complex in the ECGs in vivo. Epicardial mapping indicated a changed and prolonged activation of the RV that could be attributed to slow conduction in the RBB. The CVs in ventricular working myocardium, and the conduction delay in the AV node, were unaffected. The duration of the P wave was increased by about 18% in the Cx40KICx45/Cx45 mice. No decrease of the CV in the RA was found in contrast to the LA in which a significant reduction of about 23% was observed. These data may explain the increase of the P-wave duration, although a delayed activation between the right and left atrium cannot be excluded, and indicate that Cx45 cannot fully replace the function of Cx40, at least in the LA. Studies on Cx40 KO
mice have indicated that the increase in the duration of the P wave was higher, ranging from 30% to 56%, and was mainly due to a very significant reduction of the CV (30%) in the RA. These results, and the present data are suggestive of a chamber-specific role of Cx40. This hypothesis is strengthened by the fact that in the human heart the expression level of Cx40 is higher in the RA than in the LA. However, this differential expression remains to be demonstrated in the mouse heart.

No differences were found in the various parameters that characterize the AV conduction in the Cx40+/+, Cx40+/KICx45, and Cx40KICx45/KICx45 mice, indicating that the AV node func-
tions normally when Cx40 is replaced by Cx45. Numerous studies have described an increase in the PR interval and the Wenckebach cycle length in the Cx40 KO mice, which indicated an impairment of the AV conduction. Our results indicate that the absence of Cx40 in the AV node can be corrected by the expression of Cx45 despite differences in their intrinsic properties.

The delayed activation of the RV, but not that of the LV, during SR, which increases the activation time of the heart, accounts for the increase of the duration of the QRS complex.

Figure 6. Representative paced activation maps of the RA and LA (A and B), the RV and LV (C and D), and the RBB and LBB (E and F) of Cx40+/+, Cx40+/KICx45, and Cx40/KICx45/KICx45 mice. Position of the electrode array on the epicardium (A through D) or the septal myocardium (E and F) is indicated in the pictograms. Activation times are relative to the pacing stimulus in the maps of the RA, LA, RV, and LV and relative to the atrial activation in the maps of the RBB and LBB. CVs were calculated from these maps. Activation times (ms) are indicated by color codes. In the examples shown in A and B, the CVs in the RA and the LA were 36 and 29, 32 and 32, and 41 and 19 cm/s for Cx40+/+, Cx40+/KICx45, and Cx40/KICx45/KICx45 mice, respectively. In the examples shown in C and D, the CVs in the RV and the LV were 40 and 38, 40 and 33, and 30 and 34 cm/s for Cx40+/+, Cx40+/KICx45, and Cx40/KICx45/KICx45 mice, respectively. Note that the anisotropy of the conduction in the ventricles is independent of the genotype. CVs determined from the activation maps of the RBB and LBB illustrated in E (RS, right side of the septum) and F (LS, left side of the septum) were 42 and 47, 54 and 51, and 17 and 54 cm/s for Cx40+/+, Cx40+/KICx45, and Cx40/KICx45/KICx45 mice, respectively. Note that the activation patterns in the right and left BB are asymmetrical. Electrograms recorded in 2 different sites of both bundle branches are shown for each genotype.
The CV in the ventricular working myocardium was unaffected by the replacement of Cx40 by Cx45, because Cx40 is not expressed in the ventricular working myocardium of the adult mouse heart. The slow CV measured in the RBB of Cx40KO/Cx45KO/Cx45 mice, but not in the LBB, explains the abnormal activation of the RV, in these animals. In the absence of Cx40, Cx45 is the only known Cx which is expressed in the proximal parts of the bundle branches. Assuming that Cx45 was upregulated in a similar way in both bundle branches, its expression supports a normal conduction in the LBB, but fails to do so in the RBB, indicating that electrical and/or structural differences between LBB and RBB account for the observed effect. Analysis of mice in which one allele of the Cx40 gene was replaced by eGFP (Cx40^RKO mice) has shown that the LBB was in fact constituted with 20 or so parallel strands coming from the His bundle, and covering entirely the left side of the interventricular septum. In contrast, on the right flank the branch (RBB) emerging from the His bundle was constituted by only one thin fiber in its proximal part. Thus, the LBB is more extended and much more complex than the RBB, and this morphology is in agreement with the activation patterns which were recorded (Figures 6E and 6F). Consequently, variations of the gap junctional coupling in the ventricular CS will affect RBB conduction more severely than LBB conduction. Furthermore, the relationship between the gap junctional coupling and the CV in a strand of model cells is not linear. Assuming that Cx45 was upregulated in a similar way in both bundle branches, which were recorded (Figures 6E and 6F). Consequently, variations of the gap junctional coupling in the ventricular CS will affect RBB conduction more severely than LBB conduction. Furthermore, the relationship between the gap junctional coupling and the CV in a strand of model cells is not linear. At high levels of junctional conductance the CV saturates, and significant reductions of the conductance from very high values do not necessarily result in a significant reduction of the CV. In contrast, small reductions of the conductance from low values will lead to drastic reductions of the CV. If one assumes that the coupling is higher in the LBB than in the RBB, which is not unlikely because the CV is higher in the LBB (47.8 cm/s) than in the RBB (36.5 cm/s), a similar reduction of the intercellular coupling in both bundle branches would be more prominent in the RBB than in the LBB. The analysis of Cx40KO mice confirmed that the RBB is much more sensitive to changes in the Cx expression than the LBB. Indeed, the deletion of Cx40 without replacement resulted in conduction block in the RBB, but induced only a slowing down of the conduction in the LBB. The higher vulnerability of the RBB is also reflected in the clinical data that indicate that the frequency of conduction impairment in patients with cardiovascular diseases is much higher in the RBB than in the LBB (see for example Newby et al). In summary, the replacement of Cx40 by Cx45 resulted in the heart in a significant reduction of the CV in the LA and a normal CV in the RA. The conduction delay in the AV node was unaffected, whereas a partial loss of function became apparent in the RBB, but not in the LBB.

Acknowledgments

This work was supported by the CNRS, the Université de la Méditerranée, the European Community (contract QLGI-1999-00516), The Netherlands Heart Foundation (grant 99-200), and the French Ministry of Education (ACI Biologie du Développement et Physiologie Intégrative). S.A. was supported (fellowships) by the Association Française contre les Myopathies, and the Groupe de Réflexion sur la Recherche Cardiovasculaire.

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Replacement of Connexin40 by Connexin45 in the Mouse: Impact on Cardiac Electrical Conduction

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Circ Res. 2004;94:100-109; originally published online November 20, 2003;
doi: 10.1161/01.RES.0000108261.67979.2A

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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EXPENDED MATERIALS AND METHODS.

Construction of the targeting vector.

A fragment spanning about 12 kb of the locus of the Cx40 gene was isolated from the EMBL3 129Sv mouse genomic library. This fragment (a gift from Dr. Théveniau-Ruissy), which included exon 2 (containing the complete coding sequence) flanked by a 4 kb region upstream, and a 8 kb region downstream, was mapped. Various parts were subcloned for use to engineer the targeting vector. A subfragment comprising 2.7 kb of 5' genomic sequence followed by the first 300 bp of the Cx40 exon 2 was cloned in pBluescript (Stratagene). An NcoI site (CCATGG) was created by polymerase chain reaction (PCR)-based site directed mutagenesis at the translation start site (...AAGATGGGT...) of the exon.

Mouse Cx45 cDNA cloned in pBluescript was kindly provided by Dr. Willecke (plasmid F9-7). A NcoI site was created at the level of the ATG codon of Cx45 (...ACCATGAGT...) by PCR-based site directed mutagenesis with the aim of replacing the exon 2 of the Cx40 gene by the coding exon 3 of the Cx45 gene while maintaining the reading frame. The creation of this site mutated the second codon, which is a serine (S) codon, into a glycine (G) codon but it is worth mentioning that S and G have conserved properties. This modification also has given rise to a ACCATGG Kozak sequence identical to that of Cx40, and liable to induce a translational efficiency of Cx45 close to that of Cx40.

The exon 3 of Cx45 with its 3'UTR including its polyadenylation site was cloned in frame at the previously created NcoI site at the Cx40 start codon. The resulting 4.7 kb long construct was inserted at the XbaI site of the vector pKOLL 1-3 (kindly provided by Dr. Pattyn) upstream of the LoxP-Neo<sup>R</sup>-LoxP domain.

A 3'-homology genomic subfragment (5.5 kb) from the Cx40 locus, including the last 800 bp of exon 2, was inserted downstream of the LoxP-Neo<sup>R</sup>-LoxP domain. An additional LoxP site followed by a XbaI site (used for Southern blot genotyping) was introduced at the Eco47III site of the Cx40 exon 2 to delete with Cre recombinase, after homologous recombination, most of the exon 2 at the same time as the Neo<sup>R</sup> cassette.

The construct was checked by sequencing (Genome Express); its structure is shown in Figure 1.

Electrophysiological characterization of Cx45 channels.

The second amino-acid of wild type (wt) mouse Cx45 is a serine. The strategy used to construct the targeting vector required replacement of the second codon of Cx45, which is a serine codon, by a glycine codon, and consequently the vector encoded for a mutated Cx45 (Cx45(G)). The properties of the channels constituted with Cx45(G) were investigated and compared with those of wtCx45.
The ability of Cx45(G) to form functional gap junctional channels alone, or in heterotypic combination with wtCx45 and Cx43, was assessed in the *Xenopus* oocytes pair system. Mouse Cx45 and Cx45(G), and rat Cx43 were expressed in *Xenopus* oocytes and the level of electrical coupling developed between the homo- or heterotypic oocytes pairs was estimated by measuring the macroscopical junctional conductance (G_j) as described previously. The results (Table 1) indicated that homotypic Cx45(G) channels were formed with an efficiency similar to that of wtCx45, and that the mutation did not significantly change the ability of the Cx45 protein to form heterotypic channels with either wtCx45 or Cx43. The sensitivity of the steady state conductance (G_{ijss}) of Cx45(G) channels to the transjunctional voltage (V_j) was determined and compared with that of wtCx45 channels. The Boltzmann parameters fitting the G_j/V_j relationships for each polarity of V_j were similar in both cases (A = -0.069/0.074, V_0 = -30.8/30.7, G_{ijmax} = 1.14/1.11, G_{ijmin} = 0.08/0.06, for wtCx45 channels, and A = -0.083/0.087, V_0 = -22.4/36.2, G_{ijmax} = 1.10/1.06, G_{ijmin} = 0.01/0.03, for Cx45(G) channels), indicating that the behavior of the two types of channels were not significantly different.

HeLa cells (which are communication-deficient cells) were electroporated as described for ES cells (see below) with Cx45(G) cDNA cloned in pCMV-Script (Stratagene), cultured, and selected for integration with G418 (800 μg/mL). Expression of Cx45(G) in stably-transfected HeLa cells was first confirmed using the RT-PCR technique (not shown) (see Figure 1A of the paper, and Table 2 for the primers specific of Cx45(G)). In addition, immunofluorescence investigations carried out on these cells indicated that the expression pattern of Cx45(G) (not shown) was indistinguishable from that of wtCx45 in the same cells, and that consequently it was correctly targeted to the plasma membrane. Single channel measurements were carried out with pairs of HeLa cells stably transfected with either wtCx45 or Cx45(G) using the double whole cell patch-clamp technique. Both types of channels had a small current transition (γ_{i1}), and a large current transition (γ_{i2}) (not shown). Their values (γ_{i1} = 17.2 ± 1.3 pS and γ_{i2} = 34.7 ± 0.3 pS, with N = 5, n = 710, for wtCx45 channels; γ_{i1} = 22.1 ± 0.4 pS, and γ_{i2} = 35.3 ± 0.6 pS, with N = 5, n = 942, for Cx45(G) channels) indicated that the unitary conductances of both types of channels were similar.

As a whole, these results showed that the electrophysiological properties of wtCx45 and Cx45(G) channels were not significantly different for any of the parameters tested. Consequently no distinction has been further made between wtCx45 and Cx45(G) proteins.

**Generation of transgenic mice.**

The strategy used to generate the transgenic mice is summarized in Figure 1. CK35 ES cells (129Sv strain) were electroporated with linearized targeting vector, and selected for integration
with G418 (350 μg/mL). In the ES clones resistant to G418, the frequency of homologous recombination events at the locus of the Cx40 gene was 1:600. About 10 recombined ES cells were injected into 3.5 days postcoitum C57Bl/6 blastocysts or aggregated with CD1 morulae, then transferred to pseudopregnant CBA/C57Bl/6 foster mothers which gave birth to chimera mice. Male chimeras which had transmitted the mutated allele through the germ line were crossed with either CAG-Cre CD-1\(^8\) or Cre-deleter C57Bl/6\(^o\) females to delete Neo\(^R\)-E2Cx40 cassette, and generate mice with either a Sv129/CD1 (50%/50%) or a 129Sw/C57Bl/6 (50%/50%) background (Figure 1). Heterozygous offspring were intercrossed to obtain homozygously mutated progeny.

**Genotyping of ES cells and mice.**

Southern blot technique was employed to analyse ES cell and mouse genotypes using 3 types of probes (3', del and Neo\(^R\) probes). The 3' and del probes were restriction fragments generated by digesting cloned genomic DNA of the Cx40 gene with HindIII and XbaI, and PvuII and BglII, respectively. The Neo\(^R\) probe was a AvaII restriction fragment of the pMC1Neo vector (Stratagene). DNA was extracted from ES cell lysates or mouse tails according to standard procedures. The correct integration of the targeting vector was checked by probing XbaI-digested genomic DNA with the 3' probe (400 bp) located downstream of the 3'-homology region (Figure 1). The absence of additional random integrations of the vector was confirmed by stripping and rehybridizing the membrane with the internal Neo\(^R\) probe (720 bp) specific for the Neo\(^R\) cassette. The deletion of this cassette after Cre recombinase action was checked by hybridizing the 3'UTR Cx40 probe (del probe, 360 bp) with BglII-digested DNA.

The results of these experiments (Figure 2) indicated that the targeting vector was correctly integrated (Figures 2A and 2C), and that the Neo\(^R\)-E2Cx40 cassette was deleted (Figures 2D and 2E). Furthermore, no additional random integration of the vector was detected (Figure 2B).

PCR was used to routinely genotype the mice with DNA extracted from ear punches. Wild type (wt) and Cx40KICx45 alleles were identified using the primers p1, p2, and p3 (Figure 1 and Table 2). The deletion of Neo\(^R\)-E2Cx40 cassette was checked using the primers p4 and p5, which flank the cassette.

**Expression of the transgene in the KI mice.**

**RT-PCR experiments.** Total RNA was extracted from the ovaries of wt adult mice (8 to 12 weeks old), or from the hearts of adult mice of various genotypes (Cx40\(^+/+\), Cx40\(^+/KICx45\), Cx40\(^+KICx45\)) using Trizol reagent (GIBCO/BRL). 1μg of RNA was reverse transcribed using First Strand cDNA Synthesis Kit (Roche Diagnostics). The reactions of amplification, and
the control experiments were carried out as described previously\textsuperscript{10}. The primers used are listed in Table 2 (Online Data Supplements).

*Northern blot experiments*. 10 μg of total RNA, extracted from mouse adult atria (wt, Cx40\textsuperscript{WT}, Cx45\textsuperscript{KICx45}, Cx40\textsuperscript{KICx45/KICx45}) were analyzed by Northern blotting as described previously\textsuperscript{11}. After the first hybridization the membrane was dehybridized, and rehybridized with a new probe. Thus, for each experiment, each of the four probes used was hybridized to the same membrane. The probes used were a mouse Cx40 cDNA (1.2 kb) containing the complete coding sequence\textsuperscript{11}, a BglII-XhoI fragment (1.4 kb) of mouse Cx45 cDNA\textsuperscript{2}, a HindIII-EcoRV fragment (1.5 kb) of rat Cx43 cDNA\textsuperscript{12}, and a 1.1 kb fragment of the hypoxanthine phosphoribosyl-transferase (HPRT) gene of the mouse\textsuperscript{13}. The latter probe which overlaps the coding sequence and the 3'UTR of the HPRT gene was amplified by PCR with specific primers (Table 2).

*Immunofluorescence experiments*. The preparation of the samples, and the analysis by immunofluorescence of sections from adult mouse heart using a CLS microscope, were carried out as described previously\textsuperscript{10}. Rabbit antibodies specific for Cx43, -40, or -45 were used at 0.6 μg/mL, 4.8 μg/mL, and 10 μg/mL, respectively. Anti-Cx45 (Q14E(GP42)) guinea-pig antibodies were diluted 1:500. The specificity of these antibodies has been previously demonstrated\textsuperscript{10,14,16}. Secondary antibodies used were Texas-Red conjugated donkey anti-rabbit IgGs (Jackson ImmunoResearch Lab.), Alexa 488 conjugated donkey anti-rabbit IgGs (Molecular Probe), and Cy3 anti-guinea pig F(ab')2 fragments (Chemicon) diluted at 1:200, 1:800, 1:400, respectively.

*Western blot experiments*. Western blot experiments were carried out with rabbit polyclonal antibodies to Cx40, -43, or -45 (see above), and a mouse monoclonal antibody to desmin (Sigma Aldrich) as described previously\textsuperscript{10}.

*Morphological analysis*. The morphological analysis of adult mouse hearts was carried out as described previously\textsuperscript{17}. For each genetic background, three hearts from Cx40\textsuperscript{KICx45/KICx45} mice, coming from 3 independent litters, were investigated and compared with the heart of wt mouse.

*ECG recording and mapping of impulse propagation*. Recordings of extracellular electrograms during sinus (SR) or paced rhythm were carried out as described\textsuperscript{18,19}. Atrial mapping was performed using a 12x14 electrode grid (electrode spacing, 200 μm; frequency, 4 kHz). Atrial pacing was performed using a separate bipolar stimulus electrode positioned directly superior to the electrode grid. Ventricular recordings were made using a 13x19 grid (spacing, 300 μm; frequency, 2 kHz). Two central electrodes of the grid were used for ventricular pacing. Both the atria and the ventricles were paced at 100 ms basic cycle length (BCL) at twice the stimulus threshold. AV nodal conduction curves were determined from
recordings on the right ventricle (RV), while stimulating the right atrium (RA) with a separate bipolar stimulus electrode. The RA pacing protocol was composed of 16 basic stimuli of 150 ms, followed by one premature stimulus of 140 ms, which was decremented by 10 ms steps until the AV effective refractory period (ERP) was reached. For mapping of the His bundle branches, the RV free wall was removed, and the left ventricle (LV) free wall was cut open apically and laterally, leaving it attached to the base of the heart. The 13x19 grid (frequency, 4 kHz) was positioned on the septum and recordings were carried out in SR. Activation maps were constructed from the activation times using custom-written software.\textsuperscript{20} Maximal negative dV/dt in the unipolar electrograms was selected as the time of local activation. Maximum CVs were determined by hand from the activation maps constructed from paced electrograms during basic stimulation. The AV conduction curves were fitted to equation (1): $D^\infty + A \cdot \exp(-t/\tau)$, using a least-square method, with $D^\infty$ being the AV delay at infinitely long coupling intervals ($t=\infty$), $A$, the AV delay at $t = 0$, and $\tau$ (ms), the time constant of the conduction curves.

**LEGENDS OF FIGURES.**

*Figure 1.* Generation of Cx40KICx45 mice.

The position of probes and PCR primer pairs used to screen DNA for homologous recombination and deletion events are indicated as bars and arrowheads, respectively.

(a) Wild type locus of the mouse Cx40 gene with the non-coding exon 1(E1) and the exon 2 (E2). Black box: coding region of E2. Arrow: translation start site. B, X and Xm: restrictions sites for BglII, XbaI, and XmnI, respectively.

(b) Targeting vector. The targeting vector contained: i) 8.2 kb of DNA of the Cx40 gene (arms of 2.7 and 5.5 kb); ii) the exon 3 of the mouse Cx45 gene (dotted box indicated E3Cx45) inserted at the start codon of Cx40; iii) a cassette with the neomycin resistance gene (box Neo$^R$) flanked with two LoxP sites (stripped triangles), and 800 bp of the exon 2 of the Cx40 gene (black boxes E2Cx40). This 800 bp fragment was interrupted with a LoxP site (stripped triangle in the box E2Cx40). Exon 3 of the Cx45 gene included only the coding sequence and the 3'UTR with polyadenylation sites. The domains of homologous recombination between the locus of the Cx40 gene and the targeting vector are indicated with crossed lines.

(c) Recombined Cx40 locus.

(d) Recombined Cx40 locus after Cre-deletion. The cassette Neo$^R$-E2Cx40 was deleted by crossing heterozygous Cx40$^{+/KICx45}$ mice with mice constitutively expressing Cre recombinase (CAG-Cre or Cre-deleter mice).

*Figure 2.* Genotyping of mice.
A. XbaI-digested genomic DNA was hybridized with the downstream 3' probe (Figure 1). Southern blotting revealed fragments of 7.2 and 6 kb which identify wt (+/+ ) and knock-in (+/KI, KI/KI) alleles, respectively.

B. XmnI-digested DNA was hybridized with the internal NeoR probe. A single 12.5 kb fragment was detected by Southern blotting which confirmed that no additional random integration of the targeting vector occurred.

C. For the genotyping of the mice using PCR, primers located in the coding sequences of Cx40 (p2) and Cx45 (p3) (Figure 1), and the intron of the Cx40 gene (p1) (Figure 1) were used. Wild type (+/+) and Cx40KICx45 (+/KI, KI/KI) alleles were identified by the detection of amplicons of 475 and 660 bp, respectively.

D. BglIII-digested DNA was hybridized with a 3'UTR Cx40 probe (del probe, Figure 1). Fragments of 5.5, 4.8, and 3.5 kb revealed by Southern blotting identified wt alleles, and recombined alleles before (- Cre) and after (+ Cre) the action of recombinase, respectively, indicating that the NeoR-E2Cx40 cassette was deleted in Cx40+/KICx45 mice.

E. Characterization by PCR of Cx40+/KICx45 mice deleted for the NeoR-E2Cx40 cassette. Using the primer pair p4 and p5 (Figure 1) a 1.1 kb long fragment was amplified when the cassette was excised (lane : + Cre). With the same experimental conditions no fragment was amplified from DNA of control Cx40+/KICx45 mice which have not been crossed with mice expressing Cre recombinase (lane : - Cre).

A, C, and E illustrate experiments with DNA from 129Sv/CD1 mice; B and D, with DNA from 129Sv/C57Bl/6 mice.
REFERENCES.


Table 1. Measurements of macroscopic conductances (G_j) in paired Xenopus oocytes.

<table>
<thead>
<tr>
<th></th>
<th>wtCx45/wtCx45</th>
<th>Cx45(G)/wtCx45</th>
<th>Cx45(G)/Cx45(G)</th>
<th>wtCx45/wtCx43</th>
<th>Cx45(G)/wtCx43</th>
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</thead>
<tbody>
<tr>
<td>24 h</td>
<td>0.39 ± 0.42</td>
<td>0.56 ± 0.31</td>
<td>0.52 ± 0.74</td>
<td>2.51 ± 1.57</td>
<td>3.6 ± 1.57</td>
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<td>N = 3, n = 14</td>
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<td>N = 3, n = 16</td>
<td>N = 3, n = 18</td>
<td>N = 2, n = 12</td>
<td>N = 2, n = 13</td>
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<tr>
<td>48 h</td>
<td>3.61 ± 1.41</td>
<td>2.75 ± 2.63</td>
<td>3.04 ± 2.42</td>
<td>9.11 ± 4.32</td>
<td>12.16 ± 8.61</td>
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<tr>
<td>N = 3, n = 12</td>
<td></td>
<td>N = 3, n = 13</td>
<td>N = 3, n = 17</td>
<td>N = 2, n = 10</td>
<td>N = 2, n = 11</td>
</tr>
</tbody>
</table>

Xenopus oocytes were injected with cRNAs encoding for wtCx45, or Cx45(G) or Cx43, then paired, as described previously. 24 or 48 h after pairing the macroscopic junctional conductance induced between oocytes by the formation of homotypic (wtCx45/wtCx45, Cx45(G)/Cx45(G)) or heterotypic (Cx45(G)/wtCx45, wtCx45/wtCx43, Cx45(G)/wtCx43) channels was measured. Data represent the mean G_j values (in µS) ± SD. N, number of independent experiments; n, total number of pairs tested.

The statistical analysis of the values of G_j with an unpaired t-test indicated that the means between Cx45(G)/Cx45(G) versus wtCx45/wtCx45, Cx45(G)/Cx45(G) versus wtCx45/Cx45(G), and wtCx45/wtCx45 versus wtCx45/Cx45(G), or wtCx45/wtCx43 versus Cx45(G)/wtCx43 were not significantly different at P = 0.05.
Table 2. Oligonucleotide primers used for RT-PCR experiments and genotyping (PCR).

<table>
<thead>
<tr>
<th>Application</th>
<th>Sequences</th>
<th>Positions</th>
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<th>Expected size of amplicons (bp)</th>
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<td><strong>RT-PCR</strong></td>
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<td>Cx40</td>
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<tr>
<td>E1 (S)*</td>
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<td>Cx45</td>
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<td>WT and KI alleles</td>
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<td>p1 (S)</td>
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<td>p1/p3, 660</td>
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<td>Neo-E2Cx40 deletion</td>
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<td>p4 (S)</td>
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<td>Cx45 exon3</td>
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<td>Cx45 cds</td>
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<td>Mouse HPRT gene</td>
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<td>cds</td>
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</table>

* (S): sens; (AS): anti-sens.
† Ta: annealing temperature (°C).
‡ cds: coding sequence.
For the sequence and the structure of the Cx40 and Cx45 genes, see references ¹⁻³, ²¹.
(a) Wild type Cx40 locus

(b) Targeting vector

(c) Recombined Cx40 locus

(d) Recombined Cx40 locus after Cre-deletion

Figure 1