Connexin45 Provides Optimal Atrioventricular Nodal Conduction in the Adult Mouse Heart

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Rationale: The gap junctional protein connexin (Cx) 45 is strongly expressed in the early embryonic myocardium. In the adult hearts of mice and humans, the expression mainly is restricted to the cardiac conduction system. Cx45 plays an essential role for development and function of the embryonic heart because general and cardiomyocyte-directed deficiencies of Cx45 in mice lead to atrioventricular lethality attributable to morphological and functional cardiovascular defects. The function of Cx45 in the adult mouse has not yet been cleared.

Objective: To clarify the function of Cx45 in the adult mouse heart.

Methods and Results: To circumvent the embryonic lethality resulting from Cx45 deficiency, mice were generated in which deletion of Cx45 specifically was induced in cardiomyocytes of adult mice. These Cx45-deficient mice were viable but showed a decrease in atrioventricular nodal conductivity. In addition, the Cx30.2 protein that is coexpressed with Cx45 in the cardiac conduction system was posttranscriptionally reduced by 70% in mutant hearts. Furthermore, deletion of both Cx45 and Cx30.2 resulted in viable mice that, however, showed stronger impairment of atrioventricular nodal conduction than the single Cx45-deficient mice.

Conclusions: Cx45 is required for optimal impulse propagation in the atrioventricular node and stabilizes the level of the coexpressed Cx30.2 protein in the adult mouse heart. In contrast to the embryo, Cx45 is not essential for the viability of adult mice. (Circ Res. 2012;111:1528-1538.)

Key Words: adult mouse heart ▫ atrioventricular node ▫ conduction velocity ▫ connexin30.2 ▫ connexin45 ▫ gap junction

In the mammalian heart, gap junction channels between cardiomyocytes are crucial for electric impulse propagation and coordinated contraction of the chambers. A gap junction channel is composed of 2 hemichannels called connexons, with each contributed by 1 of 2 adjacent cells. One connexon consists of 6 connexin (Cx) proteins that are encoded by a multigene family with 20 members in the mouse genome and 12 members in the human genome.10 Dependent on their Cx composition, gap junction channels show a high variety of conductance and gating properties; homotypic gap junctions and homomeric hemichannels are composed of the same Cx isotype, whereas heterotypic gap junctions and heteromeric hemichannels consist of different isotypes.2

Five Cx isotypes are expressed in the mouse heart. Cx43 is the major cardiac Cx and is found in the working myocardium of atria and ventricles as well as in peripheral Purkinje fibers.3,4 Cx40 is strongly expressed in atria, the ventricular conduction system, and, to a lesser extent, in the atrioventricular (AV) node,5,6 whereas Cx45 and Cx30.2 are the predominant Cx in the atrial conduction system consisting of sinus node and AV node.7,8 Beside the nodes, Cx30.2 and Cx45 also are expressed in the His bundle and bundle branches.9,10 Furthermore, Cx45 is found at lower levels in proximal Purkinje fibers9 and in the working myocardium of atria and ventricles.7,11,12 More recently, Cx30 was found as the fifth cardiac Cx in the mouse heart but was only expressed in small amounts in the sinus node.13 In the human heart, expression of Cx40, Cx43, and Cx45 is similar to that in mice and other mammals.14,15 Cx45 is the predominant Cx expressed in the mammalian AV node16 and was shown to be expressed in the fetal and adult human conduction system.17,18 In contrast to the mouse heart, where Cx30.2 was found to be expressed in the conduction system,8 the human ortholog Cx31.9 is not expressed in the human heart.18

The function of Cx in the heart has been extensively studied using Cx-deficient mice.5,6,10,13,19,20 However, the function of Cx45 in the adult mouse heart has not been investigated.

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so far because both general and cardiac-restricted ablation of Cx45 cause embryonic lethality attributable to cardiovascular defects.\(^1\)\(^,\)\(^2\)\(^,\)\(^1\)\(^,\)\(^2\)\(^,\)\(^3\)\(^,\)\(^2\)\(^,\)\(^3\)\(^,\)\(^4\)\(^,\)\(^5\)\(^,\)\(^2\)\(^3\)\(^,\)\(^2\)\(^4\)\(^,\)\(^3\)\(^5\)\(^,\)\(^2\)\(^6\)\(^,\)\(^2\)\(^7\)\(^,\)\(^2\)\(^8\)\(^,\)\(^2\)\(^9\)\(^,\)\(^3\)\(^0\) Cx45 is the only cardiac Cx essential for cardiogenesis and embryonic survival.

To study the role of Cx45 in the adult heart and to circumvent embryonic lethality, we generated tamoxifen-inducible, cardiomyocyte-restricted, Cx45-deficient mice. These mice were viable but showed impaired AV nodal conduction, indicating that Cx45 in the adult mouse heart is not essential for survival but is relevant for AV nodal function. To investigate whether the AV node can still execute its function when both Cx45 and Cx30.2 are absent and to better understand the general role and interactions of Cx in AV nodal conduction, we generated Cx45//Cx30.2 double-deficient mice. These mice also were viable but showed a stronger AV nodal conduction delay, indicating that Cx45 and Cx30.2 are crucial for optimal impulse propagation and function of the AV node.

### Results

Tamoxifen-Induced Deletion of Cx45

Tamoxifen-inducible, cardiac-specific Cx45KO mice were viable and showed no conspicuous alterations in heart morphology. To prove that tamoxifen-induced deletion of Cx45 had been achieved, immunofluorescence analyses were performed. Cx45 was strongly expressed in the conduction system of the control heart (Figure 1A–C; n=6). In Cx45KO hearts (n=5), no Cx45 immunosignals were found, but instead the green fluorescent protein (GFP) was expressed (Figure 1D–F). HCN4 was used as marker for the conduction system.\(^2\)\(^7\) There was partially overlapping expression between HCN4 and Cx45 (Figure 1A′–F′) in both control and Cx45KO hearts. Although no signal for Cx45 was found with immunofluorescence analyses of Cx45KO hearts, there was a weak signal detected in immunoblots (Figures 2A and 2C; protein ratio mean±SD: control: 1, n=4; Cx45KO: 0.28±0.16, n=4; P<0.05). The remaining 30% expression level is attributable to Cx45-positive smooth muscle cells of blood vessels\(^1\)\(^1\) (Figure 2B) and fibroblasts\(^2\)\(^9\) that still express Cx45 in Cx45KO hearts, because the αMyHC promoter is not active in these cell types. To further test the efficacy of the αMyHC-CreER(T2) recombinase, tamoxifen was injected into pregnant females at early embryonic stages. Because Cx45 is essential for early embryonic heart development and because its deletion leads to heart defects and death on embryonic day 10.5,\(^2\)\(^1\)\(^–\)\(^2\)\(^3\) tamoxifen-induced Cre activity also should cause these effects in embryos. In fact, Cx45KO embryos died after tamoxifen-induced Cre activity and ablation of Cx45, whereas control littersmates without Cre allele, still expressing Cx45, were normally developed (Figure 2D; control: n=20; Cx45KO: n=17).

Effect of Cx45 Deletion on Other Cardiac Cx

To investigate whether deletion of Cx45 had any effect on the expression level of other cardiac Cx, immunofluorescence analyses were performed. Because Cx45 and Cx30.2 have a similar expression pattern in the heart,\(^8\)\(^,\)\(^2\)\(^9\) Cx30.2 was the most interesting candidate. In comparison with controls (Figures 3A and 3G; n=3), Cx45KO hearts showed a slight decrease of Cx30.2 immunosignals in both nodal regions (Figures 3D and 3J; n=3). In addition to Cx30.2, Cx40 occurs in low amounts in blood vessels, which were located inside the node. In His bundle and bundle branches, which strongly express Cx40,\(^5\)\(^,\)\(^6\) no differences were observed between control and Cx45KO mice (data not shown). The level of Cx3, expressed in the working myocardium but not in the conduction system, was not changed either (data not shown). To quantify the reduction of Cx30.2 protein indicated by immunohistological stainings (Figures 3D and 3J), immunoblot analyses of Cx30.2...
were performed with Cx45KO heart lysates. These analyses showed a reduction of Cx30.2 protein by nearly 80% in Cx45KO hearts (Figure 4A–B; protein ratio mean±SD: control: 1, n=4; Cx45KO: 0.2±0.17, n=4; P<0.05). To elucidate whether the reduction of Cx30.2 expression occurred on a protein level only or also on transcript level, semiquantitative real-time reverse-transcriptase polymerase chain reaction analyses were performed. However, the results showed that the transcript level of Cx30.2 was not significantly changed in Cx45KO hearts (Figure 4C; transcript ratio mean±SD: control: 1±0.19, n=6; Cx45KO: 1.41±0.62, n=6; P=0.21), indicating a posttranslational reduction.

Electrophysiological Characterization of Cx45KO Mice

To identify the role of Cx45 in the adult mouse heart, Cx45KO and control littermates were investigated by in vivo electrophysiological measurements. No complications caused by anesthesia or operative preparation of the jugular vein were observed. One Cx45KO mouse had developed a third-degree A V block during positioning of the electrophysiological catheter in the right ventricle, likely attributable to mechanical alteration of the AV node. No AV block was observed in control or the other Cx45KO mice. All further animals (control: n=19, 15±2.1 weeks, 9 males; Cx45KO: n=15, 16.5±2.4 weeks, 7 males) underwent the entire stimulation protocol. The analyzed parameters of the surface ECG and intracardiac recordings are shown in a schematic overview (Figure 5A). Figure 5B depicts representative ECG and intracardiac recordings of control and Cx45KO mice and shows distinct prolongation of PQ interval and AH interval. The surface ECG analyses under conditions of inhalative anesthesia showed equal heart rates (Figure 5C; 419±29 bpm [control] versus 383±43 bpm [Cx45KO]; P>0.05) and P-wave durations (12.5±1.7 ms [control] versus 13.8±2.1 ms [Cx45KO]; P>0.05). The PQ interval was significantly prolonged in Cx45KO mice (Figure 5D; 37.4±4.5 ms [control] versus 43.1±3.4 ms [Cx45KO]; P=0.01) at equal QRS intervals (Figure 5E; 13.8±2.1 ms [control] versus 13.6±2.1 ms [Cx45KO]; P>0.05) and QTc (9.1±1.0 ms [control] versus 8.7±1.1 ms [Cx45KO]; P>0.05). Neither spontaneous AV block nor supraventricular or ventricular ectopic beats or arrhythmia occurred among the groups.
Analyses of intracardiac electrophysiological recordings of atrial, AV nodal, and ventricular levels are shown in Figures 5F and 5G. The AH interval as surrogate of AV nodal supraventricular conduction was significantly prolonged in Cx45KO mice (Figure 5F; 26.5±1.7 ms [control] versus 39.4±2.1 ms [Cx45KO]; P<0.001), whereas HV interval was unchanged (Figure 5G; 7.3±1.2 ms [control] versus 6.6±1.3 ms [Cx45KO]; P>0.05). Pacing threshold currents at 1 ms stimulus duration were 0.43±0.09 mA at atrial level and 0.30±0.10 mA at ventricular level. Stimulation maneuvers on atrial and ventricular levels were performed. Wenckebach periodicity is a functional parameter of AV nodal conduction capacity and was elongated in Cx45KO mice, pointing toward impaired AV nodal pulse transition under conditions of fast atrial frequencies (Figure 5H; 80.4±2.2 ms [control] versus 86.3±3.8 ms [Cx45KO]; P=0.02). The AV nodal refractory period showed a tendency toward prolongation in Cx45KO mice without reaching statistical significance (42.7±8.8 ms [control] versus 45.4±6.6 ms [Cx45KO]; P>0.15). Testing of atrial refractory period and ventricular refractory period showed no statistical significant differences (data not shown). Interestingly, the sinus node recovery time was shorter in Cx45KO mice, pointing toward an improved sinus nodal pacemaker function (Online Figure IA; 177.6±34.0 ms [control] versus 147.3±27.7 ms [Cx45KO]; P=0.03). Inducibility of arrhythmia on atrial and ventricular levels was unaltered between the genotypes.

To characterize standard ECG parameters in the absence of narcotic agents, telemetric Holter ECG recordings were performed in 3 animals of each genotype, and 16 standard average ECGs per animal were analyzed. In accordance to the data observed in surface and intracardiac ECGs, generally faster but still equal heart rates (Figure 5I; 571±72 ms [control] versus 590±65 ms [Cx45KO]; P>0.05) were detected. QRS intervals (Figure 5K; 10.7±0.6 ms [control] versus 10.4±0.6 ms [Cx45KO]; P>0.05) were not different, but PQ intervals were significantly prolonged in Cx45KO mice (Figure 5J; 36.4±0.5 ms [control] versus 41.0±0.6 ms [Cx45KO]; P=0.001). Further ECG parameters were not different among the groups, and no spontaneous arrhythmias or relevant AV block were detected (data not shown).

Cx45/Cx30.2 dKO Mice

Although AV nodal function was impaired in Cx45KO mice, conduction was still propagated via AV nodal cardiomyocytes, probably because of the residual Cx30.2 or the presence of Cx40. To investigate whether the AV node still can fulfill its function when 2 of the 3 AV nodal Cx are absent, Cx45/Cx30.2 dKO mice were generated. The breeding of these mice was complicated for 2 reasons. First, the genes for Cx45 (Gjc1) and Cx30.2 (Gjd3) are both located on mouse chromosome 11 at a distance of only ∼3.8×10⁶ base pairs (http://www.ncbi.nlm.nih.gov). Genetic distances specify the recombination frequency on a chromosome. Thus, 3.8×10⁶ base pairs correspond, on average, to 3.8 cM, indicating a recombination frequency of 3.8%. The second problem was that the previously published conditional Cx30.2LacZ mouse line10 contained 2 loxP sites, of which 1 still remained in the genome after Cre recombinase–mediated deletion of Gjd3 (Cx30.2). Because Cx45flox/flox mice also contained 2 loxP sites flanking 1 allele, breeding these mice with Cx30.2LacZ mice would result in 3 loxP sites on the same chromosome in relatively short distance. To circumvent the risk of undesirable recombination among these 3 loxP sites leading to a possible loss of nearly 4 million base pairs of chromosome 11, another nonconditional Cx30.2KO (YFP) mouse line was generated (Online Figure IIA). Polymerase chain reaction and Southern blot hybridization analyses verified correct genotypes and genomic integration (Online Figures IIB and IIC). Cx30.2KO mice were viable and showed no obvious differences in heart morphology (data not shown). Immunofluorescence analyses of heart cryosections proved that Cx30.2 was no longer expressed in the conduction system, but instead the reporter gene YFP was active (Figure 6; n=5). Because fluorescence of YFP was too weak, the reporter protein was analyzed with anti-GFP antibodies. Expression of Cx45 and Cx40 in the AV node of Cx30.2KO mice did not seem to be altered (Figures 6A and 6C; n=3) when compared with control mice (Figure 3A–B). Interestingly, although Cx30.2 was reduced in Cx45KO mice, immunoblot analyses showed no reduction of Cx45 in Cx30.2KO hearts (Online Figure III; protein ratio mean±SD: control: 1, n=6; Cx30.2KO: 0.97±0.37, n=7; P=0.4).

Interbreeding of homozygous Cx45flox/flox mice with homozygous Cx30.2KO/KO mice yielded 64 littermates, of which 2 mice had the desired genotype Cx45floxF/
Finally, after breeding these littermates with each other and with αMyHC-CreER(T2)–expressing mice, the desired Cx45/Cx30.2 dKO mice were obtained. Surprisingly, these mice were viable after administration of tamoxifen and showed normal heart morphology as well as AV nodal structure (Figure 6E–6H; n=4). The only Cx left in the AV node was Cx40, and its expression did not seem to be altered (Figure 6G; n=4) relative to control mice (Figure 3B).

Electrophysiological Characterization of Cx45/Cx30.2 dKO Mice

To characterize the cardiac impulse propagation in absence of the 2 predominant AV nodal Cx, Cx45 and Cx30.2, Cx45/Cx30.2 dKO mice were electrophysiologically investigated in vivo. The relatively small Cx30.2KO (YFP) group was chosen as an additional control group because the deletion of Cx30.2 previously has been electrophysiologically characterized in Cx30.2LacZ mice.10 After anesthesia and

Figure 3. Expression of cardiac connexins in the atrioventricular (AV) and sinus node of control and connexin (Cx) 45 knockout (KO) mice. A, Cx30.2 expression level in the AV node of control mice. B, Cx40 is only expressed in small amounts. D, The expression pattern of Cx30.2 is slightly reduced in Cx45KO mice. E, Cx40 expression in Cx45KO is similar to the control and overlaps with green fluorescent protein (GFP) expression in the distal region of the AV node. G, Expression level of Cx30.2 in the sinus node of control mice. H, Cx40 is not found to be expressed in the sinus node but only in the surrounding atrial myocytes and in nodal blood vessels (asterisk). J, Cx30.2 expression is slightly reduced in Cx45KO mice, whereas Cx40 expression (K) is similar to that described for the control. GFP expression is indicative of Cx45. Hyperpolarization-activated cyclic nucleotide-gated potassium channel 4 (HCN4) indicates the nodal regions (C, F, I, L). Nuclei are stained by Draq5. Scale bar, 50 µm.

A  B  C

Figure 4. Connexin (Cx) 45 ablation leads to decrease of Cx30.2 protein. A, Immunoblot analyses of Cx30.2 in control and Cx45 knockout (KO) hearts yield a reduction of Cx30.2 protein expression by 80% in the mutant heart (B; *P<0.05). C, Semiquantitative real-time reverse-transcriptase polymerase chain reaction analyses show that amount of Cx30.2 transcript is not significantly altered in Cx45KO mice (P=0.21).
preparation of the jugular vein, 4 dKO mice had developed a third-degree AV block during positioning of the electrophysiological catheter while passing the AV node region. No AV block was detected in control (n=12, 13.2±1.1 weeks, 3 males) or any of the other dKO (n=9, 13.1±1.2 weeks, 6 males) and Cx30.2KO (n=4, 13.2±0.5 weeks, 4 males) mice. Representative surface ECGs and intracardiac recordings of control, Cx30.2KO, and dKO mice showed shortening of the PQ intervals in Cx30.2KO mice and significant prolongation of these parameters in dKO mice (Figures 7A and 7C; control: 44.1±3.1 ms; Cx30.2KO: 37.8±3.9 ms; dKO: 53.9±3.7 ms; P<0.05). Equal heart rates (Figure 7B; control: 406±47 bpm; Cx30.2KO: 399±37 bpm; dKO: 449±54 bpm; P>0.05), P-wave durations (not shown), QRS intervals (Figure 7D; control: 14.1±2.2 ms; Cx30.2KO: 13.2±1.0 ms; dKO: 12.5±0.7 ms; P>0.05), and QTc intervals (not shown) were measured among the different genotypes. No AV block or supraventricular or ventricular arrhythmias were detected among the groups.

Intracardiac ECGs showed that the AH interval was significantly prolonged in dKO and shortened in Cx30.2KO mice (Figures 7A and 7E; control: 33.0±4.9 ms; Cx30.2KO: 27.5±0.7 ms; dKO: 48.3±4.6 ms; P<0.05). The HV period was not altered (Figure 7F; control: 6.4±2.7 ms; Cx30.2KO: 6.5±2.1 ms; dKO: 5.2±1.1 ms; P>0.05). Wenckebach periodicity was significantly shorter in Cx30.2KO and was prolonged in dKO mice relative to control mice (Figure 7G; control: 91.0±5.7 ms; Cx30.2KO: 83.8±4.8 ms; dKO: 100.2±7.1 ms; P<0.05). AV nodal refractory period, atrial refractory period, and ventricular refractory period showed no statistical significant differences (data not shown). Interestingly, sinus node recovery time was shorter in dKO compared with control mice, analogous to Cx45KO mice (Online Figure IB; control: 266.4±46.3 ms; Cx30.2KO: 242.5±24.8 ms; dKO:
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182.8±15.3 ms; P=0.004 dKO versus control). Inducibility of arrhythmia in atria and ventricles was not different among the genotypes.

Three dKO mice (16 standard average ECGs per animal) were analyzed by telemetric Holter ECG and compared with Cx45KO mice to estimate the additional effect of Cx30.2 depletion under normal conditions. In accordance with the data observed in surface and intracardiac ECGs, equal heart rates, P-wave durations, QRS, and QT intervals were observed (not shown). No spontaneous arrhythmias or relevant AV block were detected (data not shown). However, significantly prolonged PQ intervals were measured in dKO compared with Cx45KO mice (Figure 7H; 44.2±0.9 ms [dKO] versus 39.9±0.9 ms [Cx45KO]; P=0.001), pointing toward an enhanced effect of the dKO system with more severely impaired AV nodal function in awake animals. This is supported by the frequency of mechanically induced total AV block during catheter positioning, affecting 4 of 13 dKO, but affecting only 1 of 16 Cx45KO mice (Figure 7I).

Discussion
General and cardiomyocyte-directed Cx45 deficiency leads to embryonic lethality, indicating an indispensable role of Cx45 for development and function of the embryonic heart.11,21–23 In contrast, induced deletion of Cx45 in adult hearts has no effect on viability of mice, suggesting that Cx45 is not necessary for survival of adult mice. However, a recent report described (few) viable Cx45flox/flox:αMyHC-Cre mice without obvious cardiac conduction abnormalities.12 The reason for survival of these mice could be an incomplete deletion efficacy of the αMyHC-Cre recombinase12 or possibly their genetic background. The mice used by Bao et al12 were bred on a FVB/N genetic background, whereas our Cx45KO mice had a high C57BL/6 background. Several studies have pointed out that different genetic backgrounds can cause various phenotypes for the same mutation and that such phenotypic discrepancies can occur because of modifier genes that are active only in certain genetic backgrounds.30 We have tried to generate noninducible Cx45flox/flox:αMyHC-Cre mice, but these mice were embryonically lethal with high C57BL/6 background, leading us to generate inducible Cx45KO (Cx45flox/flox:αMyHC-CreER(T2)) mice to identify the role of Cx45 in the adult mouse heart.

Our results indicate that Cx45 ablation in the adult heart leads to impaired AV nodal conduction, which was shown by prolonged PQ and AH intervals in Cx45KO mice. Functional testing revealed slowing of AV nodal conductivity under fast atrial pacing maneuvers. Thus, Cx45 provides decent signal propagation through the AV node of the adult mouse heart. Additional deletion of Cx30.2 showed worsening of AV nodal conduction, which was gathered from longer PQ and AH intervals in Cx45//Cx30.2 double-deficient mice. Because Cx30.2 also was reduced by ≈80% in the Cx45 single KO mice, we cannot exclude that this might contribute to the AV nodal conduction delay measured in Cx45KO mice. A study by Krüger et al31 in which Cx45 was additionally heterozygously deleted in Cx40KO mice described that heterozygous Cx45 deficiency contributed to a further delay of the already prolonged PQ and QRS intervals in Cx40KO mice. In contrast, the absence of both alleles of Cx30.2 in a Cx40KO background (Cx30.2/Cx40 dKO mice) led to a...
milder phenotype than observed in both single KO mice. The impulse propagation was counterbalanced when both Cx30.2 and Cx40 were missing, although the single Cx30.2KO mice showed a shorter PQ interval, whereas the single Cx40KO mice exhibited prolonged PQ and QRS intervals. The remaining Cx45 in the AV node of Cx30.2/Cx40 dKO mice seemed to maintain the basal level of conduction. Furthermore, studies of Cx45-deficient embryos showed disturbed impulse propagation through the embryonic AV canal, indicating that Cx45 is indispensable for impulse propagation through the AV canal/node. Thus, we propose that the AV nodal conduction delay observed in the single Cx45KO mice is mainly based on Cx45 deficiency, because Cx45 seems to be indispensable for the basal impulse flow through the AV node. However, a contribution of reduced Cx30.2 protein amounts to this phenotype cannot be excluded with regard to the worsened conduction phenotype of Cx45//Cx30.2 dKO mice.

It also has been observed in other studies that the absence of one Cx isoform can cause the reduction of another Cx isoform. Nelles et al showed a reduction of Cx26 protein in the liver of Cx32KO mice attributable to the missing interaction partner. Another study described a reduction of Cx45 amount and distribution in Cx43-deficient ventricles, suggesting that localization of Cx45 in the ventricle depends on Cx43 expression. Our study showed decreased Cx30.2 protein expression in Cx45KO hearts but no significant change in the transcript level of Cx30.2. Cx30.2 and Cx45 are able to form both heterotypic and heteromeric gap junction channels of low conductance (≈17 pS) shown in cell culture and also in the conduction system of the heart. It remains unclear why such mixed channels are formed in the conduction system, although slow-conducting Cx45 (≈32 pS) and Cx30.2 (≈9 pS) homotypic/homomeric channels.
already exist. Cx30.2 channels show a low sensitivity of gating to transjunctional voltage (>80 mV), whereas Cx45 channels are more voltage-sensitive. Our findings point to different roles of the 2 Cx isoforms in the same gap junction channel and could help to understand why such heterotypic/ heteromeric gap junctions are formed in the conduction system. We propose that Cx45 has a stabilizing role for Cx30.2 and possibly facilitates or favors the formation of heterotypic/ heteromeric gap junctions with Cx30.2. However, Cx30.2 does not seem to be necessary for the stability of Cx45, but rather most likely for regulating the conductance of heterotypic/heteromeric channels. Cx30.2, as the slower conduction partner, could restrain the faster Cx45. Because conduction in the atria is very fast attributable to fast conducting Cx40 channels, Cx30.2 could act as a regulator of Cx45. Through the formation of heteromeric channels with Cx45, it could prevent overly fast opening of channels in the AV node triggered by fast atrial voltages, and thus it could slow AV nodal conduction. Deletion of Cx30.2 in the mouse heart causes faster AV nodal conduction, whereas the absence of Cx45 leads to slower AV nodal conduction. These results suggest that direct interaction and formation of heteromeric and heterotypic channels composed of both Cx isoforms may ensure proper impulse propagation through the AV node. This is consistent with the overlapping expression pattern of both Cxs.

In summary, Cx45 and Cx30.2 proteins in cardiac gap junction channels strongly interact and influence each other. Although Cx45 assures conduction through the AV node and seems to contribute to stability of heteromeric Cx45/Cx30.2 channels by stabilizing Cx30.2 protein expression, Cx45.2 acts as a fine-tuner and may counteract the formation of too-fast conducting and too-fast gating channels in the AV node. Deletion of these 2 Cxs allows even less impulse propagation through the AV node, as observed in Cx45/Cx30.2 double-deficient mice. Cx40 is still present in the AV node of these mice and possibly allows remaining conduction on a basal level, yet Cx40 was not upregulated in the AV node of double-deficient mice. If this were the case, then impulses likely would have been propagated very fast through the AV node because, of all cardiac Cxs, Cx40 forms gap junction channels of highest conductance.

Other channels, for example, of the family of HCN contribute to impulse generation and propagation in the conduction system. Of these channels, HCN4 is expressed in most parts of the conduction system. In our study, HCN4 was used as marker for the conduction system and we did not find any alteration of HCN4 expression by immunofluorescence. Quantitative expression studies of these and other potassium, sodium, and calcium channels could reveal further insights into impulse propagation through the AV node of Cx-deficient mice. Investigations of Cx43-deficient mice showed changes in potassium and sodium currents attributable to ion channel remodeling in their hearts. A recent study of Yanni et al in which heart failure was induced in mice, causing sinus node dysfunction, showed remodeling of several types of ion channels as well as Cx30.2 and Cx45. It also is likely that remodeling of ion channels could occur in Cx45KO and dKO mice because of uncoupling of the myocytes. A potential remodeling of ion channels also could be a reason for the shortened sinus node recovery time observed in both our mutant mice. This was surprising because the sinus node recovery time is mainly a parameter of the pace-making function of the sinus node, which is determined by spontaneous depolarizing currents in this specified compartment. Our results suggest that ablation of sinus nodal Cxs influences the function of the sinus nodal ion channel system, resulting in faster depolarization. Another reason also could be a change in coupling properties between sinus nodal cells and the surrounding atrial myocytes. Loss of slow-conducting Cx45 (and Cx30.2) channels implicates that only fast-conducting Cx40 channels remain between the sinus nodal and the neighboring atrial myocytes. This might affect the property of the sinus node to minimize the sink from these coupled neighboring cells during diastolic depolarization, resulting in a decreased sink during diastolic repolarization. The molecular mechanism of this phenotypological finding requires further extensive studies.

Furthermore, it becomes more and more evident that Cxs play several gap junction-independent roles during development and also in adulthood. Cx43 has been described to be involved in cell adhesion, morphology, motility, and migration in different cell types. Like Cx43, Cx45 also contains a PDZ-binding motif that allows binding to the zona occludens protein 1. Via zona occludens protein 1, Cx45 is linked to the actin cytoskeleton, and thus also could play a role in adhesion or morphology of cardiomyocytes. In addition, Cx45 also could recruit other adaptor proteins like the coxsackievirus and adenovirus receptor protein (CAR), which is a transmembrane protein belonging to the family of intercellular adhesion molecules and forms protein complexes with Cx45 via zona occludens protein 1. CAR was described to play an important role for AV nodal function and at intercalated discs of working myocytes. In the future, more extensive histological studies of the AV nodal structure in Cx45KO and dKO mice might yield new insights into gap junction-independent functions of Cx45.

With the present work, the function of Cx45 has been investigated in the adult mouse heart. All cardiac Cx isoforms investigated contribute to impulse generation and directed propagation in different heart regions, often in close dependence and cooperation with each other. It appears that the different types of Cx channels and possibly also hemichannels form an intrinsic functional network that seems to be highly adaptable to the different states of cardiac physiology. Our findings might prospectively contribute to a better understanding of human pathological states with alterations of AV nodal conduction (ie, in congenital or acquired higher-grade AV block or in the formation of slow pathway structures in AV nodal reentrant tachycardias).

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Disclosures

None.

References


What Is Known?

- Gap junction channels in the mammalian heart are formed by connexin (Cx) proteins and contribute to electric impulse propagation and coordinated contraction of cardiomyocytes.
- Cx45 is strongly expressed in the conduction system of the mouse and human heart.
- The lack of Cx45 in mouse embryos leads to early embryonic death attributable to cardiovascular defects.

What New Information Does This Article Contribute?

- For the first time, the functional contribution of Cx45 to impulse propagation in the conduction system of the adult mouse heart has been clarified and its interaction with Cx30.2 has been highlighted.
- Cx45 and Cx30.2 provide optimal impulse propagation through the atrioventricular (AV) node.

Cx45 is strongly expressed in the conduction system of the adult mouse and human heart, but its function there was yet unknown because of embryonic lethality resulting from Cx45 deficiency. The present study aimed to clarify this question and should contribute to better understanding of the general role of Cxs and their interactions in the AV node. Cx45 deficiency in adult mice leads to decreased AV nodal conduction capacity. Thus, Cx45 provides optimal impulse propagation through the AV node. In addition, Cx45 protein stabilizes Cx30.2 protein, which is strongly coexpressed with Cx45 in the conduction system. When both of these Cxs are absent, impulse propagation is even more impaired in the adult mouse heart. In the present work, generation of new inducible transgenie mice allowed circumventing of embryonic lethality and study of the function of Cx45 in the adult heart. Furthermore, double-deficient mice lacking the 2 major AV nodal connexins, Cx45 and Cx30.2, were generated. In the future, these mice may be useful for more detailed AV nodal studies and serve as a model system for a better understanding of human pathological states with altered AV nodal conduction, such as congenital AV blocks or AV nodal re-entrant tachycardias.
Connexin45 Provides Optimal Atrioventricular Nodal Conduction in the Adult Mouse Heart
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Supplemental Material

Detailed Methods

Animals

Mice were maintained under a 12-hour light/dark cycle and standard housing conditions, with food and water ad libidum. All experiments were carried out in accordance with the institutional guidelines for animal welfare and the German laws on experiments with animals.

Generation of transgenic mouse lines

In order to generate Cx45-deficient mice, Cx45flox/flox mice,1 having a loxP site-flanked coding region of Cx45 (Gjc1), were bred with tamoxifen-inducible and cardiac-specific αMyHC-CreER(T2) (Takefuji et al., unpublished data, 2012) mice. αMyHC-CreER(T2) mice express the Cre recombinase under control of the αMyHC (alpha myosin heavy chain) promoter which is active specifically in cardiomyocytes. Furthermore, this Cre recombinase is fused to mutated estrogen receptors that bind the estrogen analog tamoxifen (CreER(T2)). Activation of the Cre recombinase thus can be induced by donation of tamoxifen.2 The resulting Cx45flox/flox:αMyHC-CreER(T2) mice were termed Cx45KO mice while Cx45flox/flox mice were designated as control mice. Cx45flox/flox mice were generated in our lab1 and were backcrossed to C57BL/6 mice for four times until a background of 93.7% C57BL/6 was obtained. αMyHC-CreER(T2) mice were obtained from the laboratory of Dr. Stefan Offermanns (Bad Nauheim, Germany) and were also backcrossed to 93.7% C57BL/6 background.

For generation of Cx30.2KO (YFP) mice a targeting vector was designed in which the coding region of Cx30.2 (Gjd3) was replaced by a reporter gene expressing the enhanced yellow fluorescent protein (YFP). The final targeting vector pCx30.2:YFPneo included a 1.8 kb 5’ homology region (5’HR) of Cx30.2, a frt site-flanked neomycin selection cassette under control of the phosphoglycerate kinase promoter (PGK-neo), followed by an YFP reporter gene and a 1.4 kb 3’ untranslated region (3’UTR) of Cx30.2 as well as a 6.7 kb 3’ homology region (3’HR). The pCx30.2:YFPneo targeting vector was partially derived from vectors that were cloned for generation of the Cx30.2LacZ mouse.3 The final targeting vector was analyzed by restriction mapping and partial sequencing. Functionality of frt sites was verified by transformation of Flp recombinase-expressing E. coli bacteria.4 Finally, pCx30.2:YFPneo was linearized by KpnI digestion and transfected into HM1 embryonic stem (ES) cells.5 Recombinant ES cell clones were selected using 350 µg G418 (Sigma-Aldrich, Steinheim, Germany) per ml culture medium and tested for correct homologous recombination by PCR and Southern blot hybridization. Correct ES cell clones were injected into C57BL/6 blastocyst.6 Resulting chimera were mated to C57BL/6 mice and offspring was tested for the mutant allele by PCR. Four backcrossings to C57BL/6 mice were performed until a genetic background of 93.7% C57BL/6 was achieved. Breeding the offspring with deleter-Flp mice7 resulted in deletion of the frt site-flanked neomycin cassette.

Mice deficient for both Cx45 and Cx30.2 were generated by breeding of Cx45flox/flox with Cx30.2KO mice. The resulting double heterozygous offspring (Cx45flox+/Cx30.2KO+) were mated with each other to obtain double homozygous mutant mice (Cx45flox/flox/Cx30.2KO/KO). These mice were bred with αMyHC-CreER(T2) mice to finally obtain the double homozygous mutant, Cre-expressing Cx45flox/flox/Cx30.2KO/KO:αMyHC-CreER(T2) mice, termed doubleKO (dKO) mice. Littermates of the genotype Cx45flox/flox/Cx30.2KO/+ were taken as controls.
Genotyping of transgenic mice

To determine the different transgenic alleles mice were genotyped by PCR analyses using tail tip DNA. The Cx45flox PCR was performed with the primers 5'FCfor (5'-GGA TTA AAG GCA TAT GTC ACT CTT GGC) and 3'Frev (5'-CTC TAG GAA CAC TGT AAC CTG AGA TGT CCC). The αMyHC-CreER(T2) PCR was driven with the primers P1 (5'-CTT ACC CCA CAT AGA CCT CTG ACA) and P2 (5'-TGC TGT TGG ATG GTC TTC AGA G). The Cx30.2KO PCR was performed with the primers 5´HR-for (5'-CTC TCA AGG TCC TCC GCT CAG C), branch-rev (5'-CAA GAG CGG CTA CTG GAG TCT AG) and frtNeo-rev (5'-CGA GAT CAG CAG CCT CTG TTC CAC) under the following conditions: 95°C for 5 min; 95°C for 1 min, 67°C for 1 min, and 72°C for 45 sec, 30 cycles; 72°C for 10 min. An amplicon of 400 bp was obtained for the Cx30.2 wild type allele, an amplicon of 550 bp for the Cx30.2YFPneo allele and a 620 bp amplicon for the Cx30.2YFP allele without neo-cassette. Additionally, Southern blot hybridization was performed with EcoRI-digested DNA from Cx30.2KO mice with all possible alleles and a radioactively labelled probe for the 5´HR in front of the coding region of Cx30.2. The wild type allele was indicated by a band of 3.1 kb, the Cx30.2YFPneo allele including the neo-cassette by a band of 7.2 kb and the Cx30.2YFP allele without neo-cassette by a 9.1 kb band.

Tamoxifen injection

Forty mg tamoxifen (Sigma, Germany) were dissolved in 100 µl pure ethanol and 900 µl peanut oil (Oleum arachidis raffinat; Caelo, Germany) by rigorous vortexing at room temperature, maintaining a final concentration of 4 mg/100 µl. Adult mice were injected intraperitoneally once per day on five consecutive days. Mutant mice received a daily dose of 4 mg tamoxifen while control mice were injected with 100 µl ethanol/peanut oil solution. Analyses with mice were performed at least three weeks after the last tamoxifen injection. For embryo studies, pregnant females were intraperitoneally injected with 2-4 mg tamoxifen on two consecutive days and embryos were dissected two days after the last injection.

Immunofluorecence analyses

Hearts were dissected from cervically dislocated adult mice, frozen in the gas phase of liquid nitrogen and cryosectioned (16 µm). Sections were fixed for 10 minutes in 4% formaldehyde solution (Roti-Histofix, Roth, Karlsruhe, Germany), washed three times with Tris-buffered saline, pH 7.4, 0.3% Triton X-100 (TTBS) and blocked with 5% normal goat serum for 1 h at room temperature. The sections were incubated with the following primary antibodies in blocking solution at 4°C overnight: rabbit anti-Cx45 (1:500), rabbit anti-Cx30.2 (1:300), rabbit anti-Cx40 (1:500; Biocytin, Cologne, Germany), rabbit anti-HCN4 (Alomone Labs, Jerusalem, Israel), chicken anti-GFP (1:500; Abcam, Cambridge, UK) and mouse anti-smooth muscle actin (1:500; Sigma-Aldrich, Steinheim, Germany). Afterwards, sections were washed extensively in TTBS and incubated with the secondary antibodies Alexa Fluor546-conjugated goat anti-rabbit (1:1000; Invitrogen, Karlsruhe, Germany) and Cy2-conjugated goat anti-chicken (1:750; Dianova, Hamburg, Germany) in blocking solution for 1 h at room temperature. Nuclei were stained with Draq5 (1:5000; Cell Signaling Technology, Danvers, MA, USA) within the secondary antibody incubation step. Sections were mounted in Dako Glycergel mounting medium (Dako, Hamburg, Germany) and analyzed by confocal microscopy (LSM 510; Zeiss, Germany).

Immunoblot analyses

Frozen hearts were cut in the middle (transversal), the ventricle apex was discarded while the upper part of the heart was homogenized in Complete (Roche, Mannheim, Germany) and sonicated three times for 10 s. After centrifugation for 15 min at 10,000 g and 4°C, the protein concentration of the
supernatant was determined using the bicinchoninic acid protein assay (Sigma-Aldrich, Steinheim, Germany). Samples were denatured at 65°C for 10 min containing 1xLaemmli buffer. Proteins (100 µg) were separated by electrophoresis on a 12% polyacrylamide gel and transferred to Hybond ECL membrane (Amersham Bioscience/GE Health Care, Munich, Germany). The blotting membranes were preincubated in a blocking solution of 5% milk powder in TBST (50 mM Tris, 150 mM NaCl, pH 7.5, 0.1% Tween-20) for 1 h at room temperature, followed by incubation with mouse anti-Cx43 (1:500; Chemicon/Merck, Darmstadt, Germany) or rabbit anti-Cx30.2 (1:300) in blocking solution overnight at 4°C. After three wash steps with TBST, incubation with goat anti-mouse or goat anti-rabbit horseradish peroxidase-conjugated antibodies (1:10,000; Dianova, Hamburg, Germany) was performed for 1 h at room temperature. In order to prove that equal amounts of proteins were loaded, membranes were incubated with mouse anti-β-actin (1:500; Sigma-Aldrich, Steinheim, Germany). Immunoreactivity was visualized with the ECL chemiluminescence detection kit (Pierce/Thermo Scientific, Germany) and developed with X-ray films. Band intensities were quantified using the Herolab E.A.S.Y. Win32 software (Herolab GmbH, Wiesloch, Germany).

Semi-quantitative real time reverse transcriptase (RT-) PCRs

Total RNA was isolated from the upper half of adult hearts using Trizol (Invitrogen/Life Technologies, Germany) following the manufacturer’s protocol. Genomic DNA was removed by DNase treatment as described previously and mRNA was isolated using oligo(dT)25-linked Dynabeads (Invitrogen/Life Technologies, Germany) following the manufacturer’s protocol. Semi-quantitative real time RT-PCRs and data analyses were performed as described previously. TaqMan gene expression assays (Applied Biosystems/Life Technologies, Germany) were used for Cx30.2 (Gjd3) RT-PCR and the β-actin reference gene RT-PCR.

In vivo electrophysiological examination

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). Preparation, catheterization and EPI were conducted under conditions of inhalative anaesthesia with the animal breathing spontaneously (induction period 2.5 vol%, maintenance 1.0-1.2 vol% isoflurane in 70% N2O/30% O2). A constant body temperature of 37 °C was warranted by use of a heating pad. A surface 6-lead-ECG was obtained by cutaneous clips on each limb. Access to the jugular vein was achieved by lateral cervical incision under sterile conditions. The jugular vein was dissected from surrounding tissue and proximally ligated. A 2-French octapolar mouse-electrophysiological catheter (eight 0.5 mm circular electrodes; electrode pair spacing 0.5 mm (Ciber Mouse, NuMed Inc., New York, NY, USA)] was positioned transvenously in the right heart chambers under constant monitoring of the intracardiac electrograms. The catheter was forwarded, passing the tricuspid valve, until clear atrial (proximal electrode pairs) and ventricular (distal electrode pairs) electrograms were documented. The His signal was visible in 96% of the investigated animals in which catheter positioning was successful and was mainly present on the tricuspid valve electrode level.

Analyses of the surface-ECG under inhalative anesthesia

The surface ECG was monitored continuously during the experiments. Surface ECG parameters were analyzed under stable baseline conditions at least 5 min after induction of the anaesthesia and before starting preparation of the jugular vein. Heart rate, P wave duration, PQ interval, QRS duration, QT interval and rate corrected QTc were measured in lead II (mean of 3 different ranges of 5 RR complexes). Evaluation of ECG recordings was performed according to previous mouse electrophysiological studies. All data of ECG- and EPI recording were sampled at 4 kHz (Bard stamp amplifier; Bard LabSystem, C.R. Bard Inc., New Jersey, USA) and digitally stored on optical disc.
**Transvenous Electrophysiological Study Protocol**

Transvenous atrial and ventricular recording and stimulation were conducted for electrophysiological investigation. Bipolar electrograms were obtained from each pair of electrodes. For evaluation of supra- and infra-Hisian conduction, AH and HV intervals were analyzed in the intracardiac recording in which the His (H) was visible. AH as surrogate for supra-Hisian conduction time was defined as period from the starting point of the atrial (A) signal to the maximum deflection of the H signal in the intracardiac lead. HV was defined as the time-period from the maximum deflection of the H signal to first deflection of the QRS complex in surface lead II (V=ventricular).

Pacing threshold currents at 1 ms stimulus duration were 0.43±0.09 mA at atrial and 0.30±0.10 mA at ventricular level. Twice pacing threshold rectangular stimulus pulses were administered by a modified, multi-programmable stimulator with S1S1 stimulus cycle lengths (CLs) down to a duration of 10 ms (Model 5328; Medtronic, Minneapolis, MN, USA).

Atrial fixed rate (starting at S1S1 pacing CL 20 ms beneath spontaneous RR interval) as well as extrastimulus pacing were performed. The standard electrophysiological parameters analyzed were sinus node recovery time (SNRT), Wenckebach periodicity (WBP), atrial and AV-nodal refractory period (ARP, AVNRP). SNRP was defined as the interval between the last stimulation spike and first spontaneous, sinus node triggered atrial activation after 5 s fixed rate atrial pacing (maximum return-CL at S1S1 stimulation CLs of 100 ms). ARP and AVNRP were evaluated by programmed atrial extrastimulus pacing at S1S1: 100 ms with 10 ms stepwise S1S2 reduction starting at 70 ms. AVNRP was defined as the longest S1S2 pacing interval with loss of AV-nodal conduction, ARP as longest S1S2 with absent atrial capture. Ventricular refractory period (VRP) was evaluated by programmed ventricular stimulation with single extrastimulus analogous to ARP, but at S1S1: 120 ms, 110 ms and 100 ms, and starting at 90ms. Atrial and ventricular programmed and burst stimulation protocols were applied for testing inducibility of atrial fibrillation and ventricular arrhythmias, as previously described.3,13,14

**Holter ECG: Chip implantation and recording**

For detection of spontaneous arrhythmias and AV blocks, telemetric ECG recordings were performed in awake mice. For this, telemetric Holter ECG chips with two subcutaneous electrodes (Data Science International, St. Paul, USA) were implanted under the same anesthesia protocol as described above. Chips were implanted under sterile conditions on the upper back of the mouse after medial incision and subcutaneous preparation. ECG leads were implanted subcutaneously in the left-lateral and anterior part of the thorax, and connected to the telemetric chip device. After that, skin was sutured. No mice died during the procedure, 2 mice had to be excluded from further studies and sacrificed due to inflammation of the wound. Postoperative analgesia was performed for seven days, using subcutaneous injection of 5 mg/kg Carprofen. Holter ECG recordings were performed in all mice that showed normal behaviour 7-10 days after implantation of the telemetric chips. Recordings were performed for 8 h during day and night in each mouse, electrograms were recorded continuously during this period and sampled and stored digitally using standard mouse Holter equipment (PowerLab™ System, ADInstruments, Milford, MA, USA). Analyses included standard average ECG recording (SAECG). 100 ECGs every hour of recording for each mouse were averaged, resulting in 16 analyzable SAECG recording per investigated mouse. Moreover, all recordings were manually screened for spontaneous tachy- and bradyarrhythmias and alterations of AV-nodal conduction, i.e. AV blocks grade I-III. After that, mice were investigated with in vivo electrophysiological investigation and sacrificed after that.

**Statistical Analysis**

Statistical analyses were performed with a two-tailed Student’s t-test. Discrete variables were analyzed by 2-sided Fisher’s exact test. A P value <0.05 was regarded as statistically significant.
References


Online Figures and Figure Legends:

Online Figure I: Sinus node recovery times (SNRT). (A) Cx45KO mice show significantly shortened SNRT compared to control mice (*: $P=0.03$). (B) SNRT is shortened in dKO as compared to control (*: $P=0.004$) and Cx30.2KO mice (not significant).

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A

B

C

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Online Figure II: Generation and characterization of Cx30.2KO(YFP) mice. (A) Schematic representation of the targeting strategy. The targeting vector pCx30.2YFPneo contains a 1.8 kb 5’ homology region (5’HR) of Cx30.2, a frt site-flanked neomycin selection cassette under control of a PGK promoter, followed by an YFP reporter gene and a 1.4 kb 3’ untranslated region (3’UTR) of Cx30.2 as well as a 6.7 kb 3’ homology region (3’HR). After homologous recombination of the vector in the genome, mice expressing this allele were termed Cx30.2YFPneo mice. Flp recombinase activity causes deletion of the neo-cassette resulting in the Cx30.2YFP allele. Triangles: frt sites, rectangles: Southern blot hybridization probes, arrows: PCR primers, Bcl: restriction sites for BclI. (B) PCR analysis of transgenic mice. An amplicon of 400 bp shows the Cx30.2 wild type allele, an amplicon of 550 bp the Cx30.2YFPneo allele and a 620 bp amplicon for the Cx30.2YFP allele. (C) Southern blot analysis of BclI-digested mouse DNA after hybridization with a radioactively labelled probe derived from the 5’ homologous region. The wild type allele is indicated by a band of 3.1 kb, the Cx30.2YFPneo by a band of 7.2 kb and the Cx30.2YFP allele by a 9.1 kb band.

Online Figure III: Cx45 protein expression in Cx30.2KO hearts. (A) Immunoblot analyses of Cx45 in control and Cx30.2KO hearts. (B) Quantitative analyses of Cx45 immunoblots do not show significant change in Cx45 protein expression level between the two genotypes (P=0.4).