The cardiac sodium–calcium exchanger NCX1 is a key player in the initiation and maintenance of a stable heart rhythm

Stefan Herrmann1*, Peter Lipp2, Kathrina Wiesen2, Juliane Stieber1, Huong Nguyen1, Elisabeth Kaiser2, and Andreas Ludwig1

1Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen D-91054, Germany; and 2Institut für Molekulare Zellbiologie, Medizinische Fakultät, Universitätsklinikum Homburg/Saar, Universität des Saarlandes, Homburg D-66424, Germany

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Aims

The complex molecular mechanisms underlying spontaneous cardiac pacemaking are not fully understood. Recent findings point to a co-ordinated interplay between intracellular Ca2+ cycling and plasma membrane-localized cation transport determining the origin and periodicity of pacemaker potentials. The sodium–calcium exchanger (NCX1) is a key sarcolemmal protein for the maintenance of calcium homeostasis in the heart. Here, we investigated the contribution of NCX1 to cardiac pacemaking.

Methods and results

We used an inducible and sinoatrial node-specific Cre transgene to create mice lacking NCX1 selectively in cells of the cardiac pacemaking and conduction system (cpNCX1KO). RT–PCR and immunolabeling experiments confirmed the precise tissue-specific and temporally controlled deletion. Ablation of NCX1 resulted in a progressive slowing of heart rate accompanied by severe arrhythmias. Isolated sinoatrial tissue strips displayed a significantly decreased and irregular contraction rate underpinning a disturbed intrinsic pacemaker activity. Mutant animals displayed a gradual increase in the heart-to-body weight ratio and developed ventricular dilatation; however, their ventricular contractile performance was not significantly affected. Pacemaker cells from cpNCX1KO showed no NCX1 activity in response to caffeine-induced Ca2+ release, determined by Ca2+ imaging. Regular spontaneous Ca2+ discharges were frequently seen in control, but only sporadically in knockout (KO) cells. The majority of NCX1KO cells displayed an irregular and a significantly reduced frequency of spontaneous Ca2+ signals. Furthermore, Ca2+ transients measured during electrical field stimulation were of smaller magnitude and decelerated kinetics in KO cells.

Conclusions

Our results establish NCX1 as a critical target for the proper function of cardiac pacemaking.

Keywords

Sinoatrial node • Cardiac pacemaking

1. Introduction

The complex nature of cardiac pacemaking is not fully understood. Pacemaker cells are characterized by their intrinsic capability to elicit spontaneous action potentials1,2, but the molecular mechanisms underlying this property are controversially discussed.3–7 One recent hypothesis explains pacemaker activity by the complex interaction between intracellular calcium cycling and sarcolemma-localized ion transport processes.8 The key mechanism of this ‘coupled-clock’ hypothesis is a rhythmic recurrent local calcium release, which activates the sodium–calcium exchanger (NCX1). The consequence of this activation is a net inward current (three Na+ in and one Ca2+ out), which depolarizes the membrane towards the threshold required for eliciting an action potential. Indeed, calcium imaging in isolated sinoatrial node (SN) cells revealed spontaneous calcium-induced local calcium releases, which occurred shortly before phase 0 of the action potential.9

However, this ‘coupled-clock’ concept is largely based on isolated cell experiments as well as computational and mathematical models,7–12 but whole-animal in vivo experiments are largely missing. Here, we investigated the role of the cardiac NCX1—a major actor in the proposed pacemaker concept—by conditional gene deletion. NCX1 is ubiquitously expressed in the whole heart. Conventional NCX1 mutants are
not viable and die during embryogenesis. For studying the role of NCX1 in cardiac pacemaking, it would be most advantageous to restrict gene deletion to cells of the cardiac conduction system. In addition, controlling the time point of induction of the mutation should minimize potential adaptation mechanisms. We therefore used a highly selective and inducible Cre transgene that allows temporally controlled recombination solely in cells of the cardiac conduction system. This Cre line was mated with floxed NCX1 mice. The resulting cpNCX1KO mice developed severe bradycardia and lost their capability of maintaining a stable heart rhythm as demonstrated by various supraventricular and ventricular rhythm disorders, including SN arrhythmia, SN pauses, atrioventricular (AV) block, and ventricular tachycardia. The efficient deletion of NCX1 was demonstrated by single-cell immunolabeling and functional calcium imaging. The calcium transients of cpNCX1KO were altered significantly, and regular spontaneous activity was greatly reduced demonstrating the critical contribution of the NCX1 to cardiac pacemaking.

2. Methods
For further experimental details, see Supplementary material online.

2.1 Experimental animals
Mice with a pacemaker and conduction system-specific deletion of NCX1 were generated by crossing floxed NCX1 mice with animals carrying a HCN4 (hyperpolarization-activated, cyclic nucleotide-gated cation channel 4) promoter driven and tamoxifen (tam) inducible Cre transgene. Lines were first backcrossed to a C57BL/6 background for six generations. The resulting NCX1 flox/flox, HCN4 CreER.T2/+ animals were designated as cpNCX1KO. Recombination of the floxed NCX1 gene was induced by intraperitoneal (i.p.) injection of tam at a dosage of 40 mg/kg for 5 consecutive days. Non-induced cpNCX1KO or tam-induced NCX1 flox/flox, HCN4 +/+ animals were chosen as control groups. The investigation conforms to the Directive 2010/63/EU of the European Parliament and was approved by the local regulatory authority (Regierung von Mittelfranken).

2.2 Echocardiography
Animals were investigated 8–9 weeks after tam injection by using a Vevo 770 (Visualsonics). Anaesthesia was induced by 3% isoflurane and continued with 1.5% isoflurane.

2.3 ECG recordings
In isoflurane anaesthetized mice, electrocardiogram (ECG) signals were recorded by using needle electrodes. The adequate depth of anaesthesia was determined by a negative toe-pinch reflex. In conscious mice, telemetric ECG recordings were done by using radiotelemetric ECG transmitters. Mice were injected i.p. with isoproterenol (1 mg/kg), atropine (1 mg/kg), propranolol (5 mg/kg), and 2-chloro-N-cyclopentyladenosine (0.3 mg/kg). Heart rates were continuously monitored by telemetry, and rates 1 h before vs. 1 h after drug injections were compared.

2.4 Isolation of SN cells
Mice were sacrificed by cervical dislocation. Hearts were quickly removed and right atrial wall containing the SN was pinned to an agarose block and placed in a chamber perfused with carbogen-gassed Tyrode solution (35°C). Spontaneous tissue contractions were recorded by a digital camera.

2.5 Calcium imaging
Isolated SN cells plated on coverslips were loaded with fura2 and placed on the stage of a video imaging system. Three protocols were employed: (i) assessment of spontaneous activity (ii) steady-state behaviour was investigated by electrical field stimulation; (iii) sarcoplasmic reticulum (SR) Ca2+ content and NCX activity were analysed with brief caffeine pulses (10 mM). The amplitudes of Ca2+ transients were depicted as ΔFura2 ratio, which is defined as the difference between the diastolic and peak fura2 ratio while the decay was characterized by fitting a monoeponential decay to the recovery phase. Time-to-peak values were measured by analysing the time between end diastole and the peak of the Ca2+ signal. All experiments were performed at 37°C.

2.6 In vitro SN contraction rate
Mice were sacrificed by cervical dislocation. Hearts were quickly removed and right atrial wall containing the SN was pinned to an agarose block and placed in a chamber perfused with carbogen-gassed Tyrode solution (35°C). Spontaneous tissue contractions were recorded by a digital camera.

2.7 Immunofluorescence
Cryosections were fixed in 4% paraformaldehyde and permeabilized by 0.1 Triton X-100. Isolated SN cells were plated onto polylysine-coated slides and fixed with 4% paraformaldehyde. Cells were co-incubated with polyclonal HCN4 and monoclonal NCX1 antibody followed by secondary antibodies. Images were acquired using a Zeiss LSM 5 Pascal confocal microscope.

2.8 RT–PCR and western blot
Isolation of total RNA and quantitative RT–PCR analyses were done as described. For western analysis, tissue extracts were SDS–PAGE separated and probed with the following primary antibodies: anti-NCX1, anti-SERCA, anti-PMCA, anti-PLN, and anti-PLN-phospho-Ser-16.

2.9 Statistical analysis
All experiments and analyses were blinded to the experimental group. Data are presented as mean ± SEM. Data were tested for normal distribution by using the D’Agostino–Pearson omnibus normality test. Comparisons were carried out by using the Mann–Whitney or unpaired t-test. A P-value of <0.05 was considered statistically significant with *P < 0.05, **P < 0.01, ***P < 0.001.

3. Results

3.1 Conditional deletion of NCX1 in mice
We crossed floxed NCX1 animals with mice expressing the tam-inducible Cre recombinase CreER.T2 driven by the endogenous HCN4 promoter. This transgene allows temporally controlled and highly selective recombination in cells of the cardiac pacemaking and conduction system. Recombination was examined by RT–PCR in several cardiac compartments including atria, ventricle, SN, and atrioventricular node before and after tam treatment (Figure 1A, top and Supplementary material online, Figure S1). Knockout (KO) mRNA was detected exclusively in tissues isolated from the pacemaker area, whereas no recombination was observed in the ventricles and atria. In uninduced mutants, no recombination events were detected in any tissues examined.

Immunoblots of SN tissue showed a large decline of the NCX1 expression level after Cre induction (Figure 1A, bottom). Immunohistochemistry revealed that NCX1 was absent from the pacemaker centre (as delineated by HCN4 staining) of induced mutants, whereas the expression of NCX1 in the surrounding atrial tissue was not affected (Figure 1B). To investigate the specificity of the KO in more detail, we performed NCX1/HCN4 co-immunolabeling on isolated myocytes using HCN4 as a marker for pacemaker cells (Figure 1C). In uninduced mutants, virtually all cells from atrial tissue (including SN) displayed a clear NCX1 signal. Both HCN4 and NCX1 staining were mostly...
restricted to the plasma membrane, and co-immunostaining revealed that all pacemaker cells also expressed the NCX1 (Figure 1C, left). In contrast, the tam−cpNCXKO lacked NCX1 expression selectively in SN cells, whereas atrial cells still displayed NCX1 staining (Figure 1C, right). Taken together, these data confirm the highly specific and inducible deletion of NCX1 in the pacemaker system of cpNCX1KO animals.

3.2 Ablation of NCX1 results in severe bradycardia and arrhythmias

Then, we investigated if the lack of NCX1 in pacemaker cells has any effect on the cardiac rate. ECGs were measured twice a week before, during, and after tam treatment in anaesthetized mutants (NCX1 L2/L2, HCN4 KiTCre/+) and also in litter-matched controls (NCX1 L2/L2; +/+). The heart rate of mutants decreased continuously over 5 weeks and remained permanently low afterwards (Figure 2A). Minimal heart rates attained were 227 ± 4 bpm in mutants (n = 12) when compared with 448 ± 7 bpm in controls (n = 12, P < 0.001, Figure 2B). In addition, we determined ECGs in freely moving animals by telemetry and observed a similar progression and amount of bradycardia (Supplementary material online, Figure S2). The circadian modulation of the heart rate was preserved in mutants, but mean rates were significantly reduced reaching 310 ± 14 and 270 ± 16 bpm (n = 9) during night and day, respectively (Figure 2C and Supplementary material online, Figure S2).

We continued with analysing the spontaneous contraction of isolated tissue strips from the SN area prepared 5 weeks after tam treatment. As shown in Figure 2D, the contraction rate of SN tissue from cpNCXKO was reduced by ~45% when compared with controls (see Supplementary material online, Movies for representative examples). This value is similar to the whole-animal ECG data and suggests that the severe bradycardia of mutants is indeed caused by a depressed intrinsic pacemaker activity.

In cpNCXKO animals, we noticed a progressive increase in RR interval variability beginning as soon as 1 week after tam treatment (Supplementary material online, Figure S3A). Different types of rhythm disorders were observed (Figure 3). Most commonly, arrhythmias arose from the improper discharge of the primary pacemaker centre as indicated...
by SN pauses and SN dysrhythmias. Occasionally, ventricular escape rhythms with a complete lack of P-waves were observed suggesting a complete cessation of pacemaker activity (Figure 3B). Consistent with the fact that the HCN4KiT-Cre transgene shows recombinase activity in the whole cardiac pacemaking and conduction system including AV node (Supplementary material online, Figure S1),17,20 arrhythmias were not restricted to the SN. KOs developed a moderate prolongation of the PR interval (Supplementary material online, Figure S3B and C) and second-degree AV block with or without additional SN pauses were commonly found (Figure 3C). Sporadically, severe long-lasting ventricular tachyarrhythmias were observed (Figure 3D).

### 3.3 Cardiac remodelling in cpNCX1KO

We noticed a gradual increase in heart weight and size in cpNCX1KO animals (Figure 4A). Heart-to-body weight ratios determined at 1, 2 and 3 months after Cre induction were significantly augmented by 7, 15 and 67%, respectively. In addition, we analysed the expression levels of the hypertrophic marker atrial natriuretic factor (ANF), which is released from myocytes in response to cardiac distention (Figure 4B). Both ventricles of cpNCX1 KO displayed a progressive increase in ANF expression ranging from around two-fold at 1 month to more than 10-fold at 3 months after induction. Echocardiographic analysis of mice 2 months after induction revealed that cpNCX1KO animals had no impairment in systolic function (Figure 4C and Supplementary material online, Table S1). Fractional shortening and ejection fraction were unchanged indicating a proper contractile performance. Nevertheless, the diameter and mass of the left ventricle were significantly enlarged, whereas the thickness of the interventricular septum and walls were unaltered. Therefore, the increased heart-to-body weight ratio (HBR) and augmented ANF expression are probably a result of cardiac dilatation in response to an excessively decelerated heartbeat.

### 3.4 Expression of calcium handling proteins in the sinoatrial node

We analysed whether the lack of NCX1 resulted in an altered expression of other SN proteins involved in Ca$^{2+}$ handling (Figure 4D–F). Besides NCX1, the plasma membrane calcium ATPase (PMCA) represents the main mechanism for extruding Ca$^{2+}$ out of cells.21 We found no significant adaptation of PMCA protein levels in cpNCX1KO (Figure 4E and F). In contrast, expression of the sarcoplasmic reticular calcium ATPase SERCA2 was increased 2.6-fold (Figure 4D and F), while the phosphorylation level of the SERCA regulator phospholamban was reduced by ~65% (Figure 4E and F). We found no significant change in the expression of several other genes involved in pacemaking and calcium handling, including HCN4, voltage-gated Ca$^{2+}$ channels...
(Ca, 1.2, Ca, 1.3, and Ca, 3.1), and ryanodine receptors (RyR2 and RyR3) as examined by qRT–PCR (Supplementary material online, Figure S4). In addition, we measured voltage-dependent calcium currents in isolated SN cells by patch-clamp recordings (Supplementary material online, Figure S5). We detected no significant difference in the calcium current amplitude and current density.

### 3.5 Heart rate modulation in cpNCX1KO

To explore the effects of the NCX1 loss on heart rate modulation, different signal transduction pathways were manipulated by pharmacological means and heart frequency responses were recorded by telemetry (Figure 5). The β-adrenergic agonist isoproterenol caused a rise in heart rate by ~40% in both KOs and controls (Figure 5A and C). Although this relative increase was similar between genotypes, the maximal value attained in KOs was significantly lower (435 ± 25 bpm, n = 5 for KOs when compared with 662 ± 3 bpm, n = 5 for controls, P < 0.001). In addition, the duration of the response was much shorter lasting only 40 min in KOs when compared with 80 min in controls. Moreover and in contrast to controls, block of the β-adrenergic stimulatory drive by propranolol showed no effect on the rate of KOs. Taken together, these results point to an intrinsic inhibition of the β-adrenergic signalling pathway in mice-lacking NCX1.

We also investigated the heart rate response to compounds acting on inhibitory Gi-mediated transduction pathways. The A1 adenosine receptor agonist 2-chloro-N-cyclopentyladenosine (CCPA) (Figure 5B and C) caused a decrease of heart rate in both genotypes, but KOs displayed an overshooting response with rates <50 bpm for nearly 4 h. In addition, blocking muscarinic stimulation by atropine increased rates only in KOs (Figure 5C).

Taken together, these experiments suggest an imbalance between stimulatory and inhibitory cardiac transduction pathways in NCX1 KO s. The responsiveness to drugs acting on stimulatory Gs-coupled signalling pathways was reduced, whereas the effect of compounds acting on Gi-coupled inhibitory signalling was increased.

### 3.6 Ca2+ measurements in isolated pacemaker cells

NCX1 is known to play a fundamental role in extruding Ca2+ out of ventricular myocytes, but its relevance for Ca2+ removal in pacemaker cells has not been investigated directly so far. We performed Ca2+ imaging of fura2-loaded SN cells that were identified by morphological characteristics and spontaneous activity (Figure 6). SR Ca2+ content and cytosolic Ca2+ elimination were examined by brief pulses of caffeine (10 mM, Figure 6A). The amplitude of the caffeine-evoked Ca2+ transients was not significantly different between KO and control cells, indicating a similar SR Ca2+ content (Figure 6B, top). Since SR refilling is prevented in the presence of caffeine, any decline in intracellular Ca2+ during caffeine application must be due to sarcolemmal Ca2+ transport. While control cells displayed a rapid recovery to resting Ca2+ levels, myocytes from cpNCX1KO mice depicted an almost abrogated Ca2+ extrusion (Figure 6A). Statistical analysis (n = 75 control and n = 43 KO cells) revealed that KO myocytes indeed had a dramatically reduced Ca2+ extrusion (Figure 6B, top). Ca2+ removal of atrial cells was not significantly different between genotypes (data not shown), confirming the tissue selectivity of the NCX1 deletion.

Ca2+ transients concomitant with action potentials of spontaneously active pacemaker cells (n = 94 control and n = 53 KO cells) were imaged, and the resulting traces were classified into regular rhythmic,
irregular arrhythmic, or sporadic calcium discharge pattern (see Figure 6C for representative traces). The portion of myocytes with arrhythmic spontaneous activity was prominently increased in cells from KO animals when compared with control (Figure 6D). In addition, KO cells displayed a substantially reduced overall frequency of spontaneous Ca$_{2+}$ transients (Figure 6E). These results mirror data from the whole-animal studies where cpNCX1 KO mice displayed a reduced heart rate and a frequent loss of regular rhythm generation. We also found significantly altered key parameters of Ca$_{2+}$ handling in isolated KO cells including a reduced resting Ca$_{2+}$ level, a reduced peak amplitude

**Figure 5** Drug-induced heart rate modulation is altered in cpNCX1 KO. Heart rates of controls (n = 5) and cpNCX1 KO (n = 5) were continuously monitored by telemetric ECG recordings. (A) Isoproterenol-induced heart rate modulation over time. Isoproterenol (1 mg/kg, i.p.) was injected at t = 0. (B) CCPA-induced heart rate modulation. CCPA (0.3 mg/kg) was injected i.p. at t = 0. (C) Averaged heart rates of indicated genotypes 1 h before vs. 1 h after drug injection. Propranolol and atropine were injected i.p. using 5 and 1 mg/kg, respectively.

**Figure 6** Altered global Ca$_{2+}$ handling and dysregulated Ca$_{2+}$ oscillations in cpNCX1 KO SN cells. (A) Representative Ca$_{2+}$ transients from a control and KO SN cell with brief application of caffeine (10 mM). (B) Amplitude (top) and decay time (bottom) of the caffeine-evoked Ca$_{2+}$ transients. KO (n = 43 cells) and control (n = 75 cells) are indicated by black and white columns, respectively. (C) Spontaneous activity of fura2-loaded SN myocytes was classified into rhythmic, arrhythmic, or sporadic activity. (D) Distribution of the discharge patterns observed in KO (n = 53) and control (n = 94) cells. (E) Properties of spontaneous transients including Ca$_{2+}$ resting level (rest), peak amplitude (ampl), time-to-peak, and frequency. (F) Representative electrically evoked (1 Hz) Ca$_{2+}$ transients from a control (top) and KO (bottom) cell. Field stimulations are indicated by black triangles. (G) Amplitude (ampl), diastolic Ca$_{2+}$ levels (diastolic) and decay time of electrically evoked Ca$_{2+}$ transients determined under steady-state conditions.
control and cpNCX1KO mice. The decay rate in cells from NCX1-dependent component when comparing myocytes from Ca2+ reduced recovery speed (increased decay time constant, s)ients with monoexponential decay functions, we found a significantly modify the shape and amplitude of the Ca2+ time-to-peak (@). Interestingly, when fitting the decay periods of the Ca2+ transients with monoeXponential decay functions, we found a significantly reduced recovery speed (increased decay time constant, Figure 6G) for Ca2+ transients from KO mice when compared with control cells. Taken together, these data support our conclusion that Ca2+ handling is majorly altered in cpNCX1KO SN cells. These alterations not only modify the shape and amplitude of the Ca2+ transients, but most importantly also the frequency of spontaneous Ca2+ transients and their rhythmicity.

4. Discussion

In this report, we studied the impact of the NCX1 on cardiac pacemaking. NCX1 was genetically ablated in a temporally controlled and tissue selective manner. The inducibility of the genetic system we used was of particular advantage, since intracellular Ca2+ is, among other functions, involved also in gene transcription and cellular differentiation. Hence, the acute deletion in the adult organism should minimize potential genetical and developmental compensatory mechanisms. In addition, the genetic manipulation was strictly restricted to the pacemaking and conduction system without affecting atrial and ventricular myocytes. We validated the loss of NCX1 by RT–PCR, immunofluorescence, and functional Ca2+ imaging. The severe bradycardia and rhythm disturbances observed in the whole-animal studies were replicated by the decelerated and arrhythmic contraction rate of isolated nodal tissue strips. Furthermore, we established a significant reduction in diastolic Ca2+ levels were decreased and the decay was slowed down. The loss of the NCX1 function in pacemaker cells was not compensated for by an altered expression of the PMCA and/or voltage-gated Ca2+ channels. We found a significant increase in the expression of the SR Ca2+-ATPase SERCA2, but this increase in expression was not translated into an amplified Ca2+ sequestration into the SR. The contrary was the case since electrically evoked Ca2+ transients displayed an almost three-fold slower decay. The activity of SERCA is inhibited by phospholamban and this inhibition is relieved upon phospholamban phosphorylation. Therefore, the significant relieved the phosphorylation status of phospholamban, which was observed in SN preparations of NCX1 mutants, could help explain reduced SERCA activity even in the presence of increased SERCA expression.

We hypothesize that the deletion of NCX1 interferes with the protein kinase A (PKA)-dependent phosphorylation status of pacemaker cells. One of the consequences would be a reduced PKA-mediated phosphorylation of phospholamban, which might be compensated by an increased expression of SERCA2 to preserve Ca2+ uptake.

The pharmacological in vivo data support the idea of a diminished PKA activity in cpNCX1KO cells. We found an increased responsiveness to ligands acting through Gi protein-coupled receptors and a limited response to those acting on Gs-coupled receptors, these signalling pathways result in PKA inactivation and activation, respectively. A reduced basal PKA phosphorylation status of cpNCX1KO pacemaker cells shifts cellular activity from stress to rest and may lead to an imbalance between stimulatory and inhibitory signalling transduction pathways. This could explain the observed shortened β-adrenergic response and the increased effect of compounds acting via adenosinergic and muscarinic receptors.

How could the deletion of NCX influence phosphorylation? In comparison with other cardiac cell types, pacemaker cells display an elevated cyclic AMP level and a pronounced basal PKA activity, suggesting that a high basal phosphorylation level is of special importance to pacemaker cell function.22,23 The high intracellular cyclic AMP is attributed to the constitutive activity of calcium-activated adenylyl cyclases (AC type 1 and 8), which was found to be selectively expressed in SN cells.22,24 We show that pacemaker cells lacking NCX1 display reduced, decelerated, and disrhythmic Ca2+ oscillations. This may reduce basal AC activity resulting in a reduction of intracellular cyclic AMP and consequently, diminished PKA-dependent phosphorylation.

Recently, a report describing global myocardial conditional NCX1KO mice appeared.25 These mice showed a blunted heart rate response to isoproterenol, but basal heart rate and rhythm as well as cellular Ca2+ handling were unchanged. The obvious discrepancies between this study and our findings are surprising given the fact that the same floxed NCX1 transgene was used. However, there are also important differences concerning the mouse model and the study design.

Gao et al. used the inducible Mer-CreMer transgene, which principally leads to recombination in all cardiac myocyte cell types. We used a SN and conduction system-specific Cre line. Therefore, we are confident that our phenotype is directly related to the ablation of NCX1 in the pacemaking system and is not influenced by potential effects resulting from recombination outside this system. Furthermore, it was shown in this and previous studies22,25 that the Cre line we used exhibits high recombination efficiency throughout the whole SN. It is assumed that a small fraction of SN cells (~1%) suffices to build the functional leading
pacemaker site.\textsuperscript{26} In contrast, the efficiency of the MerCreMer line in the SN is not clear. In a previous study,\textsuperscript{27} we found an incomplete recombination efficiency of the MerCreMer transgene in the SN, which resulted in a blunted phenotype when compared with complete gene deletion. Hence, it is quite possible that a mosaic patterned NCX1 deletion included not enough nodal cells to elicit a more pronounced phenotype.

Furthermore, Gao et al. reported that even at the single-cell level NCX1 deletion was incomplete. About 20\% of the control-level NCX current was retained in ‘KO pacemaker cells’, which is a remarkable observation given that Cre-mediated recombination is an all-or-none process. Therefore, NCX exchange function should be completely abolished in an individual cell and not only reduced. We can only speculate why, at least in some experiments, cellular NCX deletion was not fully achieved. Maybe the time interval between induction and analysis chosen was too short with regard to the turnover of targeted protein. An example for a typical protein turnover was recently shown by Broudz et al.\textsuperscript{28} In this study, the MerCreMer transgene was used to examine the function of RyR2 in cardiac cells. Four days after tam treatment, protein was decreased just by 50\% and declined further in the following days. In general, we wait at least 1 month after tam treatment before cellular and in vivo functions were analysed to make sure that the protein turnover was fully accomplished. Possibly the remaining fraction of NCX observed by Gao et al. is sufficient for maintaining the resting heart rate and cellular calcium handling and explains why our mouse model showed a more pronounced phenotype at the cellular and whole body level.

Our findings are also supported by other studies, which show the fundamental importance of NCX1 to pacemaking in isolated SN cells by the use of a pharmacological block.\textsuperscript{29,30} In addition, a recent abstract appeared describing an atrial-specific constitutive NCX1 deletion with a reduced and disturbed heart rate,\textsuperscript{31} which is in general agreement with our results.

Concluding remarks

At this point, we cannot provide a precise cellular mechanism explaining NCX function for cardiac pacemaking in detail. Future experiments must address subcellular Ca\textsuperscript{2+} signalling by high-speed confocal imaging, patch clamp for a detailed electrophysiological investigation, and a combination of both to foster our understanding of the exact interplay of mathematical models. The present study presents in vivo data showing the importance of NCX1 to cardiac pacemaking and provides cellular correlates and explanations for such findings. We revealed that a proper pacemaker function is not possible without NCX1 activity. We can only speculate about the relevance of NCX1 for pacemaking in other species including humans given, e.g. the difference in heart rate. Nevertheless, the findings of this study may also be of clinical interest. Recently, NCX was discussed intensively as a promising target for the treatment of ventricular arrhythmias during cardiac hypertrophy and failure. As a consequence of cardiac disease, abnormal or enhanced cytosolic calcium levels in ventricular myocytes may increase NCX activity generating a depolarizing current. Occurring at the resting membrane potential, this leads to delayed afterdepolarizations, at the plateau of the action potential this promotes early afterdepolarizations. Therefore, blocking NCX might be beneficial in an attempt to reduce the incidence of extrasystoles and ventricular tachyarrhythmias in heart failure. On the other hand, our data suggest that a complete block of NCX in nodal cells may trigger severe arrhythmias challenging the concept of NCX as an antiarrhythmic drug target.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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References

8. Lagatta EK, Meltsv VA, Vinogradova TM. A coupled SYSTEM of intracellular Ca\textsuperscript{2+} clocks and surface membrane voltage clocks controls the timekeeping mechanism of the heart’s pacemaker. Circ Res 2010;106:659–673.