Sick Sinus Syndrome in HCN1-Deficient Mice

Running title: Fenske et al.; Sinus node dysrhythmia in HCN1-deficient mice

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Journal Subject Codes: Basic science research:[132] Arrhythmias - basic studies, Basic science research:[130] Animal models of human disease
Abstract

Background—Sinus node dysfunction (SND) is a major clinically relevant disease which is associated with sudden cardiac death and requires surgical implantation of electrical pacemaker devices. Frequently, SND occurs in heart failure and hypertension, conditions that lead to electrical instability of the heart. While the pathologies of acquired SND have been extensively studied, little is known about the molecular and cellular mechanisms that cause congenital SND.

Methods and Results—Here, we show that the HCN1 protein is highly expressed in the sinoatrial node and is colocalized with HCN4, the main sinoatrial pacemaker channel isoform. To characterize the cardiac phenotype of HCN1-deficient mice a detailed functional characterization of pacemaker mechanisms in single isolated sinoatrial node cells, explanted beating sinoatrial node preparation, telemetric in vivo electrocardiography, echocardiography and in vivo electrophysiology was performed. Based on these experiments we demonstrate that mice lacking the pacemaker channel HCN1 display congenital SND characterized by bradycardia, sinus dysrhythmia, prolonged sinoatrial node recovery time, increased sinoatrial conduction time and recurrent sinus pauses. As a consequence of SND HCN1-deficient mice display a severely reduced cardiac output.

Conclusions—We propose that HCN1 stabilizes the leading pacemaker region within the sinoatrial node and hence is crucial for stable heart rate and regular beat-to-beat variation. Furthermore, we suggest that HCN1-deficient mice may be a valuable genetic disease model for human SND.

Key words: arrhythmia, ion channels, sinoatrial node
Introduction

The heart beat is initiated and maintained by the generation of spontaneous action potentials in pacemaker cells of the sinoatrial node region. A hallmark of sinoatrial pacemaker action potentials is the presence of a slow diastolic depolarization phase (SDD) following repolarization. The SDD causes the cell membrane, which typically maintains a resting membrane potential of around -60 mV $^{1,2}$, to reach the threshold potential and consequently fire the next action potential. Over the last years, the molecular basis of the ionic currents involved in sinoatrial node (SAN) action potential has been analyzed in man $^3$, rabbit $^4$ and mouse $^5$. Of particular interest are ion channels and Ca$^{2+}$-handling proteins that are essential for the slow diastolic depolarization and thus for autonomous electrical activity of the heart $^{6-8}$. Mutations in ion channels in humans (Nav1.5, HCN4) and the knockout of ion channels in mice (Cav1.3, Cav3.1, HCN4 and HCN2) have been linked to sick sinus syndrome or bradycardia $^7,9-13$. Among these channels hyperpolarization-activated cyclic nucleotide-gated (HCN) channels which are the molecular correlate of hyperpolarization-activated current (I_f) $^{14}$ are considered to be of particular importance. Three out of the four members of the HCN channel family (HCN1, HCN2 and HCN4) have been identified in pacemaker cells. Quantitatively, in all vertebrates studied so far, HCN4 underlies the major fraction of SAN I_f amounting to about 70 - 80 % of total I_f. HCN4 is essential for formation of mature pacemaker cells during embryogenesis $^{15}$. Moreover, analysis of human HCN4 channelopathies $^{16}$ and genetic mouse models ($^{1,17,18}$ but see $^9,10$) suggests that this channel plays an important role in autonomic control of heart rate. Mice deficient for HCN2 display mild cardiac dysrhythmia while autonomic control of heart rate is preserved in these mice $^{11,19}$. In contrast to HCN4 and HCN2, the role of HCN1 in heart has not been examined so far. HCN1 was originally cloned from mouse brain $^{20}$. Indeed, analysis of HCN1 knockout mice
revealed that this channel is involved in the control of numerous neuronal functions, including the control of rhythmic activity in neuronal circuits, the control of the resting membrane potential and dendritic integration\textsuperscript{21, 22}. However, there is also increasing evidence that HCN1 is expressed in heart. In the rabbit SAN, for example, RNase-protection assay and immunolabelling data\textsuperscript{23, 24} indicate significant expression of HCN1. Based on heterologous expression data and electrophysiological studies it was speculated that HCN1 together with HCN4 contributes to native I\textsubscript{f} in rabbit SAN\textsuperscript{25}. Finally, recent studies identified profound expression of HCN1 protein in mouse cardiac conduction system\textsuperscript{19, 26}. Taken together, these findings suggest that HCN1 may be involved in pacemaker function. In the present study we addressed this important question by analyzing the HCN1-deficient mouse line. To this end we performed telemetric in vivo ECG recordings and in vivo electrophysiology, electrophysiological experiments in the intact beating SAN preparation and in isolated sinoatrial pacemaker cells. We show that mice lacking the pacemaker channel HCN1 display congenital SAN dysfunction characterized by bradycardia accompanied by low cardiac output, sinus dysrhythmia and recurrent sinus pauses.

Material and Methods

Animals

HCN1-deficient (HCN1\textsuperscript{1\textdagger}) mice were obtained from the Jackson Laboratory (Maine, USA; B6;129-HCN1\textsuperscript{tm2Kndl/J}\textsuperscript{27}) and maintained on a mixed C57BL/6N and 129/SvJ background. 6-12 weeks-old HCN1\textsuperscript{1\textdagger} mice and wild-type littermates (WT) deriving from heterozygous breeding pairs were randomly assigned to the experimental procedures. Care was taken that at least one wild-type littermate was tested together with one of its HCN1\textsuperscript{1\textdagger} littermate in a given test series. Each group consisted of animals taken from at least 3 different litters. All animal studies were
approved by the Regierung von Oberbayern, were in accordance with German laws on animal experimentation and were performed according to the Guide for the Care and Use of Laboratory Animals as published by the US National Institutes of Health. Effort was taken to keep the number of animals at a minimum.

**Morphology**

Hearts from 12 week old wild type (n=5) and HCN1−/− (n=6) mice were removed and fixed in 4% paraformaldehyde for 2 h and incubated in sucrose at 4°C overnight. 16 µm cryosections were stained with H&E according to standard protocols.

**Echocardiography**

Echocardiographic images were obtained using an ultrasound imaging system for rodents (Vevo 2100, FUJIFILM VisualSonics, Toronto, Canada) utilizing the systems 22-55 MHz transducer (MS550D). 12 wild type and 12 HCN1−/− mice were analyzed.

**SAN whole mount dissection**

The hearts of 6 - 12 weeks old, littermatched mice were put in a dish filled with Tyrode III solution and the SAN region was cut out along the superior vena cava and the crista terminalis. The dissected SANs were pinned on a silicone block and rinsed with Tyrode III solution.

**Western Blot**

For protein isolation mouse SAN and atrial tissues of wild type (n=6) and HCN1−/− (n=6) mice were homogenized. After heating at 95°C for 15 min followed by centrifugation at 1000 g for 10 min to remove cell debris, the resulting supernatant was used in western blot analysis as previously described 28. The following antibodies were used: mouse anti-HCN1 (1:1000; Abcam, Cambridge, UK), rat anti-HCN4 (1:500; Santa Cruz Biotechnology, CA, USA), rabbit anti-HCN2 L (1:500; 11 and mouse anti-Tubulin (1:2000; Dianova, Hamburg, Germany).
Immunofluorescence whole mount SAN

Tissues of wild type (n=5) and HCN1 deficient (n=5) mice were stained with rat monoclonal antibody to HCN4 (1:100; Santa Cruz Biotechnology, CA, USA) and rabbit monoclonal antibody to HCN1 (1:100; Alomone labs, Jerusalem, Israel). As secondary antibodies Alexa488-conjugated anti-rat (1:400; Invitrogen, Karlsruhe, Germany) and Alexa555-conjugated anti-rabbit (1:400; Invitrogen, Karlsruhe, Germany) were used.

Electrophysiological recordings in single SAN cells

SAN cells were isolated from 15 HCN1⁻/⁻ and 16 WT as described previously 29.

Microelectrode recordings whole mount SAN

Spontaneous APs were recorded from isolated SAN preparations of wild type (n=3) and HCN1⁻/⁻ (n=4) mice by using 3 M KCl microelectrodes at 30.5 ± 0.5 °C (unless stated otherwise).

Telemetric ECG recordings in mice

Telemetry and analysis of heart rate and heart rate variability of wild type (n=3) and HCN1⁻/⁻ (n=4) mice were performed as described 30.

In vivo electrophysiological study

The EP analysis was performed in 9 wild type and 11 HCN1⁻/⁻ mice as described 31.

For detailed Materials and Methods please see Supplemental Material.

Statistical analyses

Data are expressed as mean ± SEM. They were analyzed by Grubb’s outlier test, and two outliers were excluded from the whole data analysis. Subsequently, we confirmed normal distribution by Kolmogorov-Smirnov, D'Agostino & Pearson omnibus and Shapiro-Wilk normality tests (Graph Pad Prism 5.0). If not stated otherwise, groups were compared by 2-sample t-tests, uncorrected for multiple testing. For multiple comparisons in case of factorial design, 2-way ANOVAs
(genotype, parameter/treatment/time) with or without dependent measures were employed as indicated in the text, followed by Newman-Keuls post-hoc test, if appropriate (StatSoftStatistica 5). In cases of experiments with small sample sizes (telemetric in vivo experiments and SAN whole mount and isolated hearts, we additionally performed non-parametric tests (Mann-Whitney U-tests). Relative genotype differences were assessed by expressing data as a percentage of the mean wild-type value (in case of independent data sets) or the individual basal level (in case of dependent data sets). Missing data led to a list-wise exclusion of the respective data set. All p-values were two-sided, except for the non-parametric analyses, where one-sided tests were employed, given the fact that on the basis of our own findings we expected a decrease (or no change) in heart rate (single cells, echocardiography, SAN whole mount, in vivo electrophysiological study) or an increase in heart rate fluctuation (or no change; in vivo electrophysiological study). p ≤ 0.05 was accepted as statistically significant.

Results

HCN1 is a major component of I$_{f}$ in the SAN

To dissect the particular role of HCN1 channels in sinoatrial pacemaking we analyzed a mouse line in which HCN1 was globally deleted. A microarray analysis of the SAN (wild type (WT): n=3; HCN1 deficient (HCN1$^{-/-}$): n=3; Supplemental Tables 1 and 2) confirmed that the expression levels of ion channels or proteins that were shown to contribute to sinoatrial pacemaker and action potential are not altered in response to the global deletion of HCN1. Importantly, the transcripts for the major depolarizing and repolarizing ion channels are unchanged in the HCN1$^{-/-}$ mice. In line with the unaffected gene expression profile an echocardiographic analysis demonstrated a normal cardiac structure, diastolic and systolic
function of HCN1<sup>−/−</sup> (n=12) and WT (n=12) mice (Figure 1, Supplemental Table 3). In addition, cardiac slice preparation confirmed the finding that cardiac morphology is unchanged. Together these results indicate that compensatory remodeling or changes in gene expression profile are not a relevant issue in the HCN1-deficient heart.

We first investigated the expression of HCN channels in cardiac tissue using specific antibodies (Figure 2). A 120 kDa band corresponding to the mature glycosylated HCN1 protein was present in the SAN of WT mice while it was absent in HCN1<sup>−/−</sup> mice (Figure 2A). A 115 kDa band that was detected by the antibody represents an unspecific band since it was present in both WT and HCN1<sup>−/−</sup> mice. In line with this interpretation, no truncated HCN1 protein has been reported in another study using this HCN1<sup>−/−</sup> mouse line 27. Comparable amounts of HCN4 protein were detected in SAN of WT and HCN1<sup>−/−</sup> mice. By contrast, neither HCN4 nor HCN1 were detectable in right atrium. The HCN2 protein was below the detection limit in SAN and atrium.

Using whole mount immunohistochemistry we found overlapping expression of HCN1 and HCN4 in the central region of the SAN (Figure 2B-C). At higher magnification it was evident, that both channels are expressed within single pacemaker cells of the central region of the SAN (Figure 2D). Given the robust HCN1 expression in the SAN we next asked to which extent HCN1 contributes to I<sub>f</sub> in single isolated primary pacemaker cells. We developed an optimized protocol for the isolation of central pacemaker cells and focused on two major subtypes of primary sinoatrial cells: spindle cells and elongated cells which make up 90-95% of non atrial pacemaker cells (Figure 3A). In both cell types hyperpolarizing voltage steps activated robust I<sub>f</sub> in WT mice (Figure 3B). Deletion of HCN1 reduced the amplitude of I<sub>f</sub> by 36 ± 14 % in spindle (n=18) (p < 0.05 vs. 100 ± 16 % in WT (n=15)) and by 40 ± 19 % in elongated cells.
(n=22) (p < 0.05 vs. 100 ± 16 % in WT (n=20)) (Figure 3C). Deletion of HCN1 also dramatically slowed down activation kinetics of If (Figure 3D-F). The activation time course of If from WT mice required a sum of two exponentials for an adequate fit, whereas If from HCN1−/− mice was generally well fit by a single exponential. The slower kinetics of residual If in HCN1−/− mice were similar to those of cloned HCN4 channels 32. These findings indicated that HCN1 and HCN4 are the major determinants of If in spindle and elongated SAN pacemaker cells.

**Reduced beating frequency in isolated pacemaker cells of HCN1−/− mice**

We next investigated the contribution of HCN1 to the generation of spontaneous action potentials in isolated sinoatrial pacemaker cells using current clamp recordings. Isolated pacemaker cells from either WT or HCN1−/− mice fired rhythmical, spontaneous pacemaker potentials (Figure 4A). Deletion of HCN1 shifted the resting membrane potential to more hyperpolarized potentials in spindle and elongated cells (WT: -59.9 ± 1.3 mV, n=11; HCN1−/−: -63.9 ± 1.1 mV, n=8; p < 0.05). Furthermore, in HCN1−/− mice, the firing rate was significantly reduced by 13 ± 4% as compared to WT mice (100 ± 5%; WT: n=19, KO: n=18; p<0.05;

**Supplemental Table 4A**. Some recordings were performed in the presence of submaximal (2 nM) and saturating (10 μM) doses of isoproterenol (Figure 4B). In these experiments the maximal firing rate was consistently lower in HCN1−/− mice as compared to WT mice (Supplemental Table 4A). The relative increase in the firing rate induced by isoproterenol was the same in the two groups of mice (Supplemental Table 4A).

**Pronounced fluctuations of the beat-to-beat interval in intact SAN preparations of HCN1−/− mice**

We employed an explanted SAN preparation to investigate how the effects observed on the level of isolated pacemaker cells influence the properties of the cellular network of the SAN. Like
isolated sinoatrial pacemaker cells the intact SAN preparation from either WT or HCN1<sup>-/-</sup> mice, fired spontaneous pacemaker potentials (Figure 4C). The firing rate was significantly reduced in HCN1<sup>-/-</sup> mice by 29 ± 2% as compared to WT mice (100 ± 7%; WT: n=3, KO: n=4; p < 0.05; Supplemental Table 4B). A comparable reduction of the beating rate by 20.2 ± 2.8% was observed in isolated perfused hearts (WT: 100 ± 6 %; WT: n=5, KO: n=6; p < 0.05; Supplemental Table 4C), indicating that deletion of HCN1 leads to a reduction of the intrinsic heart rate and thereby to bradycardia originating within the SAN. In agreement with the findings in isolated SAN cells, the maximal firing rate in the presence of low doses of isoproterenol was consistently lower in HCN1<sup>-/-</sup> than in WT preparations (Figure 4D, Supplemental Table 4B). The firing rate of HCN1<sup>-/-</sup> SAN preparations increased by the same relative amount in the presence of isoproterenol as compared to wild type SAN preparations (Supplemental Table 4B).

In WT SANs, the interval between consecutive beats was regular in the absence and in the presence of isoproterenol (Figure 4E-F). By contrast, unlike in the WT the interbeat interval (IBI) rapidly fluctuated (range between 200 and 400 ms) in HCN1<sup>-/-</sup> preparations (Figure 4E). In the presence of 10 μM isoproterenol these rapid fluctuations were still significantly higher than in WT mice (Supplemental Table 4D). To rule out the possibility that the irregular firing rate of the HCN1<sup>-/-</sup> SAN resulted from a lower overall firing rate, we compared the WT and HCN1<sup>-/-</sup> nodes at similar slow firing rates. Since in WT slow rates were not achieved under normal recording conditions (30.5 ± 0.5°C), we intentionally slowed the rate by cooling down the SAN to 28°C (Figure 4E). Under these conditions the firing rate was still very regular. These findings strongly suggest that the observed increase in beat to beat fluctuation in HCN1<sup>-/-</sup> mice is intrinsic to the SAN itself. To confirm this hypothesis we determined SAN function using atrial pacing of...
isolated right atrial preparation containing the SAN. Sinus node automaticity was assessed by analyzing the sinus node recovery time (SNRT) in WT (n=13) and HCN1^{-/-}(n=9) mice. The preparation was continuously paced for 10 s at 10 Hz. Before and after pacing normal spontaneous sinus cycles were recorded (SCL). SNRT is defined as the duration of the return cycle which is the interval from the last paced atrial activation to the first postpacing spontaneous beat (Figure 5A). There was a clear increase in SNRT of HCN1^{-/-} mice as compared to WT mice. This difference was also evident after adjustment for the cycle length (corrected SNRT; Figure 5B, Supplemental Table 5).

**SAN dysfunction in HCN1^{-/-} mice**

The rapidly fluctuating IBIs of HCN1^{-/-} SANs together with the increased SNRT are highly suggestive of sinus node dysfunction. To test for this possibility we recorded in vivo telemetric long-term electrocardiography (ECG) in freely-moving WT (n=3) and HCN1^{-/-}(n=4) mice (Figure 6). In long-term measurements over 72 hours, HCN1^{-/-} mice revealed a marked sinus bradycardia (Figure 6A) characterized by lower mean heart rates by 16 ± 2% as compared to the WT (100 ± 2%; WT: n=3; KO: n=4; Supplemental Table 6A; Figure 6B) and more frequent episodes of low heart rate in HCN1^{-/-} mice compared to controls (Figure 6C). Marked sinus bradycardia was also observed in an echocardiographic analysis (Supplemental Table 3). The lower overall heart rates in WT and HCN1^{-/-} mice in echocardiographic analysis as compared to ECG telemetry are due to isoflurane anaesthesia\(^\text{33}\). Bradycardia in HCN1^{-/-} mice was not due to an increase in resting phases since HCN1^{-/-} and WT mice displayed the same activity and resting behavior. In addition, heart rates above 600 bpm were almost absent in HCN1^{-/-} mice (Figure 6C). The minimum, mean and maximum heart rates were both shifted to lower values in the HCN1^{-/-} mouse as compared to WT mice. By contrast, the dynamic range of heart rate regulation
(relative differences between minimal and maximal heart rates) as well as the relative degree of heart rate regulation (maximal heart rate/minimal heart rate) was comparable between the two groups of animals (Supplemental Table 6B). The reduction was consistently observed in HCN1−/− mice during 12 hour periods of low and high activity (Figure 6D-E). Upon beta-adrenergic stimulation achieved by intraperitoneal injection of isoproterenol (0.1mg/kg) the maximal heart rate was lower in HCN1−/− than in WT preparations. The heart rate in HCN1−/− mice increased by the same relative amount in the presence of isoproterenol as compared to wild type mice (Figure 6B, Supplemental Table 6A). Injection of carbachol (0.15 mg/kg) reduced the heart rate to the same level in WT and HCN1−/− mice (Figure 6B, Supplemental Table 6A). Beside bradycardia and reduced maximum heart rate, the ECG of HCN1−/− mice revealed periods of recurrent sinus pauses which were in some cases accompanied by escape beats (Figure 6F-G). Atrioventricular conduction was normal in HCN1−/− mice as compared to WT mice (PQ-interval: WT: 36.0 ± 2.9 ms; KO: 37.6 ± 0.8 ms).

Increased HRV in HCN1−/− mice

To further investigate the dynamics of heart rate oscillations we performed a heart rate variability (HRV) analysis. To assess variability of the heart with respect to time we determined the mean RR interval (Supplemental Figure 1A), SDRR (standard deviation of all RR intervals) and RMSD (root-mean square of the difference of successive RR intervals) during a time period of 2 hours during low physical activity (time domain parameters; Supplemental Figure 1B). In WT mice these parameters were consistent with only narrow fluctuations of the RR intervals indicating a relatively stable heart rhythm. In line with this finding, Poincaré plots of WT mice displayed a low beat-to-beat dispersion that is characteristic for a stable heart rate leading to an elliptic shaped configuration (Figure 7A). In contrast, in HCN1−/− mice time domain parameters
of HRV were markedly increased, consistent with dramatic fluctuations of the RR interval and a
markedly increased HRV (Supplemental Figure 1B). Accordingly, Poincaré plots determined
for HCN1<sup>−/−</sup> mice displayed a broad comet shaped pattern caused by high beat-to-beat dispersion
typically observed in SAN dysfunction (Figure 7B).

For frequency domain analysis of HRV, we compared HRV of WT and HCN1<sup>−/−</sup> mice
during a phase of relatively low heart rate (average heart rate ~ 400 bpm) corresponding to a RR-
interval of 150 ms and during a phase of relatively high heart rate (average heart rate ~600 bpm)
corresponding to an average RR interval of 100 ms. Tachograms of WT mice showed only a
slight variation of the RR interval (Figure 7C). In the absence of SAN pathology as in WT mice
these beat-to-beat fluctuations in heart rate are generated by the opposing effects of sympathetic
and parasympathetic nervous system. In contrast to WT mice, HCN1<sup>−/−</sup> mice displayed broad
range fluctuations of the RR interval consistent with a markedly increased HRV (Figure 7D).
These fluctuations were more pronounced during phases of slow heart rate (Supplemental
Figure 2). The pronounced increase in HRV in HCN1<sup>−/−</sup> mice as compared to WT controls is
reflected in HRV spectra (Figure 7E-F and Supplemental Figure 2A). The spectra were
subdivided into three frequency ranges: high frequency (HF, 1.5 - 4 Hz), low frequency (LF, 0.4
- 1.5 Hz) and very low frequency (VLF, <0.4 Hz). In contrast to HRV spectra of WT mice
(Figure 7E), the HRV spectra of HCN1<sup>−/−</sup> mice had a markedly increased power in all frequency
bands during periods of low heart rate (Figure 7F and Supplemental Figure 2). The heart rate
fluctuations of HCN1<sup>−/−</sup> animals were in the same range as observed in the intact isolated SAN
preparation (Figure 4E). This finding indicates that the SAN is the origin of pronounced
increased heart rate fluctuations rather than the autonomic nervous system.

In order to analyze the SAN function in vivo an intracardiac electrophysiological study
was performed (WT: n=9; KO: n=11). In HCN1−/− mice the SAN recovery time SNRT and SNRT corrected for the spontaneous cycle lengths (cSNRT) was decreased at pacing cycles ranging from 110-80 ms as compared to WT mice (Figure 8, Supplemental Table 7A). In line with similar experiments in the literature at the fastest pacing cycle, the SNRT decreased in WT and HCN1−/− mice 34. The increased SNRT in HCN1−/− mice is in line with the in vitro microelectrode experiments in explanted atrial preparations containing the SAN (Figure 5) and confirms a delayed impulse formation within the SAN of HCN1−/−. In addition, premature atrial stimulation revealed a prolonged sinoatrial conduction time (SACT; WT: 16.1 ± 1.9 ms; n= 9; HCN1−/−: 28.34 ± 2.4; n= 10; p ≤ 0.01; Supplemental Figures 3 and 4; Supplemental Table 7A). We also calculated a range for the SACT to account for the baseline sinus dysrhythmia, which was present in HCN1−/− mice (Supplemental Table 7B). To this end we used the longest and shortest spontaneous sinus cycle length. The SACT range was 13.6 - 17.5 ms (n=9) for WT mice and 25.5-38.8ms (n=10) for HCN1−/− mice, indicating that the difference in the SACT is independent of changes in sinus cycle lengths. This result is consistent with impaired impulse propagation and with a sinoatrial exit block in HCN1−/− and fits to overall clinical picture of sinus node dysfunction. There was no difference in the refractory periods of the atrium, the AV node and the ventricle as well as the Wenckbach point between control mice and HCN1−/− mice (Supplemental Table 7C and D; Supplemental Figure 5).

Finally, we analyzed the hemodynamic consequences of the observed cardiac phenotype in vivo. Echocardiographic in vivo testing revealed that the bradycardia of HCN1−/− mice leads to a hemodynamically relevant reduction in cardiac output to 69% as compared to WT mice (Supplemental Table 3). The stroke volume was not different between the two groups of mice. This indicates that there is no compensation for the reduced heart rate at least on the level of the
stroke volume. *In vivo* pressure measurements revealed no difference in systolic and diastolic blood pressure between the two groups of mice (systolic pressure: WT: 118 ± 5 mmHg vs KO 114 ± 3 mmHg; diastolic pressure: WT: 74 ± 3 mmHg vs KO: 67 ± 8 mmHg; WT: n=6, KO n=3). Together, these results suggest that the reduced heart is the primary cause of the decreased cardiac output in HCN1<sup>-/-</sup> mice.

**Discussion**

Here, we show that HCN1 channels make up a physiologically relevant component of *I*<sub>f</sub> in the SAN. *I*<sub>f</sub> was reduced by ~ 30% in HCN1<sup>-/-</sup> SAN cells and the activation kinetics of the remaining *I*<sub>f</sub> was significantly slowed down. Our results also suggest that HCN1 is functionally important in spindle and elongated primary pacemaker cells of the central SAN. The main cardiac phenotype of the HCN1<sup>-/-</sup> mouse is a pronounced sinus node dysfunction characterized by impaired impulse formation and sinoatrial conduction resulting in bradycardia, sinus arrhythmia and recurrent sinus pauses. Hemodynamically, these changes lead to a relevant reduction of cardiac output.

Several mechanisms may contribute to this complex phenotype. First of all, reduction of *I*<sub>f</sub> density is an important factor which could contribute or explain the bradycardia observed in HCN1<sup>-/-</sup> mice. Heterologous expression studies as well as analysis of neuronal hyperpolarization-activated currents predominantly carried by HCN1 revealed that the HCN1 channel activates with much faster kinetics and at more positive voltages than other HCN channel types, including HCN4. Deletion of HCN1 would lead to a remaining *I*<sub>f</sub> which is reduced and largely determined by HCN4. Therefore, one would expect that activation curve of *I*<sub>f</sub> in HCN1<sup>-/-</sup> mice is shifted to more hyperpolarized potentials as compared to WT mice. Moreover, HCN1 is only slightly, if at all, upregulated when the cAMP concentration is increased from low micromolar to millimolar
cAMP concentrations. Our finding that in HCN1−/− mice chronotropic competence is almost preserved is in line with that property. This particular biophysical profile implies that a major fraction of the HCN1 channels will be opened at rest. Thus, one function of HCN1 channels may consist in providing a basal depolarizing current in the central region of the SAN which facilitates opening of the other channels contributing to the SDD. This mechanism would well explain the bradycardia resulting from HCN1 deletion.

In addition to bradycardia, HCN1−/− mice revealed pronounced sinus dysrhythmia characterized by dramatic fluctuations in the heart rate and recurrent sinus pauses. Similar fluctuations of the beat-to-beat interval were observed in intact SAN preparations. The observation of pronounced heart rate fluctuations were not only observed in in vivo telemetric ECG recordings and experiments using intact SAN preparations which were performed using small sample size (WT: n=3, HCN1−/−: n=4) but were also consistently found in a third and independent set of experiments, in in vivo electrophysiological study, which was performed using a larger sample size (WT: n=9; HCN1−/−: n=11). The presence of heart rate fluctuations in in vivo experiments and in intact SAN preparation favours the SAN as possible origin of the observed dysrhythmia and virtually excludes autonomic modulation of the SAN. In line with this conclusion, SNRT was increased in HCN1−/− mice indicating that the sinus dysrhythmia and the sinus pauses are caused by a failure of impulse formation in the SAN. In addition, we also provide evidence for a prolonged sinoatrial conduction. The prolonged sinoatrial conduction time in HCN1−/− mice could result from a more negative maximal diastolic potential (MDP) which increases the distance to the threshold at which an action potential is generated. In this situation, more current and more time is required for a cell to charge up the cell membrane of an adjacent cell to reach the threshold potential for an action potential, and therefore slows down the action...
potential conduction. This effect overweights the competing effect of a more negative MDP to increase the availability of L-type Ca\(^{2+}\) channels and voltage gated Na\(^{+}\) channels which increase dV/dt and thereby the conduction velocity. Our results suggest that cardiac HCN channels in addition to impulse formation are also important for cardiac excitability and impulse propagation.

The SND of HCN1\(^{-/-}\) mice could develop in the extensively distributed cellular network of the intact SAN itself. Within the SAN a frequent exchange of dominance among multiple pacemakers coincide with changes in heart rate and beat-to-beat cycle lengths 35. A stable monofocal position of the leading pacemaker could account for stable heart rate observed in WT animals. By contrast, disorganized shift of the leading pacemaker focus without any predominant direction, and/or competing multiple pacemakers could be responsible for enhanced beat-to-beat variability and pronounced sinus node dysrhythmia and sinus pauses in HCN1\(^{-/-}\) mice. Such a mechanism has been shown for SAN dysrhythmia 36. It is well possible that in WT mice HCN1 channel activity at the resting membrane potential increases the membrane conductance and stabilizes one leading pacemaker focus and thus decreases beat-to-beat fluctuations in the heart. In addition, HCN1 channels could protect the SAN of excess hyperpolarizing electrotonic loading by the surrounding atrium, which could significantly slow pacemaking 37 and also could impair sinoatrial conduction. Such a role has been postulated for I\(_f\) in the SAN 37. Strands of atrial myocytes have been shown to extend into the central SAN38. It is possible that HCN1 plays an important role in protecting the central SAN from the hyperpolarization at these atrio-sinoatrial contact sites. Sinus node dysrhythmia and the recurrent sinus pauses were most pronounced at low heart rates and nearly absent at high heart rates observed at high physical activity or after pharmacological stimulation by isoproterenol. These findings suggest that a fully
functional $I_f$ is of particular relevance under these vulnerable conditions.

In humans, SND is a relevant disease which affects 1 in 600 cardiac patients older than 65 years and which is responsible for more than 50% of surgical implantation of permanent pacemakers per year worldwide\textsuperscript{35,39}. SAN dysfunction commonly occurs in the setting of heart failure and arterial hypertension but also is caused as genetic disease. Since HCN1 is expressed in the human SAN\textsuperscript{3,40,41} this channel may be added to the list of candidate genes associated with human SAN dysfunction. Mutations in HCN1 could be well tolerated, and thus could be relevant for human disease. HCN1 selective blockers have been considered for the treatment of epileptic disorders, chronic pain and depression\textsuperscript{42,43}. Given the functional importance of HCN1 for cardiac pacemaking selective HCN1 blockers should be carefully tested with respect to potential adverse effects on SAN function.

**Acknowledgments:** We thank Dr. Robert Fischer for the help during the setup of the \textit{in vivo} intra cardiac measurements and for the extensive discussions during the preparation of the manuscript. We thank Katrin Roetzer for excellent technical assistance.

**Funding Sources:** This study was supported by a research scholarship for S.I.H.H. according to the Bayerische Eliteförderungsgesetz (BayEFG). S.F. was supported by the LMU Mentoring programme and S.C.K. was supported by Deutsche Forschungsgemeinschaft (DFG).

**Conflicts of Interest Disclosures:** None.

**References:**


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Siegelbaum SA, Kandel ER, Morozov A. The hyperpolarization-activated hcn1 channel is important for motor learning and neuronal integration by cerebellar purkinje cells. Cell. 2003;115:551-564.


**Figure Legends:**

**Figure 1.** Cardiac structure and function of HCN1−/− (KO) mice are indistinguishable from WT hearts. (A-B) Representative short axis echocardiogram. Dotted line indicates axis for M-mode measurements in C and D. (E-F) Long axis echocardiogram. Inset: HE stained heart sections from WT (left) and HCN1−/− mice (right). Scale bars: 1mm.

**Figure 2.** HCN1 is a major component of I_f in the SAN. (A) HCN channel expression in SAN and atrial tissue. HCN1 protein is present in WT and absent in HCN1−/− SAN (upper panel). The same western blot was reprobed for HCN4 and HCN2 (middle). Tubulin served as loading control (lower panel). (B) Whole mount SAN preparation of a WT mouse depicting the central SAN area (SAN), crista terminalis (CT) superior vena cava (SVC) right atrium (RA) and septum (SEP); scale bar: 500 μm. (C) SAN preparations from a WT mouse (upper panel) and HCN1−/− mouse (lower panel) stained for HCN4 (green, a marker for the SAN) and HCN1 (red). scale bar: 500 μm. (D) Magnification of the central SAN area of a WT mouse (upper panel) and HCN1−/−
mouse (lower panel) stained for HCN4 (green) and HCN1 (red). scale bar: 20 μm.

Figure 3. Markedly reduced $I_f$ current densities and slowed $I_f$ activation kinetics in SAN cells isolated from HCN1$^{-/-}$ (KO) mice. (A) Morphology of spindle and elongated pacemaker cells. The surface area of spindle cells were $247 \pm 17$ μm$^2$ (n=15) and that of elongated cells was $516 \pm 45$ μm$^2$ (n=20). (B) Representative $I_f$ traces measured in spindle (top) and elongated (bottom) cells of WT mice. Currents were activated from a holding potential of -35 mV by stepping to a test potential of -130 mV for 3.5 s. (C) Current density (cd) of $I_f$ in spindle (top) and elongated (bottom) cells of WT and HCN1$^{-/-}$ mice. (D-E) Representative current traces (bottom) recorded from spindle-shaped (D) and elongated (E) SAN cells of WT (left) and HCN1$^{-/-}$ (right) mice. Residuals obtained from fitting current activation with a single exponential (top) or the sum of two exponential functions (middle). (F) Mean time constants received from fitting the activation of $I_f$ with a single exponential function for HCN1$^{-/-}$ cells and with a sum of two exponentials for the WT cells. All values are given as mean $\pm$ SEM; * p < 0.05.

Figure 4. Reduced beating frequency and pronounced variations of the beat-to-beat interval in isolated pacemaker cells and intact SAN preparations of HCN1$^{-/-}$ (KO) mice. (A) Representative spontaneous APs recorded from isolated SAN cells of WT (upper panel) and HCN1$^{-/-}$ mice (lower panel). (B) HCN1$^{-/-}$ SAN cells show a significant decrease in beating rate under control conditions and after perfusion with 2 nM or 10 μM isoproterenol (ISO). For detailed results of the 2-way ANOVA please see supplemental Table 4A. (C) Representative action potentials recorded in SAN preparation of WT (upper panel) and HCN1$^{-/-}$ mice (lower panel). (D) HCN1$^{-/-}$ SANs show a significant decrease in beating rate under control conditions and after perfusion.
with 2 nM or 10 μM isoproterenol. (E-F) Interbeat interval (IBI) of WT (upper panels) and HCN1−/− (lower panels) SAN preparations under control conditions (E). For detailed results of the 2-way ANOVA please see supplemental Table 4B. and after application of 10 μM isoproterenol (F). IBI is defined as the time interval (in ms) between two consecutive action potentials. In all panels mean heart rate of all mice is shown as light grey line. Cellular arrhythmia is evident as irregular IBI duration in HCN1−/− SAN preparations (E-F). WT IBI recorded at 28°C is shown as dark grey line (E, upper panel). Furthermore, negative chronotropism in HCN1−/− preparations is evident as an increased mean IBI (light grey line). All values are given as mean ± SEM; the number of cell and SAN preparations are given in parentheses; * p < 0.05.

**Figure 5.** Prolonged sinus node recovery time (SNRT) in HCN1−/− (KO) mice. (A)

Representative action potentials recorded in right atrial preparations containing the SAN of wild-type mice during and after SNRT protocol. (B) Consecutive sinus cycle lengths (SCL), SNRT and corrected SNRT (cSNRT) in WT and HCN1−/− mice. For detailed results of the 2-way ANOVA please see supplemental Table 5. All values are given as mean ± SEM; the number of SAN preparations are given in parentheses; * p < 0.05; ** p < 0.01.

**Figure 6.** HCN1−/− (KO) mice display sinus node dysfunction characterized by pronounced bradycardia, and dysrhythmia. (A) Representative telemetric ECG traces obtained from a conscious freely moving WT (upper panel) and HCN1−/− mouse (lower panel) recorded during a phase of averaged mean heart rate (WT: ~ 550 bpm; HCN1−/−: ~ 460 bpm). (B) Mean basal heart rate in WT and HCN1−/− mice determined from ECG traces recorded over a time period of 72 h and mean heart rate after injection of isoproterenol (ISO; 0.1 mg/kg or carbachol (0.15 mg/kg).
For detailed results of the 2-way ANOVA please see supplemental Table 6A. (C-E) Mean heart rate histograms of WT (light grey) and HCN1−/− mice (dark grey) over a period of 72 h (C), during low activity periods (D) and high activity periods (E). Bin width was 12 bpm. (F) Representative sinus pauses and escape beats observed in HCN1−/− mice at rest. (G) Mean number of sinus pauses in WT and HCN1−/− mice during a period of 6 h. All values are given as mean ± SEM; the number of animals is given in parentheses; * p ≤ 0.05.

**Figure 7.** Sinus node dysrhythmia in HCN1−/− mice. (A-B) Representative Poincaré plots of wild type mice (A) and of HCN1−/− mice (B). HCN1−/− mice display a comet-shaped pattern resulting from an increase in beat-to-beat dispersion observed at slow heart rates. Note the reduced maximal heart rate in HCN1−/− mice. (C-D) Raw tachogram of R-R interval recorded over 60 seconds during a phase of low heart rate (~400 bpm) from control (C) and HCN1−/− mice (D). (E-F) Heart rate variability in HCN1−/− mice is markedly increased. Representative frequency domain power spectral density plots obtained from WT (E) and HCN1−/− mice (F). VLF: very low frequency band 0 - 0.4 Hz; LF: low frequency band 0.4 - 1.5 Hz; HF: high frequency band 1.5 - 4 Hz.

**Figure 8.** Sinus node recovery time (SNRT) is prolonged in HCN1−/−(KO) mice. (A) The SNRT was measured by pacing for 30 s at a cycle lengths S1S1 of 100 ms, and defined as the time interval between the end of the last stimulus and the first spontaneous atrial activity. upper panel: WT, lower panel: HCN1−/−. In each panel lead II of the surface ECG (top) and the intracardiac atrial electrogram (bottom) are shown. (B) Averaged SNRT values for WT (open circle; n = 7-8) and HCN1−/− mice (black square; n = 7-10) determined at different pacing cycle lengths S1S1 as
indicated on the x axes. All values are given as mean ± SEM.**: p≤0.01. 2-way ANOVA for repeated measures, followed by Newman-Keuls post-hoc test; for detailed results of the statistical analyses please see supplemental Table 7A.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Sick Sinus Syndrome in HCN1-Deficient Mice

Circulation. published online November 11, 2013;
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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SUPPLEMENTAL MATERIAL

Supplemental Methods

RNA isolation and microarray processing

RNA from sinoatrial node of 3 HCN1⁻/⁻ mice and 3 WT mice was isolated using the RNeasy Micro Kit (Qiagen). Quality of RNA specimen was checked on an Agilent BioAnalyzer 2100 (Agilent, Germany) and processed for Affymetrix Gene Chips using Affymetrix whole transcript sense target labeling kit (Affymetrix, Santa Clara). Fragmented and labeled cDNA was hybridized onto murine MouseGene1.1-ST Gene Chips (Affymetrix). Staining of biotinylated cDNA and scanning of arrays were performed according to the manufacturer's recommendations.

Bioinformatic Analysis

Raw CEL-files were imported into Expression Console 1.0 (Affymetrix, Santa Clara). RMA was used for array normalization and signal calculation. Normalized signal values were imported into Partek Genomics Suite 6.5 (Partek). The probe sets were used to calculate differentially expressed transcripts using Welch's t-test with a p-value cut-off of 0.05 and a Fold change of 1.5.
**Morphology and Echocardiography**

Hearts from 12 week old WT and HCN1<sup>−/−</sup> mice were removed and fixed in 4% paraformaldehyde for 2 h and incubated in sucrose at 4°C overnight. 16 μm cryosections were stained with H&E according to standard protocols. Echocardiographic images were obtained using an ultrasound imaging system for rodents (Vevo 2100, FUJIFILM VisualSonics, Toronto, Canada) utilizing the systems 22-55 MHz transducer (MS550D). Prior to the measurement the mice were sedated by inhalation of isoflurane (Abbott, Wiesbaden). After achieving sedation the animals were placed on the systems mouse handling table, to monitor body temperature, heart rate and respiratory rate. Long axis, M-Mode and PW-Doppler images were taken and analyzed using the system software.

**SAN whole mount dissection**

The hearts of 6 - 10 weeks old, littermatched mice were put in a dish filled with Tyrode III solution and the SAN region was cut out along the superior vena cava and the crista terminalis. The dissected SANs were pinned on a silicone block and rinsed with Tyrode III solution.

**Western Blot**

For protein isolation mouse sinoatrial node and atrial tissue was snap-frozen in liquid nitrogen. Samples were homogenized on dry ice using a mortar and pestle and suspended in homogenization buffer (2% sodium dodecyl sulfate, 50 mm Tris and proteinase inhibitor cocktail mix). After heating at 95°C for 15 min followed by centrifugation at 1000g for 10 min to remove cell debris, the resulting supernatant was used in western blot analysis as previously described<sup>2</sup>. The following antibodies were used: mouse anti-HCN1 (1:1000; Abcam, Cambridge, UK), rat anti-HCN4 (1:500; Santa Cruz Biotechnology, CA, USA), rabbit anti-HCN2 L (1:500; <sup>3</sup> and mouse anti-Tubulin (1:2000; Dianova, Hamburg, Germany).
**Immunofluorescence whole mount SAN**

SAN whole mounts were fixed in PBS containing 80% methanol / 20% DMSO, permeabilized in 1% Triton X-100 in PBS and incubated in blocking buffer (PBS, 10% normal goat serum, 1% fetal calf serum, 1% bovine serum albumin, 1% Triton X-100). Tissues were stained with rat monoclonal antibody to HCN4 (1:100; Santa Cruz Biotechnology, CA, USA) and rabbit monoclonal antibody to HCN1 (1:100; Alomone labs, Jerusalem, Israel). As secondary antibodies Alexa488-conjugated anti-rat (1:400; Invitrogen, Karlsruhe, Germany) and Alexa555-conjugated anti-rabbit (1:400; Invitrogen, Karlsruhe, Germany) were used. Images were acquired using a LSM 510 meta microscope (Carl Zeiss, Jena, Germany).

**Electrophysiological recordings in single SAN cells**

Sinoatrial node cells were isolated and plated onto poly-L-lysine-coated coverslips as described previously⁴. The SAN was digested with collagenase, elastase and protease. To increase the yield of cells from the central SAN, digestion time and concentration of enzymes were increased as needed. If measurements were performed in the whole-cell configuration at room temperature. The pipette solution contained (in mM): K-aspartate 90, NaCl 10, CaCl₂ 2, MgCl₂ 2, EGTA 5, Na-ATP 2, Na-GTP 0.1, creatine phosphate 5, pH 7.2 adjusted with KOH. The bath solution contained (in mM): NaCl 140, KCl 5.4, CaCl₂ 1, MgCl₂ 1, HEPES 5, glucose 5, BaCl₂ 2, CdCl₂ 0.3, pH 7.4 adjusted with NaOH. If current amplitudes were measured by a 3.5 s hyperpolarizing voltage step to -130 mV from a holding potential of -35mV. The identity of If was tested by applying 4 mM Cs⁺. The current amplitude was defined as the amplitude at the end of the activation pulse minus the amplitude of the initial lag. The current density was calculated as the amplitude divided by the area of the cell. The area was determined with AxioVision4.8 (Carl Zeiss, Jena, Germany) and ImageJ. The surface area of spindle cells were 247 ± 17 µm² (n=15) and that of elongated cells was 516 ± 45 µm² (n=20). This corresponds to a mean capacitance of 31 ± 2pF (n=15) for spindle cells and 35 ± 3 pF (n=20) for elongated cells. Time constants of activation were determined by fitting the current traces after the initial lag with a monoexponential or biexponential function. Action potential measurements were performed at 32 ± 1 °C using the perforated patch technique with 200 µg /ml Amphotericin B added to
the intracellular solution. The pipette solution contained (in mM): K-aspartate 130, NaCl 10, CaCl$_2$ 0.04, Na-GTP 0.1, Mg-ATP 2, creatine phosphate 6.6, HEPES 10, pH 7.2 adjusted with KOH. Bath solution contained (in mM): NaCl 140, KCl 5.4, CaCl$_2$ 1.8, MgCl$_2$ 1, HEPES 10, glucose 10, pH 7.4 adjusted with NaOH.

In experiments involving single SAN cells data were acquired using an EPC 10 amplifier and PatchMaster software (HEKA instruments, USA). Voltage clamp data were analyzed off-line by using PatchMaster and Origin 6.1 software (Microcal).

**Microelectrode recordings whole mount SAN**

For the microelectrode recordings an IX2-700 Dual Intracellular Preamplifier (Dagan Corporation) attached to a Digidata 1320A (Axon instruments) and pClamp 8 software (Axon instruments) were used. Spontaneous APs were recorded from isolated SAN preparations by using 3 M KCl microelectrodes at 30.5 ± 0.5 °C (unless otherwise stated). SANs were continuously superfused with Tyrode III solution containing (in mM): NaCl 140, KCl 5.4, MgCl$_2$ 1, CaCl$_2$ 1.8, glucose 5.5, HEPES 5, pH 7.4 adjusted with NaOH. The recording electrode was impaled in the sinoatrial node cells or atrial cells in close proximity to the sinoatrial node. Isoproterenol was added to the external solution as indicated. Analysis was done offline with Origin6.1 software (Microcal). The beat-to-beat variability is defined as the standard deviation of the beat to beat intervals.

**Microelectrode recordings right atrial preparation containing the entire sinoatrial node**

In a subset of experiments right atrial preparations containing the whole sinoatrial node were used to determine the sinoatrial node function. The recording solutions and the experimental setup were the same as for the whole mount SAN. To determine the sinoatrial node recovery time (SNRT) the atrial preparation was paced through a pacing electrode located in the atrial muscle close to the sinoatrial node via a stimulator (A360, WPI) at 10 Hz for 10 s (pulse duration: 10 ms, 0.1 mA). SNRT was measured as the time interval between the last pacing beat and the first spontaneous beat originating from the SAN. Corrected SNRT (cSNRT) was calculated as the difference between the SNRT and an average of 10 consecutive sinus cycle lengths (SCL) immediately before each run of atrial pacing.

5.7.
Telemetric ECG recordings in mice

Littermatched mice were housed in single cages in a 12 hour darklight-cycle environment with food and water ad libitum. Male littermates (3 WT and 4 HCN1$^-$ mice) were anaesthetized with Ketamine/Xylazine and radiotelemetric ECG transmitters (ETA-F20, Data Sciences International, USA) were implanted into the intraperitoneal cavity. The ECG leads were sutured subcutaneously onto the upper right chest muscle and the upper left abdominal wall muscle, approximately representing ECG lead II. The animals were allowed to recover for at least 10 days before ECG measurements started. The analog telemetric signals were digitized at 1 kHz and recorded by Dataquest A.R.T. data acquisition software (Data Sciences International, USA). Data were sampled over the whole period of the recording in freely moving animals. Basal heart rate and activity analysis has been performed over 72 hours. For pharmacological interventions drugs were injected intraperitoneally after 1 hour prerun. The ECGs were recorded for 3 to 24 hours thereafter. The animals were allowed to recover for at least 48 hour between injections.

Analysis of heart rate and heart rate variability

Mean HR was determined over 72 h from continuous recordings using the parameter analysis module of Dataquest A.R.T. 4.0 data analysis software. To determine differences in heart rate dynamics, histograms were calculated from 72 h intervals or averaged 12 h intervals during light- and dark-condition, corresponding to high and low activity periods, respectively. Heart rate values were binned using 30 equal distributed windows in the range from 200 to 800 bpm.

Heart rate variability (HRV) was determined using an analysis based on previous reports. For analysis in the frequency domain, raw ECG strip was manually inspected to confirm stable sinus rhythm. 60 s time series of RR intervals were plotted as tachograms. These tachograms were interpolated by cubic spline interpolation at 50 ms intervals to create equidistant points suitable for Fast Fourier Transform (FFT). After mean-detrending, FFT was performed using 512 spectral points and multiplying with two half overlapping Hanning windows and power spectral density plots were determined. For each time segment, the total power (TP) ($s^2$/Hz) was calculated as the integral sum of
total variability after FFT over the frequency range recorded (0 - 4.0 Hz). In addition, for each time segment, the cutoff frequencies previously determined to be accurate for mice were used to divide signal into three major components, very low frequency (VLF: 0.0 - 0.4 Hz), low frequency (LF: 0.4 - 1.5 Hz), and high frequency (HF: 1.5 - 4.0 Hz). The data obtained for each time segment were averaged.

For time domain analysis, 2 hours ECG recordings were recorded during low activity period. RR intervals were determined and ectopic beats and other artifacts removed. Mean heart rate, mean RR interval, standard deviation (SD) of all normal RR intervals in sinus rhythm (SDNN) and the square root of mean of squared differences between successive normal RR intervals (RMSSD) were calculated. In addition, 20 000 data points from the cleaned RR time series were used for Poincaré Plots. Here, RR intervals (n; x-axis) were plotted against the next RR interval (n+1; y-axis).

**Intracardiac electrophysiological study**

9 WT and 11 HCN1<sup>−/−</sup> mice (age: 6-10 weeks) were anesthetized with isoflurane (1.6 Vol% isoflorane/air) and ECG standard intervals were measured in 6-limb leads. For intracardiacelectrogram recordings we used a digital electrophysiology lab (EP Tracer, CardioTek, Netherlands). An octapolar 0.54 mm (1.7 French) electrode catheter (CIBer mouse cath, NuMed Inc.) was placed via the right jugular vein into the right atrium and ventricle, guided by the morphology of intracardiac electrical signals. The eight electrodes which are spaced 0.5 mm apart directly contact the endocardial surface of the heart. Bipolar recordings of the atrial and ventricular depolarizations were obtained from adjacent electrodes in the superior right atrium just past the superior vena cava and right ventricular apex, respectively. Standard clinical electrophysiological pacing protocols were used to determine the electrophysiologic parameters, including sinus node recovery time, sinoatrial conduction time, sinoatrial node effective refractory period, refractory periods of the atria, the AV node, and the ventricle as well as AV nodal conduction properties including Wenckebach periodicity (WBP) 10. Impulses were delivered at twice diastolic threshold (1mA) using a pulse duration of 1.0 ms. Each mouse underwent an identical pacing and programmed stimulation protocol.
**Sinus node recovery time (SNRT):** The sinus node function was evaluated by indirect measurement of SNRT by pacing for 30 seconds at various cycle lengths starting from a pacing cycle length just below the intrinsic sinus cycle length and measuring the duration of the return cycle which corresponds to the interval between the last stimulation spike and first spontaneous, sinus node triggered atrial activation. After a pause of 60 s the protocol was repeated by progressively reducing the pacing cycle length in 10 ms steps until a pacing cycle length of 80 ms was reached. A range of pacing cycle length from 110-80 ms was tested. Rate corrected SNRT (cSNRT) was calculated by subtracting the averaged sinus cycle length (SCL) from SNRT.

**Sinoatrial conduction time (SACT)** was indirectly determined by premature atrial stimulation technique which was carried out in analogy to human studies \(^{11,12}\). The protocol and the responses are shown in Supplemental Figure 3. Premature atrial stimuli were introduced via the stimulation electrode during spontaneous sinus rhythm. The entire sinus cycle was scanned by up to 80 extrastimuli. Spontaneous sinus cycle length (A1A1 interval), coupling interval of the premature atrial stimulus (A1A2), the atrial return cycle length (A2A3) and the post return cycle length (A3A4) were measured. Supplemental Figure 4 illustrates the response of the sinoatrial node to premature atrial stimulation. Upon progressively decreasing the coupling interval of the premature atrial stimulus (A1A2), the return cycles A2A3 progressively prolongs. The corresponding A2A3 data points fall on the upper diagonal line indicating fully compensatory pauses \([A1A2+A2A3=2(A1A1)]\). The pause is compensatory because late diastolic atrial depolarizations do not penetrate and reset the sinoatrial node before it fires spontaneously. Accordingly, this portion of the graph is designated "zone of no reset". As soon as the coupling interval A1A2 is decreased below a certain point of the spontaneous cycle length (80% in Supplemental Figure 4), the return cycles A2A3 is no longer fully compensatory. The data points fall below the line of full compensatory pause but remain greater than one expected sinus cycle length (A1A1; horizontal line). In some animals during this phase A2A3s remained constant yielding a plateau. This zone is called "zone of reset" (A1A2 plus A2A3 is shorter than twice the A1A1 interval) because the premature atrial depolarization penetrates, depolarizes and resets the sinoatrial node prior to its next expected spontaneous firing.
In Supplemental Figure 4 the post-return cycles A3A4 which is the first spontaneous cycle after the return cycle was also plotted (closed circles). By comparing A3A4 with A1A1 intervals it is possible to assess the sinus cycle variability and sinoatrial node automaticity. During zone of reset and the zone of no reset the post-return cycles A3A4 fall very close to the horizontal line of identity (closed circles). This finding suggests that the premature atrial stimulation did not induce overdrive suppression or other alterations of the sinoatrial node automaticity 12, 13.

For calculation of sinoatrial conduction time we determined the A2A3 interval which was exactly at the borderline between the "zone of non reset" and "zone of reset" 14, 15. The A1A2 interval at this borderline represents the shortest premature beat interval whose retrograde excitation front does not reach the sinus node, whereas earlier premature beats reset the pacemaker 16, 17. Therefore, the return interval A2A3 at this borderline, minus the spontaneous atrial cycle A1A1, is equivalent to the sum of conduction time from atrium to the sinus node plus from sinus node to atrium. Half of the total sum of conduction time gives the sinoatrial conduction time. This calculation is based on the assumptions that the SACT reflects the time for the paced impulse A1 to enter the SAN which is then reset plus the spontaneous sinoatrial cycle lengths plus the time it takes for the subsequent spontaneous beat to exit the sinoatrial node, and that the times into and out of the sinoatrial node are approximately equivalent. The SACT value for WT mice was 16.1 ± 1.9 ms; n=9 and is well consistent with results from the literature 18, 19.

Atrial depolarizations even earlier in atrial diastole are followed by a sudden transition from the zone of reset to a third zone which is designated "zone of interpolation". This zone corresponds to the area between the horizontal line and above the lower diagonal line. The lower diagonal line is termed line of complete interpolation [A1A2+A2A3=A1A1]. This line indicates where A2A3 values would fall if A2 failed to enter the sinoatrial node because the tissue surrounding the sinoatrial node is refractory. In this case the atrial response is interpolated between two normal sinus beats. Sinus node effective
refractory period (SNERP) was determined as the A1A2 interval, at which the abrupt transition from zone of no reset to the zone of interpolation occurred.

**Effective refractory periods:** Atrioventricular nodal refractory period (AVNERP) was evaluated by programmed atrial stimulation. To allow for reasonable stabilization of refractoriness the premature atrial stimulus (S2) was preceded by a train of 8 paced beats (S1). The train of 8 stimuli was applied at a S1S1 cycle length of 100 ms followed by one extrastimulus (S2). The coupling interval S1S2 was stepwise reduced in 2 ms steps to 20 ms. Subsequently, the protocol was repeated after a recovery time of 30 s using S1S1 cycle length of 90 ms and 80 ms. The AVNERP was defined as longest S1S2 pacing interval with loss of AV-nodal conduction. The minimum cycle length required to maintain 1:1 AV conduction, the Wenckebach paced cycle length, and the maximum paced cycle length causing 2:1 AV block were determined for each animal. The ventricular effective refractory period (VERP) was evaluated analogous to the AVNERP. The S1S1 intervals were 100 ms, 90 ms and 80 ms. The coupling interval S1S2 was stepwise reduced in 2 ms steps to 30 ms. Using a similar protocol, the right atrial effective refractory period (AERP) could not reliably be determined due to superposition of atrial and ventricular electrograms at premature coupling intervals below 40 ms. To circumvent this problem the following three-step protocol was used: After applying a train of 8 S1 stimuli, an extra stimulus (S2) was given to induce AV conduction block at an S1S2 coupling interval 5 ms shorter than the determined AVNERP, followed by an increasingly premature S3. AERP was determined as longest S2S3 with absent atrial response.

**AV nodal conduction curves:** AV conduction curves were determined from data obtained by the AVERP protocol described above. Distinct His bundle electrograms could be reliably identified in intracardiac recordings of 3 WT and 6 HCN1+− mice (Supplemental Figure 5A; H: His signal). AV nodal conduction curves were constructed by plotting V1V2 or H1H2 intervals versus A1A2. Latency curves were constructed by plotting A2V2 intervals versus A1A2 intervals as in 21.
Supplemental Figure 5B shows the responses to premature stimulation in a single experiment. As atrial responses occurred progressively earlier in the cardiac cycle, ventricular intervals also progressively decreased. Up to a coupling interval of 85 ms, the decrease of V1V2 is proportional to that of A1A2 and therefore the points fall on the line, which represents the theoretical curve of no AV conduction delay. For points on this curve V1V2 is equal to A1A2. At shorter A1A2 intervals, V1V2 decrease less than A1A2. The points lie above the line, indicating that AV conduction of the premature impulse A2 is delayed. The point at which the points start to deviate from the line is marks the beginning of the relative refractory period of the AV conduction system. At a critical A1A2 interval of 62 ms V1V2 reached a minimum (75 ms) and then began to increase even though the atrial interval shortened further until complete block of conduction of the premature impulse occurred (at A1A2 interval of 58 ms; Supplemental Figure 5B). From this graph functional refractory period of the AV conduction system was determined as the shortest V1V2 interval. We also plotted the intervals between His bundle responses (H1H2 intervals) and premature atrial stimulations (A1A2) for the same experiment (Supplemental Figure 5B). The H1H2 intervals correspond exactly to the V1V2 values indicating that the increase in AV conduction time occurring with premature atrial stimulation was confined entirely to the region of the AV node, e.g. between the atrial and His bundle electrogram.

Finally, A2V2 latencies were plotted versus A1A2 intervals. As A1A2 intervals decreases, the A2V2 latency increases (Supplemental Figure 5C). The A2V2 lengthening is slight for relatively long A1A2 intervals and becomes larger as these intervals shorten. The diagonal line indicates A2V2 lengthening equal to the A1A2 shortenings (slope=-1). There was usually a segment of the curves that coincided with this line. The horizontal broken line represents a base line corresponding to the diagonal line in Supplemental Figure 5B.

**Arterial blood pressure measurements**

Mice were anesthesized by injecting a mixture of midazolam (Midazolam-Ratiopharm, Ratiopharm, 0,05 mg/kg), medetomidine (Dorbene vet., Pfizer, 0,5 mg/kg) and fentanyl (Fentanyl-Jansen, Jansen-Cilag 0,005 mg/kg). A Millar Tip Pressure Transducer Catheter (Millar Instruments) was inserted in
the right carotic artery and subsequently placed in the left ventricle. The catheter was connected to a
A/D Card (DT301, Data Translation) via a transducer control unit (TC-510, Millar Instruments), a
signal amplifier (DC-bridge amplifier, Type 660, Hugo Sachs Elektronik) and a BNC connector block
(Hugo Sachs Elektronik). The signal was analysed utilizing the DasyLab software (DasyLab 8.0,
National Instruments Ireland Resources Limited). The systolic and diastolic aortic pressure was
determined.
### Supplemental Table 1

**Downregulated transcripts in sinoatrial node tissue of HCN1−/− mice**

<table>
<thead>
<tr>
<th>Accession</th>
<th>Gene Symbol// Gen Name</th>
<th>p-value KO vs. WT</th>
<th>Fold-change KO vs. WT</th>
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<td>-1.5523</td>
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<td>NM_001252057</td>
<td>Ly6c1 // lymphocyte antigen 6 complex, locus C1</td>
<td>0.0245944</td>
<td>-1.61768</td>
</tr>
<tr>
<td>NR_037297</td>
<td>Mir3110 // microRNA 3110</td>
<td>0.0380998</td>
<td>-1.62142</td>
</tr>
<tr>
<td>NM_153502</td>
<td>Ankrd23 // ankyrinrepeatsdomain 23</td>
<td>0.0413793</td>
<td>-1.64579</td>
</tr>
<tr>
<td>ENSMUST00000121887</td>
<td>Fam107a // family with sequence similarity 107, member A</td>
<td>0.0202383</td>
<td>-1.6873</td>
</tr>
<tr>
<td>ENSMUST00000114792</td>
<td>Fkbp5 // FK506 bindingprotein 5</td>
<td>0.0449762</td>
<td>-1.71764</td>
</tr>
<tr>
<td>AK034234</td>
<td>Fut11 // fucosyltransferase 11</td>
<td>0.0128125</td>
<td>-1.72975</td>
</tr>
<tr>
<td>NM_007669</td>
<td>Cdkn1a // cyclin-dependent kinase inhibitor 1A (P21)</td>
<td>0.0138576</td>
<td>-1.73095</td>
</tr>
<tr>
<td>NM_172648</td>
<td>Ifi205 // interferonactivatedgene 205</td>
<td>0.00468679</td>
<td>-1.76978</td>
</tr>
<tr>
<td>NM_007749</td>
<td>Cox7c // cytochrome c oxidase, subunit VIIc</td>
<td>0.0126134</td>
<td>-1.97584</td>
</tr>
<tr>
<td>XR_108081</td>
<td>LOC100504898 // uncharacterized LOC100504898</td>
<td>0.0305362</td>
<td>-2.33442</td>
</tr>
<tr>
<td>NM_001013373</td>
<td>Tmprss13 // transmembraneprotease, serine 13</td>
<td>0.0198162</td>
<td>-2.46718</td>
</tr>
</tbody>
</table>

Note: p-values were not corrected for multiple testing.
Supplemental Table 2
Upregulated transcripts in sinoatrial node tissue of HCN1<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th>Accession</th>
<th>Gene Symbol//Gen Name</th>
<th>p-value KO vs. WT</th>
<th>Fold-change KO vs. WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_029001</td>
<td>Elovl7 // ELOVL family member 7, elongation of long chain fatty acids</td>
<td>0.000926335</td>
<td>2.86973</td>
</tr>
<tr>
<td>NM_001085530</td>
<td>Gm13298 // predictedgene 13298</td>
<td>0.0412558</td>
<td>1.80908</td>
</tr>
<tr>
<td>ENSMUST00000160534</td>
<td>Gm4477 // predictedgene 4477</td>
<td>0.00724953</td>
<td>1.75278</td>
</tr>
<tr>
<td>NM_010934</td>
<td>Npy1r // neuropeptide Y receptor Y1</td>
<td>0.0364213</td>
<td>1.58377</td>
</tr>
<tr>
<td>NM_172807</td>
<td>Ppwd1 // peptidylprolyl-isomerase domain and WD repeat containing 1</td>
<td>0.000204777</td>
<td>1.56153</td>
</tr>
<tr>
<td>NM_001146007</td>
<td>Trim12c // tripartite motif-containing 12C</td>
<td>0.0182562</td>
<td>1.55974</td>
</tr>
</tbody>
</table>

Note: p-values were not corrected for multiple testing.
Supplemental Table 3
Echocardiographic Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>n (WT)</th>
<th>KO</th>
<th>n (KO)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVSd [mm]</td>
<td>0.74 ± 0.04</td>
<td>12</td>
<td>0.81 ± 0.04</td>
<td>12</td>
<td>ns</td>
</tr>
<tr>
<td>IVSs [mm]</td>
<td>1.11 ± 0.06</td>
<td>12</td>
<td>1.16 ± 0.04</td>
<td>12</td>
<td>ns</td>
</tr>
<tr>
<td>LVIDd [mm]</td>
<td>4.26 ± 0.06</td>
<td>12</td>
<td>4.25 ± 0.13</td>
<td>12</td>
<td>ns</td>
</tr>
<tr>
<td>LVIDs [mm]</td>
<td>3.01 ± 0.11</td>
<td>12</td>
<td>2.97 ± 0.14</td>
<td>12</td>
<td>ns</td>
</tr>
<tr>
<td>LVPWd [mm]</td>
<td>0.80 ± 0.06</td>
<td>12</td>
<td>0.82 ± 0.05</td>
<td>12</td>
<td>ns</td>
</tr>
<tr>
<td>LVPWs [mm]</td>
<td>1.07 ± 0.09</td>
<td>12</td>
<td>1.08 ± 0.06</td>
<td>12</td>
<td>ns</td>
</tr>
<tr>
<td>LV Mass [mg]</td>
<td>123.64 ± 5.75</td>
<td>12</td>
<td>133.33 ± 6.41</td>
<td>12</td>
<td>ns</td>
</tr>
<tr>
<td>EF [%]</td>
<td>50.79 ± 2.44</td>
<td>12</td>
<td>49.49 ± 1.66</td>
<td>12</td>
<td>ns</td>
</tr>
<tr>
<td>FS [%]</td>
<td>17.08 ± 1.71</td>
<td>12</td>
<td>16.46 ± 1.22</td>
<td>12</td>
<td>ns</td>
</tr>
<tr>
<td>CO [ml/min]</td>
<td>14.54 ± 1.62</td>
<td>12</td>
<td>10.00 ± 0.76</td>
<td>12</td>
<td>*</td>
</tr>
<tr>
<td>SV [µl]</td>
<td>33.15 ± 2.10</td>
<td>12</td>
<td>30.70 ± 2.10</td>
<td>12</td>
<td>ns</td>
</tr>
<tr>
<td>HR [bpm]</td>
<td>432.15 ± 35.07</td>
<td>12</td>
<td>323.99 ± 8.39</td>
<td>12</td>
<td>**</td>
</tr>
<tr>
<td>MV A</td>
<td>400.22 ± 56.20</td>
<td>7</td>
<td>372.65 ± 41.33</td>
<td>6</td>
<td>ns</td>
</tr>
<tr>
<td>MV E</td>
<td>528.27 ± 51.43</td>
<td>7</td>
<td>538.79 ± 88.01</td>
<td>6</td>
<td>ns</td>
</tr>
<tr>
<td>MV E/A</td>
<td>1.40 ± 0.12</td>
<td>7</td>
<td>1.45 ± 0.18</td>
<td>6</td>
<td>ns</td>
</tr>
</tbody>
</table>

mean ± SEM * p < 0.05; ** p < 0.01; *** p < 0.001; ns: not significant. Note: p-values were not corrected for multiple testing.

IVSd intraventricular septal thickness (diastole)
IVSs intraventricular septal thickness (systole)
LVIDd left ventricular dimension (diastole)
LVIDs left ventricular dimension (systole)
LVPWd left ventricular posterior wall dimension (diastole)
LVPWs left ventricular posterior wall dimension (systole)
LV Mass mass of left ventricle
EF ejection fraction
FS fracional shortening
CO cardiac output
SV stroke volume
HR heart rate
MV A flow velocity through mitral valve (ventricular relaxation)
MV E flow velocity through mitral valve (atrial contraction)
**Supplemental Table 4A**

**Beating rate in single sinoatrial node cells**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>n (WT)</th>
<th>KO</th>
<th>n (KO)</th>
<th>rate reduction in KO</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>basal</td>
<td>289.4 ± 12.9</td>
<td>19</td>
<td>251.3 ± 10.6</td>
<td>18</td>
<td>13.2 ± 3.7%</td>
<td>*</td>
</tr>
<tr>
<td>2 nM isoproterenol</td>
<td>334.5 ± 15.3</td>
<td>18</td>
<td>293.7 ± 8.9</td>
<td>22</td>
<td>12.2 ± 2.7%</td>
<td>*</td>
</tr>
<tr>
<td>10 µM isoproterenol</td>
<td>367.9 ± 20.2</td>
<td>11</td>
<td>319.8 ± 8.4</td>
<td>18</td>
<td>13.1 ± 2.3%</td>
<td>*</td>
</tr>
</tbody>
</table>

A 2-way ANOVA (genotype, dose) revealed main effect of genotype (F_{1,100} = 17.87, p < 0.0001) and dose (F_{2,100} = 17.53, p < 0.0001), but no factorial interaction (F_{2,100} = 0.0829, p = 0.9204). mean ± SEM * p< 0.05 (t-test)

**Beating rate in single sinoatrial node cells (relative to basal)**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>n (WT)</th>
<th>KO</th>
<th>n (KO)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>basal</td>
<td>100 ± 4%</td>
<td>19</td>
<td>100 ± 4%</td>
<td>18</td>
<td>ns</td>
</tr>
<tr>
<td>2 nM isoproterenol</td>
<td>115 ± 5%</td>
<td>18</td>
<td>117 ± 4%</td>
<td>22</td>
<td>ns</td>
</tr>
<tr>
<td>10 µM isoproterenol</td>
<td>127 ± 7%</td>
<td>11</td>
<td>127 ± 3%</td>
<td>18</td>
<td>ns</td>
</tr>
</tbody>
</table>

**Supplemental Table 4B**

**Beating rate in sinoatrial preparation**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>n (WT)</th>
<th>KO</th>
<th>n (KO)</th>
<th>rate reduction in KO</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>basal</td>
<td>289.6 ± 20.0</td>
<td>3</td>
<td>205.1 ± 6.0</td>
<td>4</td>
<td>29.2 ± 2.1%</td>
<td>*</td>
</tr>
<tr>
<td>2 nM isoproterenol</td>
<td>300.6 ± 2.9</td>
<td>3</td>
<td>209.9 ± 2.0</td>
<td>4</td>
<td>30.0 ± 6.6%</td>
<td>*</td>
</tr>
<tr>
<td>10 µM isoproterenol</td>
<td>366.9 ± 15.6</td>
<td>3</td>
<td>289.0 ± 19.2</td>
<td>4</td>
<td>21.2 ± 5.2%</td>
<td>ns</td>
</tr>
</tbody>
</table>

A 2-way ANOVA (genotype, dose) for repeated measures (dose) revealed main effect of genotype (F_{1,15} = 40.53, p < 0.0001) and dose (F_{2,15} = 14.99, p < 0.001), but no factorial interaction (F_{2,15} = 0.0775, p = 0.925). mean ± SEM * p≤ 0.05 (MWU test)

**Beating rate in sinoatrial preparation (relative to basal)**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>n (WT)</th>
<th>KO</th>
<th>n (KO)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>basal</td>
<td>100%</td>
<td>3</td>
<td>100%</td>
<td>4</td>
<td>ns</td>
</tr>
<tr>
<td>2 nM isoproterenol</td>
<td>105 ± 9%</td>
<td>3</td>
<td>103 ± 11%</td>
<td>4</td>
<td>ns</td>
</tr>
<tr>
<td>10 µM isoproterenol</td>
<td>128 ± 13%</td>
<td>3</td>
<td>140 ± 5%</td>
<td>4</td>
<td>ns</td>
</tr>
</tbody>
</table>
Supplemental Table 4C
Beating rate of isolated perfused hearts

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>n (WT)</th>
<th>KO</th>
<th>n (KO)</th>
<th>rate reduction in KO</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>rate</td>
<td>392.9 ± 23.1</td>
<td>5</td>
<td>313.4 ± 11</td>
<td>6</td>
<td>20.2 ± 2.8%</td>
<td>*</td>
</tr>
</tbody>
</table>

mean ± SEM * p<0.05 (MWU test)

Supplemental Table 4D
Beat-to-beat variability in sinoatrial preparation

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>n (WT)</th>
<th>KO</th>
<th>n (KO)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>basal</td>
<td>5.7 ± 0.5</td>
<td>3</td>
<td>29.0 ± 7.5</td>
<td>3</td>
<td>*</td>
</tr>
<tr>
<td>2 nM isoproterenol</td>
<td>1.7 ± 0.3</td>
<td>3</td>
<td>32.8 ± 3.8</td>
<td>3</td>
<td>*</td>
</tr>
<tr>
<td>10 µM isoproterenol</td>
<td>1.6 ± 0.7</td>
<td>3</td>
<td>7.8 ± 1.5</td>
<td>3</td>
<td>*</td>
</tr>
</tbody>
</table>

A 2-way ANOVA (genotype, dose) for repeated measures (dose) revealed main effect of genotype (F_{1,4} = 35.17, p = 0.004) and dose (F_{2,8} = 8.497, p = 0.010), and a significant factorial interaction (F_{2,8} = 6.4845, p = 0.0211). mean ± SEM * p<0.05 (MWU test)
### Supplemental Table 5

**Sinoatrial recovery time in sinoatrial preparation**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>n (WT)</th>
<th>KO</th>
<th>n (KO)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCL</td>
<td>266.22 ± 12.4</td>
<td>13</td>
<td>333.0 ± 34.2</td>
<td>9</td>
<td>*</td>
</tr>
<tr>
<td>SNRT</td>
<td>369.5 ± 21.9</td>
<td>13</td>
<td>520.7 ± 43.9</td>
<td>9</td>
<td>**</td>
</tr>
<tr>
<td>cSNRT</td>
<td>98.0 ± 16.7</td>
<td>13</td>
<td>187.7 ± 20.8</td>
<td>9</td>
<td>**</td>
</tr>
</tbody>
</table>

A 2-way ANOVA (genotype, dose) for repeated measures (SCL, SNRT) revealed main effects of genotype \((F_{1,19} = 10.27, p=0.004)\) and Test \((F_{1,19} = 171.83, p < 0.0001)\) and a significant factorial interaction \((F_{1,15} = 21.99, p < 0.005)\), indicating that the genotype differences became more pronounced during SNRT despite the a priori lower SCL observed in KO. This could be confirmed by comparison of corrected SNRT (cSNRT) values.

Mean ± SEM * p< 0.05, ** p < 0.01 (t-test)
Supplemental Table 6A

Basal heart rate and after pharmaca injection

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>n (WT)</th>
<th>KO</th>
<th>n (KO)</th>
<th>rate of reduction in KO</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>basal</td>
<td>547.9 ± 10.0</td>
<td>3</td>
<td>459.6 ± 9.3</td>
<td>4</td>
<td>16.1 ± 1.7%</td>
<td>*</td>
</tr>
<tr>
<td>isoproterenol</td>
<td>743.5 ± 9.7</td>
<td>3</td>
<td>604.7 ± 12.4</td>
<td>4</td>
<td>18.7 ± 1.7%</td>
<td>*</td>
</tr>
<tr>
<td>carbachol</td>
<td>254.7 ± 35.6</td>
<td>3</td>
<td>233.3 ± 8.28</td>
<td>4</td>
<td>9.1 ± 1.4%</td>
<td>ns</td>
</tr>
</tbody>
</table>

*basal vs. isoproterenol*: A 2-way ANOVA (genotype, drug) for repeated measures (drug) revealed main effect of genotype ($F_{1,5} = 128.1$, $p < 0.0001$) and drug ($F_{1,5} = 247.3$, $p < 0.0001$), but no significant factorial interaction ($F_{1,5} = 6.0978$, $p > 0.05$). mean ± SEM  * p<0.05 (MWU test)

*basal vs. carbachol*: A 2-way ANOVA (genotype, drug) for repeated measures (drug) revealed main effect of genotype ($F_{1,5} = 9.6340$, $p = 0.0267$) and drug ($F_{1,5} = 226.1$, $p < 0.0001$), but no significant factorial interaction ($F_{1,5} = 4.077$, $p = 0.10$), indicating that carbachol was similarly efficient in KO and WT mice. mean ± SEM

Supplemental Table 6B

dynamic range and degree of heart rate regulation

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>n (WT)</th>
<th>KO</th>
<th>n (KO)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>dynamic range (max HR-min HR)</td>
<td>448.3 ± 22.9</td>
<td>3</td>
<td>426.0 ± 92.6</td>
<td>4</td>
<td>ns</td>
</tr>
<tr>
<td>degree of HR regulation (max HR/min HR)</td>
<td>2.3 ± 0.1</td>
<td>3</td>
<td>2.1 ± 0.2</td>
<td>4</td>
<td>ns</td>
</tr>
</tbody>
</table>

mean ± SEM  ns: not significant (t-test)
### Supplemental Table 7A

**Intracardiac Parameters of Sinoatrial Node Function**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>KO</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (WT)</td>
<td>n (KO)</td>
<td></td>
</tr>
<tr>
<td>SNRT₁₁₀</td>
<td>172.21 ± 15.23</td>
<td>282.30 ± 24.65</td>
<td>9</td>
</tr>
<tr>
<td>SNRT₁₀₀</td>
<td>185.38 ± 27.50</td>
<td>286.45 ± 20.77</td>
<td>10</td>
</tr>
<tr>
<td>SNRT₉₀</td>
<td>183.69 ± 30.63</td>
<td>287.19 ± 26.55</td>
<td>8</td>
</tr>
<tr>
<td>SNRT₈₀</td>
<td>169.50 ± 12.83</td>
<td>220.08 ± 34.96</td>
<td>7</td>
</tr>
<tr>
<td>cSNRT₁₁₀</td>
<td>43.45 ± 10.13</td>
<td>114.12 ± 17.65</td>
<td>10</td>
</tr>
<tr>
<td>cSNRT₁₀₀</td>
<td>53.23 ± 14.27</td>
<td>119.59 ± 11.78</td>
<td>11</td>
</tr>
<tr>
<td>cSNRT₉₀</td>
<td>50.98 ± 19.86</td>
<td>125.89 ± 14.79</td>
<td>10</td>
</tr>
<tr>
<td>cSNRT₈₀</td>
<td>51.14 ± 12.34</td>
<td>104.83 ± 17.08</td>
<td>8</td>
</tr>
<tr>
<td>SACT</td>
<td>16.10 ± 1.90</td>
<td>28.34 ± 2.38</td>
<td>10</td>
</tr>
<tr>
<td>SNERP</td>
<td>48.80 ± 3.10</td>
<td>51.20 ± 2.40</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>1</sup> Data were compared by 2-way ANOVA (genotype, pacing cycle) for repeated measures (pacing cycle), which revealed a significant main effect of genotype ($F_{1,11} = 7.711, p = 0.018$) as well as a genotype x pacing cycle interaction ($F_{3,33} = 3.7723, p = 0.0196$). In case of missing data, the whole data set was excluded from ANOVA, but included into post-hoc t-tests. P values were obtained by Newman-Keuls post-hoc test.

<sup>2</sup> 2-way ANOVA (genotype, pacing cycle) for repeated measures (pacing cycle) revealed a significant main effect of genotype ($F_{1,10} = 10.68, p = 0.008$).

- mean ± SEM * p< 0.05; ** p < 0.01; *** p < 0.001; ns: not significant

### Supplemental Table 7B

**beat-to-beat variability (Intracardiac electrophysiological study)**

<table>
<thead>
<tr>
<th>WT</th>
<th>n (WT)</th>
<th>KO</th>
<th>n (KO)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.98 ± 0.18</td>
<td>9</td>
<td>5.41 ± 1.26</td>
<td>11</td>
<td>*</td>
</tr>
</tbody>
</table>

mean ± SEM * p < 0.5 (t-test)
Supplemental Table 7C
Intracardiac Parameters of Atrioventricular Node Function

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>n (WT)</th>
<th>KO</th>
<th>n (KO)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVNERP&lt;sub&gt;100&lt;/sub&gt;</td>
<td>61.00 ± 3.80</td>
<td>9</td>
<td>57.09 ± 2.40</td>
<td>11</td>
<td>ns&lt;sup&gt;3)&lt;/sup&gt;</td>
</tr>
<tr>
<td>AVNERP&lt;sub&gt;80&lt;/sub&gt;</td>
<td>61.30 ± 3.49</td>
<td>9</td>
<td>58.36 ± 3.06</td>
<td>11</td>
<td>ns</td>
</tr>
<tr>
<td>AVNERP&lt;sub&gt;60&lt;/sub&gt;</td>
<td>62.20 ± 3.78</td>
<td>9</td>
<td>62.73 ± 2.48</td>
<td>11</td>
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<tr>
<td>WBP</td>
<td>73.00 ± 2.30</td>
<td>8</td>
<td>74.73 ± 1.69</td>
<td>11</td>
<td>ns</td>
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<tr>
<td>2:1 AV Block</td>
<td>61.50 ± 2.20</td>
<td>9</td>
<td>63.82 ± 1.25</td>
<td>11</td>
<td>ns</td>
</tr>
<tr>
<td>AVFRP</td>
<td>79.57 ± 2.81</td>
<td>8</td>
<td>81.5 ± 1.91</td>
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<td>ns</td>
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<tr>
<td>A2V2&lt;sub&gt;100&lt;/sub&gt;</td>
<td>41.75 ± 1.90</td>
<td>8</td>
<td>44.55 ± 1.66</td>
<td>11</td>
<td>ns&lt;sup&gt;3)&lt;/sup&gt;</td>
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<tr>
<td>A2V2&lt;sub&gt;80&lt;/sub&gt;</td>
<td>46.63 ± 2.73</td>
<td>8</td>
<td>51.09 ± 2.75</td>
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</table>

3) Confirmed by 2-way ANOVAs for repeated measurements, which failed to reveal significant genotype or genotype x pacing cycle interactions.

mean ± SEM  ns: not significant

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
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<tr>
<td>AVNERP</td>
<td>atrioventricular node effective refractory period</td>
</tr>
<tr>
<td>WBP</td>
<td>Wenckebachpoint</td>
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<tr>
<td>AVFRP</td>
<td>atrioventricular node functional refractory period</td>
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## Supplemental Table 7D

**Intracardiac Parameters of the refractory periods of atrium and ventricle**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>n (WT)</th>
<th>KO</th>
<th>n (KO)</th>
<th>p</th>
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<td>AERP&lt;sub&gt;100&lt;/sub&gt;</td>
<td>25.33 ± 3.04</td>
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<td>24.45 ± 2.70</td>
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<td>ns&lt;sup&gt;4)&lt;/sup&gt;</td>
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<td>AERP&lt;sub&gt;80&lt;/sub&gt;</td>
<td>27.14 ± 3.65</td>
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<td>24.33 ± 1.53</td>
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<tr>
<td>VERP&lt;sub&gt;100&lt;/sub&gt;</td>
<td>51.11 ± 2.95</td>
<td>9</td>
<td>49.64 ± 4.81</td>
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<td>ns&lt;sup&gt;4)&lt;/sup&gt;</td>
</tr>
<tr>
<td>VERP&lt;sub&gt;90&lt;/sub&gt;</td>
<td>51.56 ± 3.16</td>
<td>9</td>
<td>49.64 ± 4.23</td>
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<tr>
<td>VERP&lt;sub&gt;80&lt;/sub&gt;</td>
<td>50.89 ± 2.31</td>
<td>9</td>
<td>49.82 ± 3.98</td>
<td>11</td>
<td>ns</td>
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</table>

<sup>4)</sup> Confirmed by 2-way ANOVAs for repeated measurements, which failed to reveal significant genotype or genotype x pacing cycle interactions.

mean ± SEM; ns: not significant

<table>
<thead>
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<th>Parameter</th>
<th>Description</th>
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<td>AERP</td>
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<tr>
<td>VERP</td>
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Supplemental Figures

Supplemental Figure 1

Supplemental Figure 2
Supplemental Figure 3

A

\[ \text{II} \quad \begin{array}{ccccccc}
R & R & R & R & R \\
A1 & A1 & A2 & A3 & A4
\end{array} \]

HR\(_{A1}\) 

100 ms

B

zone of non reset

Sinus node

SA-atrial junction

Atrium

A1 A1 A2 A3 A4

zone of reset

Sinus node

SA-atrial junction

Atrium

A1 A1 A2 A3 A4

zone of interpolation

Sinus node

SA-atrial junction

Atrium

A1 A1 A2 A3 A4
Supplemental Figure 5

A

S1S2 = 100 ms

S1S2 = 80 ms

RVd

B

C

A1A2 [ms]

AVFRP
Figure Legends:

**Supplemental Figure 1** Time domain RR interval variability parameters of WT and HCN1<sup>−/−</sup>(KO) mice. (A) Mean RR interval. (B) Square root of mean of squared differences between successive normal RR intervals (RMSSD) and standard deviation (SD) of all normal RR intervals in sinus rhythm (SDNN) were calculated from 2 hours ECG recordings recorded during low activity period. Mean ± SEM; * p ≤ 0.05.

**Supplemental Figure 2** Spectral analysis of heart rate variability. (A) HRV determined during a phase of slow heart rate for WT and HCN1<sup>−/−</sup> (KO) mice. Results are illustrated for the total power spectrum (0 - 4 Hz; TP) and specific frequency bands, i.e. very low frequency band (0 - 0.4 Hz), low frequency (0.4 - 1.5 Hz; LF) and high frequency (1.5 - 4 Hz; HF). (B) HRV determined during a phase of high heart rate for WT and HCN1<sup>−/−</sup> mice. Results are illustrated as in (A). Mean ± SEM; * p ≤ 0.05.

**Supplemental Figure 3** Illustration of the premature atrial extra stimulation technique with examples of non reset and reset responses and interpolation. (A) Upper trace: lead II surface ECG, lower trace: proximal intraatrial lead. p represents the atrial complex, R the ventricular complex. A1A1 is the spontaneous sinus cycle length (SCL). A2 is the premature stimulus. A3 und A4 are the return and the post return beat, respectively. (B) Ladder diagrams illustrating different responses to atrial premature stimulation. Premature atrial excitation propagates in two directions, to the ventricles (not shown) and to the sinoatrial junction and the sinoatrial node. The response of the SAN depends on the timing of the premature stimulus. Very late premature A2 collide with the outgoing activation A1 from the preceding sinoatrial node impulse and do not penetrate the sinoatrial node. A3 therefore occurs in time (zone of non reset). More premature A2 enter and reset the timing of the sinoatrial node (zone of reset). This results in an earlier occurrence of the next sinoatrial node activation. A2A3 is larger than A1A1 (SCL) and stays relatively constant reaching a plateau. The A2A3 interval consists of SCL plus an antegrade and a retrograde sinoatrial conduction time (SACT; grey area). The antegrade and the retrograde components are assumed to be equal. Zone of interpolation: At very early premature stimuli sinoatrial entrance block may occur due to refractoriness of the tissue surrounding the sinoatrial node (sinoatrial junction). The impulse does not penetrate and reset the sinoatrial node. A3 is interpolated so that A2A3 are less than A1A1.

**Supplemental Figure 4** Determination of sinoatrial conduction time (SACT) by programmed premature atrial stimulation. The normalized return cycle (A2A3/A1A1) and post return cycles (A3A4/A1A1) are plotted as a function of the normalized coupling interval of the premature atrial stimulus (A1A2/A1A1). Each point represents one test cycle. The horizontal line represents the mean spontaneous sinus cycle length. The upper diagonal line represents the line of full compensatory pauses [A1A2+A2A3=2A1A1]; the lower diagonal line is termed line of complete interpolation [A1A2+A2A3=A1A1]. Zones of sinoatrial node responses to premature extrastimuli are indicated on top of the figure. Zone of non reset, zone of reset and zone of interpolation. SNERP: Sinus node effective refractory period. SACT: sinoatrial conduction time. For the S1S2 intervals below the AERP no response was obtained. For details see text.

**Supplemental Figure 5** Determination of atrioventricular conduction. (A) AV nodal conduction was analyzed by premature atrial stimulation. A train of 8 stimuli (S1) were applied at a S1S1 cycle length of 100 ms followed by a premature extrastimulus (S2) at decreasing S1S2 coupling interval. left panel S1S2 = 100 ms; right panel S1S2 = 80 ms; top trace: lead II of the surface ECG; bottom trace: the intracardia atrial electrogram. (B) The AV nodal conduction curves were generated by plotting V1V2 intervals or H1H2 intervals versus the A1A2 coupling interval of the premature atrial stimulus. From this graph the atrioventricular nodal functional refractory period (AVFRP) was determined as the shortest H1H2 or V1V2 interval. (C) The AV nodal latency curve was determined by plotting the A2V2 delay versus the A1A2 interval. S: pacing stimulus artifact. A: atrial electrogram. H: His bundle signals. V1 and V2: ventricular signals.
References:

1. Nolan MF, Malleret G, Lee KH, Gibbs E, Dudman JT, Santoro B, Yin D, Thompson RF, Siegelbaum SA, Kandel ER, Morozov A. The hyperpolarization-activated hcn1 channel is important for motor learning and neuronal integration by cerebellar purkinje cells. Cell. 2003;115:551-564


