Pannexin-1 Deficient Mice Have an Increased Susceptibility for Atrial Fibrillation and Show a QT-Prolongation Phenotype

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Key Words
Cardiac ion channels • Pannexin-1 • Knock-out • \textit{In vivo} electrophysiology • Atrial fibrillation • Cardiac repolarization • QT prolongation

Abstract
Background/Aims: Pannexin-1 (Panx1) is an ATP release channel that is ubiquitously expressed and coupled to several ligand-gated receptors. In isolated cardiac myocytes, Panx1 forms large conductance channels that can be activated by Ca\textsuperscript{2+} release from the sarcoplasmic reticulum. Here we characterized the electrophysiological function of these channels in the heart \textit{in vivo}, taking recourse to mice with Panx1 ablation. Methods: Cardiac phenotyping of Panx1 knock-out mice (Panx1\textsuperscript{-/-}) was performed by employing a molecular, cellular and functional approach, including echocardiography, surface and telemetric ECG recordings with QT analysis, physical stress testing and quantification of heart rate variability. In addition, an \textit{in vivo} electrophysiological study entailed programmed electrical stimulation using an intracardiac octapolar catheter. Results: Panx1 deficiency results in a higher incidence of AV-block, delayed ventricular depolarisation, significant prolongation of QT- and rate corrected QT-interval and a higher incidence of atrial fibrillation after intraatrial burst stimulation. Conclusion: Panx1 seems to play an important role in murine cardiac electrophysiology and warrants further consideration in the context of hereditary forms of atrial fibrillation.

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Introduction

Pannexins (Panx) are channel-forming glycoproteins with structural/topological, but not sequence similarity to connexins [1, 2]. Panx contain four transmembranous domains with an intracellular N- and C-terminus [2]. The pannexin family comprises three members of which only Pannexin-1 (Panx1) is ubiquitously expressed [2]. Panx1 oligomerizes to hexameric pore-forming channels, which, inserted in the cell membrane [3], form cell-specific gap junctions [4]. Panx1 exhibits tissue-specific patterns of glycosylation, which may point to different regulatory mechanisms and functions [5]. Panx1 channel opening can be triggered by membrane depolarization, mechanical stimulation, adenosine triphosphate (ATP), intracellular calcium, and extracellular potassium [6-8]. Panx1 channel closing is initiated by carbon dioxide-mediated acidification, negative feedback from ATP, or pharmacologically by the channel blockers carbenoxolone, probenecid, and flufenamic acid [9-11]. Intercellular communication via pannexins involves the release of small molecules which act in a paracrine or endocrine fashion [12]. ATP, released through Panx1 channels, induces via activation of purinergic receptors (P2Y) further Panx1 channel opening, thereby increasing Panx1 activation [9]. In addition, Panx1 functions as a calcium permeable channel in the endoplasmic reticulum and is involved in intercellular calcium signalling [13].

There is growing evidence for a functional role of Panx1 in the cardiovascular system: Panx1 expression in smooth muscle and vascular endothelium is involved in vasoconstriction and vasodilatation as a result of ischemia or mechanical stress [14, 15]. In rat cardiac myocytes, Panx1 constitutes large conductance cation channels, which are activated by extracellular ATP and membrane stretch, leading to Ca\(^{2+}\) release from the endoplasmic reticulum [16]. In addition, activation of Panx1 induces Ca\(^{2+}\) influx and membrane depolarization in isolated cardiac myocytes, which, under certain circumstances, might trigger action potentials [17]. In the murine heart, membrane stretch induces Panx1-mediated ATP release, which activates P2Y\(_6\) receptors in a paracrine way. In turn, this results in activation of heterotrimeric Go\(_{12/13}\) proteins and Rho [18]. In a mouse model of pressure overload (induced by transverse aortic constriction), Panx1-mediated ATP release seems to trigger cardiac fibrosis via these signalling cascades [18]. In Langendorff ex vivo perfused rat hearts, brief cycles of ischemic pre- or post-conditioning induce the formation of Panx1-P2X\(_7\) complexes, which release ATP and other endogenous cardioprotectants [19]. Application of ATP prior to induction of ischemia resulted in a significantly smaller infarct size and a better recovery of left ventricular function, which could be abolished by Panx1 or P2X\(_7\) inhibition, respectively [20]. Thus, Panx1 seems to contribute and sustain distinct functions in the cardiovascular system. In the present study we aimed to reveal the role of Panx1 in cardiac electrophysiology by studying Panx1 deficient mice.

Materials and Methods

Panx1 deficient mice (C57/Bl6 X 129Sv) were provided from the Department of Clinical Neurobiology, University of Heidelberg [21]. Animal experiments were approved by a local animal care committee and are in accordance with the convention of the National Institute of Health Guide for the care and use of laboratory animals (NIH Publication No. 85-23, revised 1996). All measurements were done in adult age- and sex-matched littermate pairs of wild-type (Panx1\(^{+/+}\)) or knock-out mice (Panx1\(^{-/-}\)).

Breeding of Panx1\(^{-/-}\) mice and genotyping

Panx1\(^{-/-}\) mice (C57/Bl6 X 129Sv, more than ten backcrosses) appear healthy, have a typical life span and breed normally [21 and personal experience]. For genotyping, DNA was isolated from tails (DNeasy fibrous tissue mini kit, Qiagen, Hilden, Germany). PCR amplifications were performed with REDTaq® ReadyMix™ PCR reaction mix (Sigma Aldrich Chemie, Steinheim, Germany) using Primer 1 and Primers 2 for detecting wildtype and heterozygous mice and Primer 1 and 3 for detecting Pannexin-1 deficient and
heterozygous mice, respectively. The primer sequences (5’ to 3’) were as follows: Primer 1 GGA AAG TCA ACA GAG GTA CCC, Primer 2 CTT GCC CAC GGA GTA TGT GTT and Primer 3 GTC CCT CTC ACC ACT TTT CTT ACC. Amplification products were separated on a 2% agarose gel, stained with ethidiumbromide and visualized (Biometra, Jena, Germany).

**Histopathological analysis**

Hearts from both genotypes were formalin-fixed and paraffin-embedded. Slices (4 μm) were stained with hematoxylin/eosin (H/E) or elastica van Gieson according to standard procedures. In two different H/E stained slices of each heart encompassing the two atria and the two ventricles (resembling the four chamber view in echocardiography) the diameter of ventricular walls, width of cardiac myocytes and length of nuclei were quantified. All slices were screened for cellular disarray, inflammation and cytoplasmic lipofuscin. Elastica van Gieson stained slides were qualitatively screened for signs of fibrosis (interstitial and endocardial). All slides were scanned with a 20-40x magnification using a Leica DM2000.

**Anthropometric data**

Body weight (BW), heart weight (HW), and tibia length (TL) were analysed in adult mice and relation of HW/BW and HW/TL were compared in both genotypes.

**Echocardiography**

Mice were slightly sedated (inhaled 0.5 Vol% isoflurane/oxygen) to minimize effects on cardiac function and heart rate [22]. Two dimensional imaging and M-mode recordings were performed with a VisualSonicsVevo® 2100 Imaging ultrasound system (SonoSite, Fujifilm) equipped with a MS-400 transducer measuring the following parameters in a parasternal short axis view at the midventricular level (in mm): Interventricular septal diameter in diastole (IVSd), left ventricular diameter enddiastolic and endsystolic (LVDd, LVDs), left ventricular posterior wall in diastole and systole (LVPWd, LWPWs). Ten beats per animal were averaged. Relative wall thickness ((IVSd+LVPWd)/LVIDd) and left ventricular mass (in mg) \(1.05[(IVS+LVIDd+LVPWd)^3-LVIDd^3]\) were calculated from M-mode measurements, where 1.05 (mg/mm³) is the density of myocardium. Left ventricular systolic function was determined by calculating the shortening fraction (SF % = [LVIDd-LVIDs/LVIDd] x 100). Analyses were performed by two examiners who were blind to the genotype of the animals. All measurements were in the range of published data for C57BL/6 mice [23, 24].

**Gene expression analysis by semi-quantitative RT-PCR**

Total RNA was isolated (RNasy fibrous tissue mini kit®, Qiagen, Hilden, Germany) from ventricles and atria, respectively, and complementary DNA was obtained by reverse transcription (QuantiTect Reverse Transcription Kit®, Qiagen) followed by PCR (Taq Polymerase Kit®, Qiagen). The primer sequences were Panx1 forward 5´-TTC TTC CCC TAC ATC CTA CTG C-3`, Panx1 reverse 3´-TGT TCT CCA GCA CCT TCA GAC-5´ as previously published [25], and for the housekeeping gene (HPRT) forward 5'-GCT TCC TCA GCA CCT CTA GAC-3' and HPRT reverse 5'-TTA GGT ATA CAA AAC AAA TCT AGG TCA TAA CC-3`. Amplification products were separated on a 2% agarose gel, stained with ethidiumbromide and quantified by densitometry (TotalLab TL 100 analysis software, UK). Expression levels were quantified as percent expression of the internal standard.

**Surface Electrocardiography (ECG)**

Surface ECGs were performed in sedated mice (5% avertin (250 mg/kg) or 1.5% isoflurane/oxygen) with a PowerLab 8/30 supplemented with an animal BioAmp and analysed by the LabChart software (all ADInstruments, Spechbach, Germany). Measurements included heart rate, P wave duration, PR interval, QRS interval, rate corrected QT interval (QTc); the end of the T wave was defined as the point at which the slow component returned to the isoelectric line. For QTc calculation two different formulas were used: \(QTc = QT/(RR/100)^{1/2}\) [26] or \(QTc=QT+0.154(100-RR)\) [27, 28]. The first formula is based on the Bazett equation, and the second is a modification of the Sagie-Framingham formula. All data represent the mean of 70 consecutive beats that were manually analysed independently by two examiners who were blinded to the genotypes of the animals.
Telemetric ECGs

An ECG transmitter (Model TA10EA-F20, Data Sciences, Minneapolis, USA) was implanted into the peritoneal cavity of adult mice with such a position of the electrodes that generated a lead II on ECG. After a recovery period from surgery of two weeks, a 24 h ECG was recorded with a sampling rate of 1 kHz. Telemetric ECGs were manually analysed by two examiners who were blinded to the genotypes of the animals (LabChart Software, ADInstruments, Spechbach, Germany).

Physical stress tests by swimming and treadmill exercise

Telemetric ECG analysis during physical exercise was performed as previously described [24]. In brief, swimming mice were placed in a heated water tank for 5 min. ECGs were recorded 5 min before, during and 10 min after swimming and were analysed with regard to heart rate and arrhythmias. For strenuous exercise testing mice were physically challenged on a treadmill (Simplex II Metabolic Modular Treadmill, Columbus Instruments, Columbus, Ohio, USA) using a ramp protocol that involved an increase in velocity of 1 m/min every 2 min and an increase in gradient angle of 5° every 4 min [24]. Maximum heart rate was determined as mean of ten beats at the end of exercise.

Heart rate variability (HRV)

For heart rate variability, 20 s intervals were analysed every hour for a period of 24 h as described recently [24]. The following parameters for time domain analysis were calculated: mean normal-to-normal intervals (NN, in ms), standard deviation of all NN intervals (SDNN, in ms), square root of the mean square of successive differences between adjacent NN intervals (RMSSD, in ms), and the percentage of normal consecutive NN intervals differing by > 6 ms (pNN6, in %) was determined in at least six segments of 120 s. For frequency domain analysis, spectra were calculated with a FFT (fast Fourier transformation) size of 1024 data points, half overlap and a Welch window (HRV LabChart Module, ADInstruments, Spechbach, Germany). Cut-off frequency ranges for high-frequency (HF 1.5 – 5 Hz), low-frequency (LF 0.15 – 1.5 Hz) and very low frequency (VLF 0 – 0.15 Hz) bands were used as defined for mice [29], and the power (ms²) in these bands was calculated. According to current guidelines, LF and HF were also expressed in normalized units (nu), which represent the relative value of each power component in proportion to the total power minus the VLF component, and the LF/HF ratio was determined [30].

Electrophysiological study by intracardiac programmed electrical stimulation

Transvenous electrophysiological studies were performed in vivo as described previously by us and others [28, 31]. Briefly, mice were anesthetized with 5% Avertin® and a 2 F octapolar electrophysiology catheter was advanced via the jugular vein into the right atrium and right ventricle. The octapolar catheter is equipped with eight circular electrodes that are 0.5 mm broad and are 0.5 mm apart from each other (Ciber Mouse, NuMed Inc., NY, USA). Atrial and ventricular stimulations were performed by applying rectangular stimulus pulses with twofold intensity of the pacing threshold by an electrical stimulator and a PowerLab 8/30 (ADInstruments, Spechbach, Germany). Surface ECGs and intracardiac electromyograms were recorded simultaneously and analysed by the LabChart Software 7.0 (ADInstruments, Spechbach, Germany). Sinus node recovery time (SNRT) was measured, and rate corrected SNRTc was calculated by subtracting the steady-state cycle length from the SNRT. Atrial refractory periods (ARP), atrioventricular nodal refractory periods (AVNRP), and ventricular refractory periods (VRP) were recorded during fixed rate and extrastimulus pacing. Wenckebach periodicity was determined by eight atrial stimuli with progressive consecutive interval shortening by 2–5 ms. Atrial vulnerability was evaluated by burst stimulation for 5 s, starting with 50 ms cycle length and shortened up to 10 ms. Atrial fibrillation was defined as rapid and fragmented atrial electrogams with irregular AV-nodal conduction for >1s. Ventricular vulnerability was examined by ventricular stimulation with eight stimuli at 110 ms, 100 ms, and 80 ms intervals followed by three extrastimuli. Ventricular burst stimulation for 5 s started with 50 ms cycle length that was shortened up to 10 ms. Ventricular tachycardia was defined as four or more consecutive ventricular ectopic beats.

In order to accelerate conduction and provoke catecholamine-sensitive tachyarrhythmias, isoprenaline (1 mg/kg intraperitoneal) was given. If heart rate was less than 600 min⁻¹ or did not increase for at least 20% after isoprenaline administration, 0.5 mg/kg was given additionally. QT and QTc intervals were determined before and after isoprenaline in both genotypes, since a paradoxical QT prolongation is a hallmark for long QT syndrome type 1.
Statistical analysis

Data are presented as mean ± SD and were obtained using the indicated number of mice. Statistical significance was assessed using the (un)paired two-sided t-test, and p-values < 0.05 were considered statistically significant. All statistical calculations were done with GraphPadPrism 6® (La Jolla, CA, USA).

Results

Anthropometric, histopathologic and echocardiographic characterization of Pannexin-knock-out mice (Panx1−/−) in comparison to Pannexin-1 wild-type mice (Panx1+/+)

Anthropometric data were analysed in all mice subjected to experiments. There was no difference between Panx1+/+ and Panx1−/− mice regarding various body parameters (Table 1A).

Histopathological analysis concerning hypertrophy as quantified by measuring wall thickness of both ventricles, size of cardiomyocytes, and length of cell nuclei, did not show significant differences in Panx1−/− mice compared to Panx1+/+ mice (Table 1B). There were no signs of fibrosis, intracellular storage, inflammation or muscular disarray in Panx1−/− mice.

Table 1. Body parameter (A), histopathological (B) and echocardiographic data (C) were compared between the two genotypes and revealed no significant differences. IVS interventricular septum, LVID left ventricular internal diameter, LVPW left ventricular posterior wall, SF shortening fraction, EF ejection fraction, CO cardiac output, s systole, d diastole
Echocardiographic measurement of cardiac dimensions and functional cardiac analysis were comparable between the two genotypes (Table 1C).

**Gene expression pattern of Pannexin-1 in the murine heart**

Pannexin-1 expression was significantly higher in murine atria than in ventricles as shown by semi-quantitative RT-PCR using two different primer pairs and HPRT as an internal standard (Fig. 1). Due to the lack of a specific Panx1 antibody for the mouse, Western blot analysis and immune histochemistry to determine the exact cellular localization of Panx1 was not feasible in (data not shown).

**Table 2.** ECG parameters in Panx1+/+ and Panx1−/− mice measured in surface ECGs (sedation with isoflurane or avertin, respectively), or in telemetric ECG-recordings. * significant difference between wildtype and mutant mice. (A) Basic ECG parameters did not differ between the two genotypes and various modes of ECG recording, except for a shorter RR interval in Panx-1−/− mice when sedated with isoflurane. (B) QRS duration in telemetric recordings was significantly longer in Panx1−/− mice. (C) QT and QTc intervals were significantly prolonged in all Panx1−/− mice, independent of the mode of sedation. This was also seen in telemetric recordings.

<table>
<thead>
<tr>
<th></th>
<th>Isoflurane</th>
<th>Avertin</th>
<th>p-value</th>
<th></th>
<th>Isoflurane</th>
<th>Avertin</th>
<th>p-value</th>
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<td><strong>A</strong></td>
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<tr>
<td>RR interval (ms)</td>
<td>132 ± 8</td>
<td>120 ± 7</td>
<td>0.01</td>
<td>n = 10</td>
<td>143 ± 18</td>
<td>139 ± 27</td>
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<td>P duration (ms)</td>
<td>8.4 ± 1.2</td>
<td>9.4 ± 1.5</td>
<td>0.26</td>
<td>0.44</td>
<td>10.1 ± 0.9</td>
<td>10.2 ± 1.9</td>
<td>0.87</td>
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<td>P amplitude (mV)</td>
<td>0.06 ± 0.02</td>
<td>0.1 ± 0.04</td>
<td>0.12</td>
<td>0.57</td>
<td>0.07 ± 0.02</td>
<td>0.09 ± 0.05</td>
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<td>PR interval (ms)</td>
<td>42 ± 3.5</td>
<td>39 ± 8.4</td>
<td>0.45</td>
<td></td>
<td>45 ± 6</td>
<td>44.5 ± 4.4</td>
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<td>QRS duration (ms)</td>
<td>8.3 ± 1.5</td>
<td>9.4 ± 1.8</td>
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<td></td>
<td>10.1 ± 1</td>
<td>10.1 ± 2.7</td>
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<td>R amplitude (mV)</td>
<td>0.9 ± 0.2</td>
<td>1 ± 0.3</td>
<td>0.69</td>
<td></td>
<td>1 ± 0.2</td>
<td>0.9 ± 0.2</td>
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<td><strong>B</strong></td>
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<td>RR interval (ms)</td>
<td>113 ± 17</td>
<td>113 ± 15</td>
<td>0.97</td>
<td>0.77</td>
<td>12 ± 3.4</td>
<td>12 ± 2.9</td>
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<td>P duration (ms)</td>
<td>36 ± 1.9</td>
<td>35 ± 3.1</td>
<td>0.57</td>
<td></td>
<td>10.6 ± 0.9</td>
<td>11.7 ± 1.1</td>
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<td><strong>C</strong></td>
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<tr>
<td>QT (ms)</td>
<td>45 ± 6</td>
<td>53 ± 5</td>
<td>6/8</td>
<td>0.026</td>
<td>38 ± 3</td>
<td>45 ± 2</td>
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<td>QTc (ms)</td>
<td>35 ± 3</td>
<td>45 ± 4</td>
<td>7/7</td>
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<td>31 ± 3</td>
<td>41 ± 3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Telemetry</td>
<td>44 ± 3</td>
<td>49 ± 3</td>
<td>14/16</td>
<td>0.0001</td>
<td>42 ± 3</td>
<td>47 ± 2</td>
<td>&lt;0.0001</td>
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</table>
Analysis of surface and telemetric ECGs in Pannexin-1+/+ and Pannexin-1−/− mice

Surface ECGs were analysed in sedated mice using avertin or isoflurane. The two anesthetics were employed and the results were compared to reveal potential confounding effects that might result from the medication. No differences between the two anesthetics could be detected except for higher heart rates in Panx1−/− with isoflurane (Table 2A).

There was no difference between the two genotypes when comparing P amplitude and duration, PR interval, and R amplitude (Table 2A). QRS duration as measured in non-sedated animals during telemetric ECG recordings was significantly longer in Panx1−/− mice (Table 2B). QT and rate corrected QT intervals (QTc) in Panx1−/− mice were significantly prolonged compared to Panx1+/+ mice in telemetric and surface ECGs, irrespective of the anesthetic that was used (Table 2C, Fig. 2A). Remarkably, Panx1+/+ and Panx1−/− mice sedated with isoflurane show a significantly shorter QT and QTc interval compared to the corresponding genotypes sedated with avertin or having a telemetric ECG recording (at least p < 0.005). In contrast, the QT and QTc intervals in avertin sedated mice did not differ to QT and QTc intervals of telemetric ECGs of the same genotype. Thus, isoflurane seems to shorten QT and QTc intervals in mice irrespective of the genotypes studied here (Fig. 2A).

Isoprenaline, a selective β1-agonist, resulted in a significant increase in heart rate in both genotypes (RR intervals before and after isoprenaline: Panx1+/+ 132 ± 10 ms and 116 ± 2 ms, Panx1−/− 127 ± 15 ms and 111 ± 5 ms, n = 6, p < 0.0001). QT and QTc intervals in Panx1+/+ mice as well as QTc intervals in Panx1−/− mice did not change significantly (QT intervals before and after isoprenaline: Panx1+/+ 41 ± 4 ms and 40 ± 7 ms, QTc intervals before and after
isoprenaline: Panx1+/− 36 ± 4 ms and 37 ± 6 ms, Panx1+/− 50 ± 4 ms and 51 ± 5 ms, all n = 6). There was only a significant shortening of the QT interval in Panx1−/− mice after isoprenaline (56 ± 5 ms versus 53 ± 6 ms, n = 6, p < 0.005).

Since QRS and QT intervals were prolonged at least in telemetric ECGs of Panx1−/− mice, we tested whether QT prolongation was due to QRS prolongation. To this end we subtracted the QRS duration from QT intervals to obtain so-called JT intervals. Differences in these intervals between the two genotypes provided evidence that Panx1−/− mice had a significantly prolonged JT interval (33 ± 3 ms versus 37 ± 3 ms, p = 0.0009, in Panx1+/+, n = 14, and Panx1−/− mice, n = 16, respectively). Thus, telemetry revealed significant abnormalities of cardiac depolarisation and repolarisation in Panx1−/− mice.

Next, we correlated QT and QTc intervals from telemetric ECGs with the corresponding RR intervals in the two genotypes. In addition, we compared two correction formulas for the calculation of QTc intervals. All QT and QTc intervals of both genotypes were plotted against the corresponding RR interval and analysed by linear regression. The slope of the straight lines, which show how QT and QTc intervals vary at different RR intervals, indicate that QT and QTc intervals differ most strikingly at lower heart rates between Panx1+/+ and Panx1−/− mice. To exclude that this phenomenon is a consequence of the correction formula used (described by Mitchell, based on the Bazett formula) [26], a second formula commonly used in clinical cardiology was adapted to the mouse model [28]. Linear regression analyses showed that comparable results are obtained with both formulas (Fig. 2B).

Fig. 3. (A) The number of episodes with AV-block documented in telemetric ECG recordings over 24 h was significantly higher in Panx1+/− mice compared to Panx1+/+ mice. (B) Comparison of heart rates at times when AV-block was seen demonstrated that AV-block in Panx1+/− mice occurred predominantly at lower heart rates than AV-block detected in Panx1+/+ mice. (C) ECG recording showing the typical characteristics of an AV-block type 2, with a single P-wave that was not followed by a ventricular contraction.
Telemetric ECG analysis regarding cardiac arrhythmia in resting and exercised mice
Panx1−/− mice exhibited a significantly higher incidence of AV-block (type 2) compared to Panx1+/+ mice (Fig. 3A, C). AV-block in Panx1+/+ mice occurred significantly more frequent at higher heart rates, whereas the majority of AV-block in Panx1−/− mice was seen at lower heart rates (Fig. 3B). Premature contractions were recorded rarely, and there was no difference between genotypes.

ECG analysis during swimming showed no difference in heart rate or arrhythmia between the two genotypes throughout the experiment. Treadmill exercise was performed employing two different protocols (increase in velocity and increase in both velocity and inclination). Heart rate significantly increased to a maximum level of about 800 bpm, and there was no significant difference between the genotypes employing either of the two protocols. Furthermore, there was no significant difference when comparing the running distance reached by wildtype and mutant mice (max. distance without inclination: Panx1+/+ 360 ± 97m, Panx1−/− 377 ± 101m, n = 14; max. distance with inclination Panx1+/+ 259 ± 65m, Panx1−/− 252 ± 71m, n = 14). No arrhythmia occurred throughout the treadmill exercises.

Heart rate variability in Panx1+/+ and Panx1−/− mice
To analyse heart rate variability in both genotypes, 24 h ECGs were screened and segments without artefacts were analysed in respect of time and frequency domain parameters. There was no indication for the presence of relevant physiological alterations in Panx1+/+ mice (Table 3).

Electrophysiological studies in Panx1+/+ and Panx1−/− mice
Firstly, sinus node function was analysed by determining the sinus node recovery time (SNRT) at 100ms. There was no significant difference between both genotypes for this parameter. Secondly, atrioventricular conduction was assessed using AV interval
and Wenckebach point measurements. There was no significant difference between both genotypes. Thirdly, refractory periods of the atria, AV-node and ventricles were determined by extrastimuli at different cycle lengths. Again, there was no significant difference when comparing the two genotypes (Table 4). All parameters obtained in the two genotypes were in accordance with what has been reported in mice previously [32]. Finally, repeated atrial and ventricular burst stimulation with different cycle lengths before and after injection of isoprenaline was applied to provoke arrhythmia and test for atrial and ventricular vulnerability. We found a significantly higher proportion of atrial fibrillation in Panx1−/− mice compared to Panx1+/+ mice (Fig. 4A, B). There was no significant difference in the duration of atrial fibrillation when induced by atrial or ventricular burst stimulation (3.6 ± 4s after atrial burst stimulation vs 3.6 ± 2s after ventricular burst stimulation, not significant).

**Discussion**

The present study describes cardiac effects of Panx1 deficiency in the mouse and reveals multiple functions of Panx1 in cardiac physiology.

Panx1 deficiency did not lead to structural or functional abnormalities as seen both in histology and echocardiography, but showed diverse electrophysiological abnormalities, particularly with regard to ventricular depolarization, repolarization, and an increased susceptibility for atrial fibrillation.

Sinus node function did not seem to be affected in mice with Panx1 deficiency, since there were no differences in heart rates at rest, during and after physical activity, with different modes of anaesthesia, and after stimulation with isoprenaline when comparing

### Table 4. Electrophysiological data obtained from intracardiac studies.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pannexin-1+/+</th>
<th>Pannexin-1−/−</th>
<th>significance</th>
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<td>Sinus node function</td>
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<tr>
<td>SNRT 100 ms</td>
<td>148±24</td>
<td>164±17</td>
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<tr>
<td>SNRTc (@100 ms)</td>
<td>44±28</td>
<td>61±50</td>
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<tr>
<td>SNRT/SCL*100 (@100 ms)</td>
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<td>153±39</td>
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<td>Atrioventricular conduction</td>
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<td>AV interval (ms)</td>
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<td>AV-nodal RP 110 ms</td>
<td>52±9</td>
<td>48±10</td>
<td>4</td>
</tr>
<tr>
<td>AV-nodal RP 100 ms</td>
<td>59±8</td>
<td>55±9</td>
<td>5</td>
</tr>
<tr>
<td>ARP 120 ms</td>
<td>47±3</td>
<td>52±7</td>
<td>3</td>
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<tr>
<td>ARP 110 ms</td>
<td>48±7</td>
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<tr>
<td>ARP 100 ms</td>
<td>52±8</td>
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<td>VRP 120 ms</td>
<td>56±12</td>
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<td>VRP 110 ms</td>
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<td>VRP 100 ms</td>
<td>53±9</td>
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wild type and mutant mice. Three different modes of physical stress testing (two treadmill protocols and swimming) provoked similar maximal heart rates in both genotypes that were comparable to what has been published before [32]. In addition, sinus node recovery time tested by electrical stimulation was not altered in Panx1\(^{-/-}\) mice. However, Panx1\(^{-/-}\) showed a significantly higher incidence of second degree AV-block which was more pronounced at lower heart rates. An increase in vagotonus predisposing to a higher incidence of AV-block during lower heart rates in Panx1\(^{-/-}\) mice could not be detected by heart rate variability analysis. In addition, atrial depolarization and AV-conduction were not altered when analysing surface ECGs, ECGs recorded by telemetry and intracardiac parameters of atrial depolarization. Ventricular depolarization was significantly prolonged in Panx1\(^{-/-}\) mice as evidenced by QRS prolongation. Intermittent AV-block and prolonged ventricular depolarization point to a possible functional role of Panx1 for impulse propagation. We speculate that Panx1 might be preferentially expressed in the conduction system, and its absence might result in delayed electrical conduction. This hypothesis unfortunately could not be further substantiated by western blotting and immune histochemistry analysis using confocal laser scanning microscopy as currently available antibodies proved to be nonspecific. Therefore, the exact mechanisms of the increased incidence of AV-block and QRS prolongation in Panx1\(^{-/-}\) could not be clarified yet.
One of the two most striking findings was the significantly prolonged repolarization in Panx1-/- mice as quantified by QT and QTc measurements in surface ECGs (using different anaesthetics) and telemetry. Since QRS complexes were also significantly prolonged in Panx1-/- mice, we subtracted the QRS interval from the QT interval and compared the so-called JT interval between the two genotypes, which further supports the notion of an altered ventricular repolarization in Panx1-/- mice.

Strikingly, the QT interval prolongation in Panx1-/- mice was most evident at the lowest heart rates. We therefore wanted to exclude overestimation of QTc due to the correction formula proposed by Mitchell, which is based on the Bazett formula used in clinical cardiology [26]. When employing a modified Framingham formula for mice [27, 28] we showed a highly significant concordance of QTc intervals assessed by either formula. This is in agreement with data reported previously in another mouse model [28], thus supporting the validity and comparability of calculated QTc intervals in mice and men.

The QTc interval was mostly prolonged at lower heart rates, which is a hallmark for type 3 long QT-syndrome in humans, where the highest risk for torsades-de-pointes tachycardias and sudden cardiac death occurs during rest or sleep. This might explain why none of the animals exhibited stress-induced arrhythmia.

The clinically most relevant finding is the observation that Panx1-/- mice had a significantly higher incidence of atrial fibrillation after intracardiac burst stimulation. Ventricular vulnerability did not seem to be altered. This finding might result from higher murine expression of Panx1 in the atria compared to the ventricles.

Atrial fibrillation is the most frequent arrhythmia in humans and is associated with a significant cardiovascular morbidity and mortality [33]. Although atrial fibrillation often develops secondary to other diseases, there is increasing evidence of a genetic predisposition, and genome wide association studies have identified risk loci in many regions of the genome [34]. Genetic variants in humans have been identified in ion channels, the gap junction protein Connexin 40, and in transcription factors [35]. In addition, numerous transgenic mice models for atrial fibrillation have been characterized and have helped elucidating different pathophysiological mechanisms underlying atrial fibrillation [36]. In these models, atrial fibrillation might be the common clinical manifestation resulting from diverse mutations that alter ion channel dynamics, G-protein coupled receptor signalling, calcium homeostasis, and regulation of transcription factors [36]. Shortening or prolongation of atrial action potentials are the electrophysiological basis for atrial fibrillation resulting from mutations in potassium and sodium channels [35]. Our in vivo electrophysiological data in Panx1-/- mice did not show a difference in atrial refractory period or in the occurrence of early after depolarisations.

Mutations in the human gap junction protein Connexin 40 reduced conduction velocity throughout the atrium, which promotes re-entry circuits [37]. Although, Panx1-/- mice did not exhibit prolonged atrial depolarization in surface ECG and in in vivo electrophysiological measurements, the incidence of AV-block and QRS duration was significantly increased, pointing to an at least intermittent delay in impulse propagation, which might also contribute to the increased risk in stimulation-induced atrial fibrillation in Panx1-/- mice.

Since Panx1 channels, when expressed as a non-selective cation channel at the cell membrane of cardiomyocytes, are involved in calcium release from the endoplasmic reticulum and are subsequently activated by the released calcium [13, 16], atrial fibrillation might be provoked by altered calcium signalling and homeostasis due to Panx1 deficiency.

To clearly elucidate the mechanism of the increased susceptibility for atrial fibrillation and to strengthen the relevance of these in vivo findings, further experiments are needed. These studies should include patch-clamp experiments in atrial and ventricular cardiomyocytes of both genotypes as well as the recordings of monophasic action potentials in Langendorff-perfused hearts to measure action potential durations at different sites.

Panx1 deficiency might alter cardiac development in fetal life and result in secondary changes, which increase the risk for atrial fibrillation. Pannexin-1 deficiency in the heart might result in changes in the expression or function of other ion channels, adaptor proteins
or even signalling cascades. Some influences could be excluded by the use of a tissue-specific knock-out mouse model. In addition, for analysing the molecular effects of Panx1 deficiency in the complex interplay of signal transduction and cellular cross-talk microarray-based gene expression profiling may identify candidate genes that are secondary altered. These experiments will differentiate whether Panx1 itself or downstream molecules are the cause for the electrophysiological findings in vivo.

Although pannexins are linked to the pathogenesis of onset and progression of diverse diseases, no germ-line mutations have been identified so far [38]. Given that human and murine pannexins share up to 94% conserved sequence homology [5], our findings could be transferred to conditions in human subjects. The rapid increasing technical possibilities in next generation sequencing and data repositories might help to elucidate the potential contribution of Panx1 in the pathogenesis of atrial fibrillation in humans. An alternate approach might be the study of Panx1 expression in patients with chronic atrial fibrillation who might undergo cardiac surgery for other reasons.

In summary, Panx1 in the heart influences different electrophysiological parameters involving depolarization, repolarization and atrial vulnerability causing atrial fibrillation.

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Disclosure Statement

None of the authors have anything to disclose.

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