Connexin 30 is expressed in the mouse sino-atrial node and modulates heart rate

Daniel Gros1*, Magali Théveniau-Ruissy1, Monique Bernard2, Thierry Calmels3, Frank Kober2, Goran Söhl†, Klaus Willecke4, Joël Nargeot5, Habo J. Jongsm6, and Matteo E. Mangoni5

1Institut de Biologie du Développement de Marseille-Luminy (UMR CNRS 6216), Université de la Méditerranée, Campus Scientifique de Luminy, case 907, Avenue de Luminy, 13288 Marseille, France; 2Centre de Résonance Magnétique Biologique et Médicale (UMR CNRS 6612), Université de la Méditerranée, Marseille, France; 3Bioprojet-Biotech, Saint Grégoire, France; 4Institut für Genetik, Abteilung Molekulargenetik, Universität Bonn, Bonn, Germany; 5Institut de Génomique Fonctionnelle (UMR CNRS 5203, Unité INSERM 661), Universités de Montpellier I et II, Département de Physiologie, Montpellier, France; and 6Department of Medical Physiology, University Medical Center, Utrecht, The Netherlands

Received 1 June 2009; revised 21 July 2009; accepted 10 August 2009; online publish-ahead-of-print 13 August 2009

Time for primary review: 21 days

Aims
This study aimed at characterizing expression and the functional role of the Gjb6 gene, encoding for connexin 30 (Cx30) protein, in the adult mouse heart.

Methods and results
The expression of the Gjb6 gene in the mouse heart was investigated by RT–PCR and sequencing of amplified cDNA fragments. The sites of Gjb6 expression were identified in the adult heart using transgenic mice with reporter genes (Cx30LacZ/LacZ and Cx30 LacZ/LacZ/Cx40EGFP/EGFP mice), as well as anti-HCN4 (hyperpolarization activated cyclic nucleotide-gated potassium channel 4) or anti-connexin antibodies. Cine-magnetic resonance imaging and telemetric ECG recordings were used to evaluate the impact of Cx30 deficiency on cardiac physiology. Gjb6 was shown to be expressed in the sinoatrial (SA) node of the adult mouse heart. Eighty from 100 nuclei on average were LacZ-positive in the SA node of Cx30LacZ/LacZ mice. No significant LacZ expression was seen in other cardiac tissues. Cx30 protein was identified in low abundance in the SA node of wild-type mice, as indicated by immunofluorescence experiments. Telemetric ECG recordings indicated that Cx30-deficient mice displayed a mean daily heart rate (HR) that was 9% faster than that measured in control mice (572 ± 38 b.p.m. vs. 524 ± 23, P < 0.05). This moderate tachycardia was still observed after inhibition of the autonomic nervous system, demonstrating that Cx30 deficiency resulted in changes in the intrinsic electrical properties of the SA node. Consistent with this hypothesis, Cx30LacZ/LacZ displayed a significant reduction of SDNN (standard deviation of the interbeat interval) compared with control mice. Increase of both the cardiac index (20%) and the end-diastolic volume to body weight ratio (16%) with no deficiency in ejection fraction or stroke volume were observed in mutant mice. An increase in cardiac index was interpreted as being a direct consequence of high HR, whereas large end-diastolic volume may be an indirect consequence of prolonged high HR.

Conclusion
Cx30 is functionally expressed, in low abundance, in the SA node of the adult mouse heart where it participates in HR regulation.

Keywords
Gjb6 gene • Connexin 30 • Gap junction • Intercellular coupling • Sinoatrial node • Pacemaker • Mouse heart • Heart rate

1. Introduction
Twenty connexin (Cx) genes have been identified in the human genome, and 21 in that of the mouse.1 Proteins encoded by this gene family fulfil three types of functions: (i) intercellular communication when they are the structural components of gap junction channels spanning two adjacent plasma membranes,2 and allowing ions, small metabolites, and second messengers to translocate...
from cell to cell; (ii) intercellular signalling when they are assembled into hemi-channels (or connexons) only, that span a single plasma membrane; and (iii) other functions, including diverse phenomena such as cell growth, tumorigenesis, injury, apoptosis, etc., and for which gap junction-mediated intercellular adhesion has been suggested to play a role. Several genetic diseases caused by mutations in Cx genes have been identified in human; some of them have been modelled in the mouse.

Four connexins have been shown to be expressed in cardiomyocytes: Cx45, -43, -40, and -30.2. Their roles, both in the propagation of cardiac electrical activity, and in heart development, have been partially deciphered through investigations of transgenic mice. Cx43 is the major connexin of the mammalian heart. In the mouse, Cx43 is encoded by the Gja1 gene, and it is abundantly expressed by all cardiomyocytes with the exception of those of the sinoatrial (SA) and atrioventricular (AV) nodes, the His bundle and the proximal parts of the bundle branches. It has been demonstrated that development of the cardiac outflow tract requires expression of Gja1 in the dorsal and ventral regions of the neural tube. In addition, cardiomyocyte specific loss of Cx43 results in cardiac abnormalities and post-natal death. It has also been shown that a 90% decrease of Cx43 abundance in ventricular tissue leads to a 50% reduction of impulse conduction velocity, and induces fatal arrhythmias. Thus, Cx43 contributes to the regulation of impulse propagation in the ventricles. In the adult mouse, Cx40, encoded by Gja5, is expressed in atrial cardiomyocytes, and in those of the ventricular conduction system (CS), and the central part of the AV node. Cx40 is detected neither in adult working ventricular myocytes, nor in the SA node. Only a fraction of mice that survive deletion of Gja5 displays cardiac malformations, suggesting the existence of genetic modifiers. Investigations of cardiac electrical activity in Cx40-deficient mice have identified two types of defects: alterations of influx propagation (reduced conduction velocity or conduction blocks) at various levels of the CS (AV node, His bundle, and bundle branches), and an increased incidence of inducible atrial arrhythmias. These results, in agreement with the expression pattern of Cx40, indicate that this Cx contributes significantly to the propagation of electrical activity in the atria and the cardiac CS. Cx45, encoded by Gjc1, is expressed in all compartments of the early mouse heart (E8.5, E9.5), but, as development proceeds, expression declines, and in the adult mouse heart, Gjc1 expression is mainly restricted to cardiomyocytes of the CS, including those of the SA node, and of the most peripheral regions of the interventricular septum. Germline or conditional disruption of Gjc1 results in embryonic death around E10. The causes of the lethality have not yet been clearly established, but abnormal haemodynamics in Gjc1 knock-out embryos may explain some aspects of the cardiac and vascular morphological phenotypes. These results, however, indicate that Cx45 protein is required for the normal progress of cardiogenesis. In the adult mouse heart, Cx30.2, encoded by Gjb6, is expressed in the SA and AV nodes, and to a lesser extent in the His bundle and its branches. Cx30.2 contributes to slow down impulse propagation in the AV node, and to limit the number of beats conducted from atria to ventricles.

Here we report that Cx30, encoded by the Gjb6 gene, is expressed in the murine heart. The use of molecular markers indicates that Cx30 expression is restricted to the SA node. Comparison of heart rate (HR) of Cx30-deficient mice with that of control mice indicates that Cx30 contributes to HR regulation. These results provide the first evidence for the expression and function of a fifth connexin gene in the murine heart.

2. Methods

All experimental protocols with animals were performed in agreement with the French (decree no. 2001–486) and German laws in application of the European Council Directive (no. 86/609/EEC), with the national charts dealing with ethics of experiences with animals, and confirmed 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 1996). All experimenters were duly authorized to experiment with animals.

Mouse connexin genes are designated according to the international nomenclature (see http://www.informatics.jax.org/ then enter ‘connexin genes’, and also http://www.gene names.org /genefamily/gj.php).

2.1 Mouse genotypes

Generation and genotyping of Cx40EGFP/Cx30lacZ/LacZ, and Cx30lacZ/LacZ mice have been described. The genetic background of Cx30-deficient mice was: 50% C57BL/6/6, 50% CD1. Wild-type control mice were obtained by crossing C57BL/6 males with CD1 females, all purchased from CERJ (Le Genest St Isle, France). Double mutant mice were generated by crossing of Cx40EGFP/EGFP females with Cx30lacZ/LacZ males. Rats (Wistar) were purchased from the Charles Rivers Laboratory (L’Arbresle, France).

2.2 RT–PCR experiments

RNA was extracted from whole hearts of adult rats, and from mouse hearts at different developmental stages (E9.5, E12.5, E14.5, newborn, and adult) with Trizol reagent (Invitrogen). RNA was reverse transcribed using the oligo(dT) primers of the Superscript II amplification system (Invitrogen). The sequences of the oligonucleotide primer pairs used for amplification of rat and mouse cDNA fragments by polymerase chain reaction (PCR) or nested PCR are available in Supplementary material online, Table S1. All RNA samples were tested for DNA contamination with assays performed in the absence of reverse transcriptase. For the details of protocols, see Supplementary material online.

2.3 Cloning and sequencing

PCR-amplified fragments from mouse and rat cDNAs were extracted from gels, then cloned using standard molecular biology techniques. Sequencing was performed at Genome Express (Meylan, France).

2.4 Detection of reporter genes and immunochemistry

Beating adult hearts were removed from Cx30lacZ/LacZ mice under general anaesthesia (see Supplementary material online), processed to detect LacZ expression (see Supplementary material online), then post-fixed with paraformaldehyde for 30 min. The right atrium was dissected and prepared (see Supplementary material online) to expose the SA node region, then examined with a Zeiss Lumar stereomicroscope. Alternatively, paraformaldehyde-fixed right atrial preparations were perfused with 10% (for 2 h) and 20% (overnight) sucrose solutions at 4°C, embedded with Tissue-Tek OCT compound (Sakura Fineteck), and frozen in liquid nitrogen before cryostat sectioning at −20°C.
Sections (5 μm-thick) were either stained for 30 min with 0.2% eosin, dehydrated, mounted in Entellan (Merck), then observed with a Zeiss Axioplan II light microscope, or processed for HCN4 (hyperpolarization-activated cyclic nucleotide-gated potassium channel 4) immunodetection (see Supplementary material online). Sections, mounted in Aquatex (Merck), were observed with an Axioplan II microscope.

Hearts collected from Cx40EGFP/EGFP/Cx30LacZ/LacZ double transgenic mice were first processed for the detection of LacZ expression (see Supplementary material online). Right atria were dissected and prepared to expose the SA node region, then observed with a Zeiss Lumar stereomicroscope equipped for epifluorescence.

2.5 Immunofluorescence experiments
Paraformaldehyde-fixed right atrial preparations and entire hearts, from wild-type and Cx30LacZ/LacZ mice, were perfused with sucrose solutions, embedded with OCT compound and frozen in liquid nitrogen, as described earlier. Sections, cut in a plane perpendicular to the crista terminalis (CT), were washed three times in PBS, quenched with 100 mM NH4Cl for 10 min, washed again, and blocked with saturation solution (see Supplementary material online) for 60 min at room temperature. For single labelling experiments, sections were incubated overnight at 4°C with primary antibodies (see Supplementary material online) diluted in saturation solution, washed three times in PBS, then incubated with appropriate secondary antibodies (see Supplementary material online) for 60 min at room temperature. For double labelling experiments, sections were incubated overnight with the first primary antibodies (anti-Cx30), then either overnight (anti-Cx45) or for 60 min (anti-α-actinin) with the second primary antibodies. Sections were then successively incubated with two appropriate secondary antibodies for 60 min at room temperature. After washing, sections were stained with Hoechst 33342 (1 μg/mL) (Merck) for 20 min, mounted with Fluoromount-G (Southern Biotech), and observed with a Zeiss Axioplan II microscope equipped for epifluorescence. Control experiments were carried out by omitting the primary antibodies.

2.6 Cine-magnetic resonance imaging
Eleven wild-type mice (Cx30+/+), and 12 Cx30-deficient mice (Cx30LacZ/LacZ), with a balanced sex combination in both cases, were used for cine-magnetic resonance imaging (MRI) investigations. Their age ranged from 3 to 4 months. The protocol used for this in vivo investigation is available in Supplementary material online.

2.7 Telemetric ECG recording on conscious mice
Experiments were carried out with 10 wild-type control mice (Cx30+/+), and 10 Cx30-deficient mice (Cx30LacZ/LacZ) with 50% of females in both cases. Their age ranged from 4 to 8 months. ECG recordings by telemetry, and recording analysis were carried out as described in Supplementary material online.

3. Results
3.1 Gjb6 gene transcript is expressed in the mouse heart
The repertoire of Cx genes expressed in the rat heart was explored by RT–PCR. Besides the expression of genes previously reported, Gja1, -a1, -a4, -a5, and -c1 encoding for the Cx43, -46, -37, -40, and -45 proteins, respectively,19–22 a weak signal was detected with primer pairs deduced from the Gjb6 gene sequence (Supplementary material online, Table S1), encoding Cx30 (Figure 1A). The identity of the 301 bp amplicon, containing the 590–890-bp region of the published rat Gjb6 cDNA sequence (NM_053388), was confirmed by DNA sequencing (data not shown). This result prompted us to investigate Cx30 gene expression in the mouse heart. RT–PCR experiments were performed using intron-spanning primer combinations to detect a transcript isoform containing the three exons identified in the mouse Gjc6 gene (Figure 1B) (Supplementary material online). Nested PCR experiments yielded amplicons of the predicted sizes, whatever the developmental stage investigated, from E9.5 to the adult stage (Figure 1B). Identity of amplicons was subsequently confirmed by sequencing (not shown).

3.2 Gjb6 gene expression sites in the adult mouse heart
We subsequently aimed to identify the expression sites of the Gjb6 gene in the adult mouse heart. For this purpose, Cx30LacZ/LacZ transgenic mice were used.17 In all mutant mouse hearts, a cluster of β-galactosidase-positive cell nuclei was seen at the junction of the superior vena cava with the right atrium (Figure 2A). Examination of right atrial preparations (Supplementary material online) indicated that the clusters of β-galactosidase-positive nuclei were always located along the CT (Figure 2B–D), at the presumed site of the SA node. The number of stained nuclei was variable from one mouse to another (Figure 2A–D), with a mean of 80–100 nuclei (n = 30 atria). Occasionally a few stained nuclei were also seen scattered around the wall of the superior vena cava, near its entrance into the atrium (data not shown).

No significant β-galactosidase activity was detected in other cardiac structures. In Cx30LacZ/LacZ/Cx40EGFP/EGFP double transgenic mice, the clusters of β-galactosidase-positive nuclei were observed along the CT, within the intercaval region, that was devoid of EGFP expression (Figure 2E). Examination of sections containing β-galactosidase-positive nuclei, after immunodetection of HCN4, indicated that all labelled nuclei were localized in HCN4-positive regions. No labelled nuclei were seen outside these regions (Figure 2F). Finally, in sections of right atrial preparations from Cx30LacZ/LacZ mice, cut in a plane perpendicular to the CT, the β-galactosidase-positive nuclei were always observed in the sub-epicardial region of the tissue (Figure 2G). No stained nuclei were seen in the sub-endocardial regions.

Expression of the Cx30 protein was investigated in sections of right atrial preparations from control adult mice using rabbit anti-Cx30 antibodies. Specific signals were observed near the CT, in the sub-epicardial part of the presumed SA node region (Figure 3A and B). No signal was detected in sections subjected to control experiments. Double immunolabelling of the sections with rabbit anti-Cx30 and mouse anti-α-actinin antibodies indicated that Cx30 protein was very closely associated with α-actinin-positive cells (Figure 3C and D). Sections were also treated with both rabbit anti-Cx30 and guinea-pig anti-Cx45 antibodies. Examination of sections showed that the Cx30-positive regions were included in the Cx45-positive regions (data not shown) that extended from the sub-epicardial surface to the sub-endocardial surface of the nodal regions, as previously described.14 Detailed analysis of the sections near the epicardial surface revealed sites where both Cxs, Cx30...
and Cx45, were expressed, while in other sites either Cx30 or Cx45 was expressed (Figure 3E and F).

3.3 Cardiac phenotype of Cx30-deficient mice

To evaluate a possible impact of Cx30 deficiency on cardiac physiology, various morphological and functional parameters of the left ventricle of control (Cx30+/+) and mutant (Cx30lacZ/lacZ) mice were measured in vivo using cine-MRI. This technique required anaesthesia of mice, and under these conditions the HR of mutant mice was significantly faster than that of control mice (540 ± 55 b.p.m. vs. 488 ± 59, P < 0.05) (Table 1). The values of all measured parameters with cine-MRI, or values calculated from these parameters, are given in Table 1. The morphological parameters were normalized to body weight to take into account the significant weight difference between control and age-matched Cx30-deficient mice (28 ± 6 g vs. 24 ± 5, P < 0.05) (Table 1), considering that weight loss in the mutants might not be related to the gene deletion.23 There was no sign of hypertrophy in mutant mice as indicated by similar ventricular wall mass/body weight ratios in both genotypes (VWmass/BW, Table 1). However, there was a significantly larger end-diastolic volume as indexed to body weight (EDVol/BW) in Cx30-deficient mice than in control mice (2.8 ± 0.4 mL/g vs. 2.4 ± 0.4, P < 0.05) (Table 1). This larger normalized EDVol was driven by differences in normalized end-diastolic length (EDL/BW) (0.34 ± 0.06 mm/g vs. 0.29 ± 0.05, P < 0.05) (Table 1). Among the functional parameters, the ventricular wall thicknesses of both control and mutant mice were similar (EDWT/BW and ESWT/BW, Table 1). Among the functional
**Figure 2** Localization of β-galactosidase activity in Cx30^lacZ/lacZ adult mouse heart. (A) External (or epicardial) view of the basal region of a heart showing the right atrium (ra), the superior vena cava (svc), and a part of the right ventricle (rv). Note the cluster of β-galactosidase-positive cell nuclei (arrow) at the junction of the superior vena cava with the right atrium. Bar: 1 mm. (B) Endocardial face of the intercaval region with a cluster of stained nuclei along the CT. The dashed line delineates the entrance of the right atrial chamber. (ra) indicates the epicardial face of the right atrium. Bar: 50 μm. (C and D) illustrate two examples of β-galactosidase-positive nuclei clusters: one with only a few positive nuclei (C), the other with numerous positive nuclei (D). ct: crista terminalis; ra: endocardial face of the right atrium. Bar: 100 and 75 μm for (C) and (D), respectively. (E) Endocardial face of a right atrial preparation dissected from a Cx30^lacZ/lacZ/Cx40EGFP/EGFP adult mouse heart. The trabeculae of the atrium (ra), the crista terminalis (ct), and the interatrial septum (ias) express EGFP. The cluster of β-galactosidase-positive nuclei (arrow) is located along the CT in a region devoid of EGFP. Bar: 150 μm. (F) Section carried out in a right atrial preparation from a Cx30^lacZ/lacZ mouse after in toto β-galactosidase activity detection, and HCN4 immunodetection. The HCN4-positive area, in light brown, has clear-cut boundaries (dashed lines). Nuclei with β-galactosidase activity were observed only in the HCN4-positive region. Arrow indicates the nodal artery. Bar: 40 μm. (G) Serial eosin-stained sections, perpendicular to the CT, in the SA node region. Sections were carried out from a right atrial preparation of Cx30^lacZ/lacZ mouse heart after in toto β-galactosidase activity detection. Note that all the labelled nuclei (arrows) are localized in the sub-epicardial region. epi and endo: epicardial and endocardial surfaces, respectively. Bar: 100 μm.
Figure 3  Expression of Cx30 protein in wild-type adult mouse heart. Sections in right atrial preparations, perpendicular to the CT. (A and B) Sections incubated with anti-Cx30 antibodies and appropriate secondary antibodies. Immuno-positive signals (red) (yellow arrows) were observed in the sub-epicardial region, close to the CT, in the presumed SA node. Arrowheads point to the CT. Endo and epi: endocardial and epicardial faces, respectively. Bars: 150 μm. (C and D) Sections incubated with anti-Cx30 and anti-α-actinin antibodies, and appropriate secondary antibodies. Striated expression of α-actinin (green signal) identifies cardiomyocytes. Cx30 (red signal) was very closely associated with these cells. Nuclei were stained with Hoechst (blue signal). Bars: 40 μm. (E and F) Sections incubated with anti-Cx30 and anti-Cx45 antibodies, and appropriate secondary antibodies. Upper sub-panels: Cx45 expression (green signal) near the epicardial surface of the SA node region; middle sub-panels: Cx30 expression (red signal) in the same area; lower sub-panels: merged micrographs of the upper and middle sub-panels, with Hoechst-stained nuclei (blue signal). Stars indicate sites where Cx45 and Cx30 were both expressed; green and red arrows indicate sites where either Cx45 or Cx30 was expressed, respectively. Bars: 10 μm.
Cx30 expressed in the SA node modulates HR

**4. Discussion**

**4.1 Cx30 protein is expressed in the SA node of adult mouse heart**

RT–PCR experiments and subsequent sequencing of all amplicons demonstrated that the Gjb6 gene, encoding the Cx30 protein, was expressed in the rodent heart. Its low expression might explain the inability (HRV) were similar both in Cx30+/+ and Cx30lacZ/lacZ mice (Supplementary material online, Figure S1), suggesting that the loss of Cx30 affected rate-dependent HRV parameters without altering the autonomic regulation of HR. The QT interval of Cx30lacZ/lacZ mouse ECGs was significantly shorter than that of control mice (46 ± 8 ms vs. 53 ± 7, P < 0.05) (Table 2). However, when QT interval values were corrected (QTc) to take into account HR (see Supplementary material online), the difference between the two genotypes disappeared (42 ± 5 ms vs. 47 ± 5, P > 0.05) (Table 2), indicating that the shorter QT interval recorded in mutant mice was a consequence of HR increase. No significant differences in other ECG waveform parameters were found between control and Cx30lacZ/lacZ mice (Table 2). The moderate tachycardia observed in Cx30lacZ/lacZ mice was still present after pharmacological block of the autonomic nervous system input by injection of atropine and propanolol (Supplementary material online). After injection, HR reached 541 ± 43 b.p.m. in Cx30lacZ/lacZ mice, and 497 ± 43 b.p.m. (P < 0.05) in control animals (Figure 4C) (Table 2), indicating that tachycardia was intrinsic to HR. Shortening of the QT interval in Cx30lacZ/lacZ ECGs was observed also after injection of atropine and propanolol, while the QTc interval did not differ between the two genotypes. In contrast, under the same conditions, the PQ interval was longer in control mice than in Cx30lacZ/lacZ mice (Table 2), although the difference between the two mean values was at the limit of statistical significance (39 ± 2 ms vs. 34 ± 3, P = 0.0504).

Thus there was a trend towards reduction of PQ intervals in Cx30-deficient mice that responded to autonomic input to a lesser extent than control mice. However, it is unlikely that Cx30 directly regulates AV intranodal conduction because expression of Cx30 in the AV node has not been observed. The simplest hypothesis to explain the reduction of the PQ intervals is to consider that this phenomenon is secondarily induced by the increase of HR in Cx30-deficient mice. No atrial dysrhythmias, or episodes of ventricular arrhythmias, were observed in ECG recordings of Cx30lacZ/lacZ mice, indicating that Gjb6 gene deletion did not affect cardiac depolarization or repolarization phases.

Finally, examination of sections from right atrial preparations showed that the pattern and intensity of immunoreactive sites to anti-Cx45 antibodies in the SA node region of Cx30-deficient mice were similar to those observed in corresponding sections from control mice (see above). Likewise, immunoreactivity to anti-Cx43 and anti-Cx40 antibodies in sections of right and left atria from Cx30-deficient mice was similar to that seen in control mice (data not shown). Loss of Cx30 protein was thus not compensated by changes of other cardiac connexin genes.
why Cx30 transcript remained undetectable in heart when techniques much less sensitive than RT–PCR were applied. 24,25 Our nested PCR approach indicated that a single Gjb6 transcript isoform including exons A, B, and 1, is transcribed in the mouse heart. In Cx30LacZ/LacZ mice, β-galactosidase-positive-nuclei, indicating Gjb6 expression, were observed at the junction of the superior vena cava with the right atrium, in a discrete area enclosed in a larger region that did not express Cx40, as shown in Cx30LacZ/LacZ/Cx40EGFP/EGFP mice. Given the location of the SA node in the heart, on the one hand, and the absence of Cx40 in the mouse SA node, on the other hand, these results suggest that Gjb6 is expressed in the SA node. This conclusion was strengthened by immunochemical evidence indicating that all β-galactosidase-positive-nuclei were observed in HCN4-positive regions only. Indeed, HCN4 underlies ‘pacemaker’ f-channels, and is the only hyperpolarization-activated cyclic nucleotide-gated cation channel (HCN) isoform demonstrated to be expressed throughout the mouse SA node. 28,29 Besides HCN4, Cx45 is another molecular marker of the murine SA node. 9,14 Immuno-fluorescence experiments demonstrated expression of Cx30 protein in a Cx45-positive region, i.e. in the SA node. Cx30 abundance was low, and always associated with α-actinin-positive cells, suggesting that Cx30 was expressed in cardiomyocytes, and restricted to the sub-epicardial part of the node, consistent with the distribution of the β-galactosidase-positive-nuclei. In addition, Cx30 expression sites were also immunoreactive to anti-Cx45 antibodies. These results indicate that in the adult mouse heart, Cx30 is weakly expressed in the sub-epicardial region of the SA node, likely in cardiomyocytes. Thus, Cx30, along with Cx45 and Cx30.2, 15,16 is the third Cx identified in the murine SA node.
Table 2 Heart rate and ECG parameters in conscious and freely moving control (Cx30+/+) and age-matched Cx30-deficient (Cx30lacZ/lacZ) mice

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<th>Heart rate, b.p.m. ± SD</th>
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<td></td>
<td>Mean HR (24 h)</td>
<td>SDNN (ms)</td>
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<tr>
<td>Cx30+/+ (n = 10)</td>
<td>524 ± 23</td>
<td>25 ± 5</td>
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<tr>
<td>Cx30lacZ/lacZ (n = 10)</td>
<td>572 ± 38</td>
<td>18 ± 4</td>
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Recordings under baseline conditions and after injection of atropine and propanolol were performed on the same mice. Data are expressed as mean ± SD. SDNN, standard deviation of the interbeat (RR) interval. Statistical significance was assessed by the one-way ANOVA followed by a post hoc Tukey test. P-values < 0.05 were considered as significant. *QTc = QT/(RR/100).35

4.2 Cx30 slows down HR in the mouse

Cx30 deficiency induced moderate tachycardia in the mouse. Under anaesthesia (cine-MRI analysis), HR of Cx30lacZ/lacZ mice was 10% faster than that of control mice (Table 1). In conscious freely moving mice (telemetric recordings), a similar increase in HR (9%) was observed between Cx30lacZ/lacZ and control animals (Table 2, Figure 4). HR increase in Cx30lacZ/lacZ mice is not due to a change in the activity of the autonomic nervous system because Cx30lacZ/lacZ mice displayed higher HR than control mice even after injection of atropine and propranolol, inhibitors of muscarinic and β-adrenergic input in pacemaker cells.30 (Table 2). These observations indicate that tachycardia was due to a change in the intrinsic electrical properties of the SA node. While the HRV spectra were similar both in Cx30+/+ and Cx30lacZ/lacZ mice, the increase in HR in Cx30lacZ/lacZ mice was accompanied by a reduction of SDNN. Because SDNN has been proposed to be an index of SA node rate,31 our observations are consistent with the hypothesis that Cx30 regulates HR by influencing SA node activity. Furthermore, no ventricular conduction defects or arrhythmias were detected in Cx30lacZ/lacZ mice, indicating that the lack of Cx30 had no physiological impact on heart excitability.

How can Cx30 protein, present in the SA node, influence HR? The SA nodal pacemaker is connected to the surrounding atrial tissue by Cx junctional channels which allow enough current flow to the atrial cells to drive them but not to the extent that pacemaking is abolished by too much current flow from the source (SA nodal cells) to the sink (atrial cells). Several hypotheses have been put forward to explain the successful pacing and driving of atrial tissue by SA nodal cells. One of them proposed a gradient of coupling (by means of junctional channels) between the node and the surrounding atrial tissue, with a low coupling in the node centre, and an increasingly higher coupling in the direction of atrial muscle.9,26 Another hypothesis described a model, involving both junctional coupling and tissue geometry, in which strands of atrial cells are intertwined with strands of nodal cells.32 In the case of the mouse, this model has to be slightly modified because in this species the junction between the SA node and the right atrium is limited to a small region on the epicardial side of the node where a few strands of atrial muscle contact the nodal cells.15 It is tempting to speculate that the Cx30-expressing cells, identified in the present work in the sub-epicardial region of the node, are connected to atrial working myocardial cells. HR changes in the mouse are largely dependent on the maximal diastolic potential (MDP) of the pacemaker cells: the more negative the MDP the lower the HR. Cx30 junctional channels show a high conductance (single channel conductance: γj = 179 pS; residual conductance: 48 pS),33 and the Cx30-expressing nodal cells would be more sensitive to the hyperpolarizing influence of the atrial cells than the nodal cells expressing low-conductance junctional channels only (like Cx45 and Cx2.0 channels with γj values of 32 and 9 pS, respectively).10 Consequently, in the Cx30 null hearts, the MDP of the sub-epicardial nodal cells adjoining the atrial cells would be less negative than that of the Cx30 containing wild-type cells, resulting in an increase in HR. Further investigations will be needed to validate this hypothesis, such as establishing the precise relationship between Cx30-expressing nodal cells and atrial cells, and defining the structure of junctional channels between atrial and nodal cells.

4.3 Cx30 deficiency induced ventricular remodelling

CI was increased by 20% in Cx30-deficient mice, when compared with control mice. This increase can first be related to HR increase in mutant mice (9%), since CI is directly proportional to HR. A second contributor to CI increase is the body-weight-corrected stroke volume (SVol/BW) which was 10% higher in Cx30-deficient mice (not statistically significant). Meanwhile, Cx30-deficient mice show ventricular dilatation, with a 16% increase of the end-diastolic volume/body weight (EDVol/BW) ratio, without any sign of hypertrophy. A permanently increased HR can contribute to an increased EDVol without hypertrophy. This has been described earlier in experimental models of tachycardia-induced
heart failure and in patients,\textsuperscript{34} in which increased EDVol was additionally associated with altered EF. In the above investigations, pacing was used to increase HR by more than 20\%, which had led to significant functional alterations. We hypothesize that similar mechanisms were involved in our finding, but to a lesser extent given the moderate increase in HR (9\%). Cardiac function therefore remained intact at the time the Cx30-deficient mice were investigated.

4.4 Conclusions

Cx30 slows down HR in the mouse. This situation is reminiscent of the role of Cx30.2 that decelerates impulse propagation through the mouse AV node.\textsuperscript{15,35} One could expect that the human ortholog of murine Cx30.2, Cx31.9, could play a role similar to that of Cx30.2 in the mouse. However, this is not the case. Cx31.9 has not been detected either in the AV node of the human heart, or in other compartments of the cardiac CS.\textsuperscript{36} The authors of these findings indicate that the variation in Cx31.9/Cx30.2 expression in the AV node could be considered as a specialization that maintains species specific conduction velocities in this CS compartment, and thus imply that Cx expression in the mouse heart has unique characteristics that may not be shared by all mammals. This leads us to ask about Cx30 expression in the SA node of mammals other than mouse.

The size and the geometry of the mammalian SA node vary from species to species\textsuperscript{66} but it is organized in such a way that the suppressive effect of the large atrial muscle mass on its pacemaking activity is minimized. HR also varies from species to species, but the total number of heart beats in the whole life of a mammal (except for man) is rather constant (between 1 and 2 billion).\textsuperscript{37} Obviously, these constants require that each species elaborate mechanisms that maintains species specific conduction velocities in this CS compartment, and thus imply that Cx expression in the mouse heart has unique characteristics that may not be shared by all mammals. This leads us to ask about Cx30 expression in the SA node of mammals other than mouse.

The Supplementary material is available at \textit{Cardiovascular Research} online.

Acknowledgements

Guinea-pig anti-Cx45 antibodies were a generous gift of E. Dupont and N.J. Severs (Imperial College, London). We thank M. Chanson (University Hospitals, Geneva) for his invaluable help, and R. Kelly (IBML, Université de la Méditerranée, Marseille) for reading the typescript.

Conflict of interest: none declared.

Funding

This work was supported by the CNRS (UMR6216 and 5203) and INSERM, the Fondation de France (grant 2003-005710 to D.G.), the Agence Nationale pour la Recherche (grant ANR-06-PHYSIO-004-01 to M.E.M.), the Association Française contre les Myopathies (grant MNN1-2006-11814 to M.T.-R.), and the German Research Association (grants to K.W.).

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