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Triggered activity in atrial myocytes is influenced by $\text{Na}^+/\text{Ca}^{2+}$ exchanger activity in genetically altered mice

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Introduction

Triggered activity is an initiator of cardiac arrhythmia. It results from spontaneous Ca^{2+} release events leading to a sudden elevation of subsarcolemmal Ca^{2+} concentration. Previous studies have described increased function and in some cases increased protein levels of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) as part of the molecular remodeling process in tissue from patients or animal models with atrial fibrillation[1–5].

NCX is a transmembrane exchanger protein that generates an electrical inward current during systolic sarcoplasmic Ca^{2+} release by extruding 1 Ca^{2+} ion in exchange for 3 Na^+ ions. During a diastolic spontaneous sarcoplasmic Ca^{2+} release event, NCX becomes active generating a transient inward current that may form the underlying mechanism for a delayed afterdepolarization (DAD) of the resting membrane potential (for review see[6]). This again may initiate a spontaneous action potential (sAP) which may then generate an atrial extrasystole initiating atrial arrhythmias.

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Conflict of interest

None.

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Currently, it is unknown whether in atrial myocytes, increased NCX function alone may be sufficient to promote atrial triggered activity – independent of other molecular or structural remodeling processes associated for example with atrial fibrillation. It is also unclear, whether - vice versa - reduced NCX expression may inhibit atrial triggered activity. We therefore investigated proarrhythmic mechanisms in atrial myocytes from NCX-overexpressor (OE) and heterozygous knockout (KO) mice that do not show evidence for arrhythmic or structural heart disease.

Methods

All methods listed below are described in detail in the online supplement.

Mouse models with altered NCX expression

Generation of the homozygous NCX-overexpressor (OE) and the heterozygous NCX knockout (KO) mouse model has been described previously[7, 8]. Adult male (54.7 %) and female (45.3 %) mice were used for experimentation. All mice were 7.5 – 12.5 weeks of age.

Cell surface area

Microscopic assessment of the cell surface area was performed via planimetric quantification in non-fixed isolated atrial myocytes.

Masson-Goldmann fibrosis staining

The extent of myocardial collagen deposit was quantified with Masson-Goldmann fibrosis staining.

Immunoblot analysis

The protocol for immunoblot analysis and the quantification related to the housekeeping protein calsequestrin, was conducted as previously reported with minor modifications[9, 10].

Immunofluorescence stainings

The cellular distribution pattern of NCX was analyzed in OE vs. WT_{OE} by immunofluorescence stainings in fixed isolated atrial myocytes using a NCX-specific antibody as previously reported[11]. Immunofluorescence staining was not applied to assess overall protein amount per cell.

Isolation of atrial cardiomyocytes

Atrial myocytes were isolated using a protocol previously described by Lemoine et al.[12] with minor modifications. Investigated cells were rod-shaped and exhibited an intact plasma membrane with a prominent cross-striation in absence of major intracellular inclusions or deposits.

Cellular electrophysiology

The patch-clamp setup and technical aspects of the measurement have been described previously [9]. Experimental protocols and solutions used for voltage- and current-clamp recordings are given in the online supplement.

Measurement of the cellular Ca^{2+} transient

An Olympus IX50 microscope was connected to a dual-emission microfluorescence system (Photon Technologies Inc., South Brunswick, USA). Data was acquired and processed with Felix 1.42 (Photon Technologies Inc., South Brunswick, USA) as described previously[9]. Solutions and experimental protocols are given in the online material supplement.

Statistics

Data are expressed as mean $\pm$ standard error of the mean (SEM). Student's unpaired two-tailed t-test was used for direct comparison between the OE vs. the corresponding wild-type (WT_{OE}) or KO vs. the corresponding wild-type (WT_{KO}) mouse lines. Whenever the normality test failed in data sets, statistical analyses were performed using the Mann-Whitney Rank Sum test. Repeated measures were calculated using a Two Way ANOVA test followed by a Holm-Sidak post hoc test. To calculate rates and proportions, the z-test was applied as indicated with the data. A p-value < 0.05 was considered to be statistically significant.

Results

KO and WT_{KO} animals are bred on mixed genetic background (C57BL/6 and CD-1) while OE and WT_{OE} are on a C57BL/6 background. Differences in electrophysiological properties between WT mouse lines based on different genetic backgrounds have been described previously[13]. This was also the case in our models, where some parameters of cellular electrophysiological and Ca^{2+} handling properties were significantly different between WT_{OE} and WT_{KO} . These are given in the online data supplement (table 1). Thus, for all following experiments, direct quantitative/statistical comparisons were only performed within the same genetic background i.e. OE vs. WT_{OE} or KO vs. WT_{KO} .

Macroscopic and cellular characteristics of atrial myocardium

Microscopic analysis revealed no differences in structure or the cell surface area between OE vs. WT_{OE} (cell surface area in μm^2 ; OE: 1190 ± 44 ; $n=81$ cells / 3 animals; WT_{OE} : 1169 ± 51 ; $n=84$ cells / 3 animals; $p>0.05$). Cell surface area was also unaltered between KO vs. WT_{KO} (cell surface area in μm^2 ; KO: 1296 ± 37 ; $n=84$ cells / 3 animals; WT_{KO} : 1351 ± 52 ; $n=84$ cells / 3 animals; $p>0.05$) (online supplement, Fig. 1A and C). There was no difference in the atrial weight to body weight ratio in OE vs. WT_{OE} (atrial weight/body weight multiplied by $1 \cdot 10^3$; OE: 0.28 ± 0.01 ; $n=8$ animals; WT_{OE} : 0.29 ± 0.01 ; $n=11$ animals). The atrial weight to body ratio was also unaltered in KO vs. WT_{KO} (atrial weight/body weight multiplied by $1 \cdot 10^3$; KO: 0.29 ± 0.03 ; $n=8$ animals; WT_{KO} : 0.27 ± 0.01 ; $n=8$ animals; $p>0.05$) (online supplement, Fig. 1B). Masson-Goldmann fibrosis staining exhibited a similar extent of collagen deposit and no evidence for atrial fibrosis in OE vs. WT_{OE} (fibrosis in %; OE: 1.85 ± 0.50 ; $n=4$ animals; WT_{OE} : 2.36 ± 0.67 ; $n=4$ animals; $p>0.05$). There was also no

evidence for an altered extent of collagen deposit in KO vs. WT_{KO} (fibrosis in %; KO: 1.49 ± 0.52 ; n=4 animals; WT_{KO}: 1.68 ± 0.18 ; n=4 animals; $p > 0.05$) (online supplement, Fig. 2A and B). Electrical cell capacitance obtained during patch-clamp experiments was analyzed as a potential hypertrophy marker. Cell capacitance was unaltered between OE vs. WT_{OE} (cell capacitance in pF; OE: 63.5 ± 2.7 ; n=103 cells / 23 animals; WT_{OE}: 59.5 ± 1.9 ; n=106 cells / 25 animals; $p > 0.05$). Cell capacitance was also similar in KO vs. WT_{KO} (cell capacitance in pF; KO: 67.7 ± 2.8 ; n=70 cells / 16 animals; WT_{KO}: 62.0 ± 4.0 ; n=61 cells / 16 animals; $p > 0.05$) (online supplement, Fig. 1D).

Expression of NCX and key Ca²⁺ handling proteins

Immunoblot analyses were performed to evaluate the protein expression level of NCX and other key Ca²⁺ handling proteins (Fig. 1A). NCX expression was confirmed to be reduced to 64 ± 8 % in KO compared to WT_{KO} (n=8 homogenates; $p < 0.05$). Conversely, NCX expression was increased to 196 ± 29 % in OE vs. WT_{OE} (n=8 homogenates; $p < 0.05$). Junctin (Jcn) expression was increased to 181 ± 16 % in OE vs. WT_{OE} (n=8 homogenates; $p < 0.05$). Junctin expression was unaltered in KO compared to WT_{KO}. Protein levels of calsequestrin (Csq), triadin (Trd), sarco(-endo)plasmic reticulum Ca²⁺ ATPase (SERCA), troponin I (Tni), phospholamban (Plb), ryanodine receptor (RyR), Ser2808 phosphorylated ryanodine receptor (RyRp) and the alpha 1C subunit of the voltage-gated L-type Ca²⁺ channel (Ca_v1.2) were unaltered between KO vs. WT_{KO} (n=8 homogenates). Also, there were no changes in the expression levels of these proteins in OE vs. WT_{OE} (n=8 homogenates).

Measurement of NCX mediated Ca²⁺ extrusion capacity and SR Ca²⁺ load

NCX activity was assessed in isolated atrial cardiomyocytes loaded with fluo-4-AM via rapid caffeine (10 mM) application. As described previously[14] the velocity of the decay of the caffeine-induced Ca²⁺ transient correlates with NCX inward mode activity. The time to decay to 50 % (T₅₀) of the peak amplitude of the caffeine-induced Ca²⁺ transient was shortened in OE vs. WT_{OE} confirming increased NCX activity (T₅₀ in s: OE: 3.1 ± 0.7 ; n=23 cells / 5 animals; WT_{OE}: 6.2 ± 0.9 ; n=23 cells / 4 animals; $p < 0.05$; Fig. 1B and C). Conversely, T₅₀ was increased in KO vs. WT_{KO} confirming reduced NCX activity (T₅₀ in s: KO: 8.2 ± 0.9 ; n=22 cells / 3 animals; WT_{KO}: 4.5 ± 0.5 ; n=24 cells / 3 animals; $p < 0.05$; Fig. 1B and C). The peak amplitude of the caffeine-induced Ca²⁺ transient was unaltered in OE vs. WT_{OE} suggesting unaltered Ca²⁺ load of sarcoplasmic reticulum (SR) (F/F₀; OE: 2.8 ± 0.3 ; WT_{OE}: 3.4 ± 0.3 ; $p > 0.05$; Fig. 1D). The peak amplitude of the caffeine-induced Ca²⁺ transient was also unaltered in KO vs. WT_{KO} (F/F₀; KO: 3.50 ± 0.21 ; WT_{KO}: 3.80 ± 0.23 ; $p > 0.05$; Fig. 1D).

Occurrence of DADs and spontaneous APs

To evaluate the proneness towards arrhythmia, patch-clamp experiments in isolated atrial cardiomyocytes were conducted in the current-clamp mode. Cells were subjected to a stimulation protocol (as reported previously with minor alterations[9]) containing abrupt changes in pacing cycle lengths (20 sweeps each; in Hz: 1, 0.5, 0.25, 0.125) followed by 25 repetitions of a step containing 5 sweeps of 5 Hz followed by a short-coupled sweep after 50 ms and 4 s of rest. The short-coupled S2 stimulus was initially designed as an additional proarrhythmic provocation. However the majority of cells was still refractory at the time of

this stimulus thus no additional depolarization could be captured. Therefore the S2 stimulus was probably ineffective as an additional provocation mechanism. However, since no EADs were observed and since only DADs/sAPs occurring after the S2 beat and after full repolarization of the membrane potential were counted, and since all genotypes were exposed to the same protocol, we do not consider this a major limitation, although the protocol may have been similarly effective without the S2 beat. Throughout the protocol, no early afterdepolarizations (EADs) could be observed in any of the mouse lines. The occurrence of delayed afterdepolarizations (DADs), defined as low amplitude depolarizations >1 mV of the resting membrane potential and of spontaneous APs defined as fully developed action potentials triggered by a DAD were quantified (Fig. 2A and 3A). Since every spontaneous AP was elicited by a DAD, each spontaneous AP was also numerically counted as a DAD.

The fraction of cells exhibiting DADs was unaltered in OE vs. WT_{OE} (% of cells exhibiting one or more DADs; OE: 35 out of 37 cells / 11 animals (95 %); WT_{OE}: 36 out of 38 cells / 13 animals (95 %); $p>0.05$ (z-test)). In KO vs. WT_{KO} the fraction of cells exhibiting DADs was also unaltered (% of cells exhibiting one or more DADs; KO: 37 out of 39 cells / 10 animals (95 %); WT_{KO}: 33 out of 34 cells / 10 animals (97 %); $p>0.05$ (z-test))

Averaged to all investigated cells, the number of DADs was also not altered in OE vs. WT_{OE} (DADs per all investigated cells; OE: 118.9 ± 20.5 ; $n=37$ cells / 11 animals; WT_{OE}: 98.9 ± 14.8 ; $n=38$ cells / 13 animals; $p>0.05$) (Fig. 2B)). KO and WT_{KO} also exhibited a similar number of DADs averaged to all investigated cells (DADs per all investigated cells; KO: 88.4 ± 12.8 ; $n=39$ cells / 10 animals; WT_{KO}: 110.0 ± 16.4 ; $n=34$ cells / 10 animals; (Fig. 3B and E))

The fraction of cells with spontaneous APs was significantly increased in OE vs. WT_{OE} (% of cells with spontaneous APs; OE: 19 out of 37 cells / 11 animals (51 %); WT_{OE}: 7 out of 38 cells / 13 animals (18 %); $p<0.05$ (z-test)). There was no difference in the fraction of cells with spontaneous APs between KO and WT_{KO} (% of cells with spontaneous APs; KO: 10 out of 39 cells / 10 animals (26 %); WT_{KO}: 15 out of 34 cells / 10 animals (44 %); $p>0.05$; (z-test)).

The number of spontaneous APs per all investigated cells was significantly increased in OE vs. WT_{OE} (spontaneous APs per all investigated cells; OE: 35.4 ± 15.3 ; $n=37$ cells / 11 animals; WT_{OE}: 1.1 ± 0.9 ; $n=38$ cells / 13 animals; $p<0.05$ OE vs. WT_{OE}; Fig. 2C). The number of spontaneous APs per all investigated cells tended to be decreased in KO vs. WT_{KO}, however this finding did not reach statistical significance (spontaneous APs per all investigated cells; KO: 0.4 ± 0.1 ; $n=39$ cells / 10 animals; WT_{KO}: 3.9 ± 1.8 ; $n=34$ cells / 10 animals; $p=0.079$ KO vs. WT_{KO}; Fig. 3C).

In those cells, which exhibited at least one or more DADs (event-positive cells) the numerical ratio of spontaneous AP/DAD was significantly increased in OE compared to WT_{OE} (OE: 0.18 ± 0.05 ; $n=35$ cells / 11 animals; WT_{OE}: 0.02 ± 0.02 ; $n=36$ cells / 12 animals; $p<0.05$; Fig. 2D). The ratio of spontaneous AP/DAD tended to be smaller in KO vs. WT_{KO}

but did not reach statistical significance (KO: 0.01 ± 0.002 ; $n=37$ cells / 10 animals; WT_{KO} : 0.09 ± 0.04 ; $n=33$ cells / 10 animals; $p=0.06$; Fig. 3D).

Additional proarrhythmic provocation in KO vs. WT_{KO}

Under the experimental conditions chosen above, the number of events in WT_{KO} was too low to establish a statistical significance for a protective effect in KO, although borderline significance was reached (Fig. 3 B–D).

Therefore, 10 μ M isoproterenol, lowered extracellular K^+ concentration (2.6 mM) and increased extracellular Ca^{2+} concentration (3.0 mM) were applied. Under these conditions, cells were paced with 1 Hz for 40 s followed by 25 repetitions of 5 sweeps on 5 Hz with a short-coupled sweep after 50 ms and 4 s of rest.

Under these conditions, in KO vs. WT_{KO} , the reduction of the number of spontaneous APs per all investigated cells as well as the reduction of the ratio of spontaneous AP/DAD in event-positive cells reached statistical significance (spontaneous APs per all investigated cells; KO: 0.8 ± 0.4 ; $n=17$ cells / 6 animals; WT_{KO} : 14.5 ± 6.2 ; $n=14$ cells / 5 animals; $p=0.013$; Fig. 3F; ratio spontaneous AP/DAD in event-positive cells: KO: 0.01 ± 0.003 ; $n=17$ cells / 6 animals; WT_{KO} : 0.12 ± 0.05 ; $n=14$ cells / 5 animals; $p=0.007$; Fig. 3G).

DAD amplitude

The DADs had a higher mean amplitude in OE vs. WT_{OE} (in mV; OE: 3.8 ± 0.1 ; $n=37$ cells / 11 animals; WT_{OE} : 2.4 ± 0.03 ; $n=38$ cells / 13 animals; $p<0.05$ OE vs. WT_{OE} ; Fig. 4A and B). Conversely, the mean DAD amplitude was significantly reduced in KO vs. WT_{KO} (in mV; KO: 2.2 ± 0.02 ; $n=39$ cells / 10 animals; WT_{KO} : 3.0 ± 0.03 ; $n=34$ cells / 10 animals; $p<0.05$ KO vs. WT_{KO} ; Fig. 4A and B).

Resting membrane and action potential

Patch-clamp experiments in isolated atrial cardiomyocytes were conducted in current-clamp mode to investigate AP kinetics. The AP duration was measured as time to 90 % decay (APD_{90}) during a pacing frequency of 1 Hz at room temperature (22 ± 2 °C) (Fig. 5A).

APD_{90} was found to be significantly increased in OE vs. WT_{OE} (in ms; OE: 118.3 ± 6.6 ; $n=37$ cells / 11 animals; WT_{OE} : 95.1 ± 5.5 ; $n=38$ cells / 13 animals; $p<0.05$ OE vs. WT_{OE}) (Fig. 5B). Conversely, APD_{90} was significantly shortened in KO vs. WT_{KO} (in ms; KO: 49.7 ± 3.6 ; $n=39$ cells / 10 animals; WT_{KO} : 78.4 ± 5.0 ; $n=34$ cells / 10 animals; $p<0.05$ (Fig. 5B)). The resting membrane potential was slightly but significantly depolarized in OE vs. WT_{OE} (in mV; OE: -61 ± 0.6 ; $n=37$ cells / 11 animals; WT_{OE} : -63 ± 0.5 ; $n=38$ cells / 13 animals; $p<0.05$ OE vs. WT_{OE}). Conversely, the resting membrane potential was hyperpolarized in KO vs. WT_{KO} (in mV; KO: -65.1 ± 0.7 ; $n=39$ cells / 10 animals; WT_{KO} : -62.7 ± 0.4 ; $n=34$ cells / 10 animals; $p<0.05$) (Fig. 5C). The AP amplitude was unaltered between OE and WT_{OE} (in mV; OE: 93.9 ± 2.6 ; $n=37$ cells / 11 animals; WT_{OE} : 93.1 ± 2.8 ; $n=38$ cells / 13 animals; $p>0.05$ OE vs. WT_{OE} Fig. 5D). The AP amplitude was slightly but significantly increased in KO compared to WT_{KO} (in mV; KO: 96.8 ± 2.3 ; $n=39$ cells / 10 animals; WT_{KO} : 88.6 ± 2.5 ; $n=34$ cells / 10 animals; $p<0.05$ KO vs. WT_{KO} ; Fig. 5D).

Spontaneous Ca²⁺ release events

The propensity for spontaneous sarcoplasmic reticular (SR) Ca²⁺ release events was evaluated in isolated single atrial cardiomyocytes subjected to a field stimulation protocol comprised of 3 min with 1 Hz pacing to achieve a steady state level, followed by 2:56 min with 0.125 Hz pacing. A spontaneous SR Ca²⁺ release event was defined as a non-field-stimulated rise of the fluorescence signal during diastole, which exceed the baseline noise fluorescence signal at least twice. The occurrence of spontaneous Ca²⁺ release events was quantified during the 0.125 Hz pacing step and averaged to all investigated cells.

The number of occurrence of spontaneous SR Ca²⁺ release events was not altered between OE vs. WT_{OE} (spontaneous SR Ca²⁺ release events per all investigated cells; OE: 10.2±4.4; n=19 cells / 3 animals; WT_{OE}: 14.7±5.6; n=18 cells / 3 animals; p>0.05 OE vs. WT_{OE}; Fig. 6A and B). The number of occurrence of spontaneous SR Ca²⁺ release events was also unaltered in KO vs. WT_{KO} (spontaneous SR Ca²⁺ release events per all investigated cells; KO: 14.2±6.1; n=17 cells / 3 animals; WT_{KO}: 10.0±3.3; n=17 cells / 3 animals; p>0.05 KO vs. WT_{KO}; Fig. 6A and B).

Diastolic Ca²⁺ level and systolic Ca²⁺ transient amplitude

The diastolic and systolic Ca²⁺ levels were obtained using indo-1-AM during 1 Hz field stimulation. For quantification of the systolic Ca²⁺ transient amplitude, the diastolic Ca²⁺ level was subtracted from the peak value of the systolic Ca²⁺ transient.

No differences regarding the diastolic Ca²⁺ level (Fig. 6C) or the systolic Ca²⁺ transient amplitude (Fig. 6D) were found between OE and WT_{OE} (diastolic Ca²⁺ level (405/495 nm): OE: 3.6±0.1; n=27 cells / 3 animals; WT_{OE}: 3.7±0.1; n=20 cells / 3 animals; p>0.05 OE vs. WT_{OE}; systolic Ca²⁺ transient amplitude (405/495 nm); OE: 1.0±0.1; n=27 cells / 3 animals; WT_{OE}: 0.8±0.1 n=20 cells / 3 animals; p>0.05 OE vs. WT_{OE}). In contrast KO revealed an increased diastolic Ca²⁺ level (Fig. 6C) and systolic Ca²⁺ transient amplitude (Fig. 6D) compared to WT_{KO} (diastolic Ca²⁺ level (405/495 nm); KO: 3.4±0.1; n=23 cells / 4 animals; WT_{KO}: 3.0±0.1 n=25 cells / 4 animals; p<0.05 KO vs. WT_{KO}; systolic Ca²⁺ transient amplitude (405/495 nm); KO: 0.7±0.1; n=23 cells / 4 animals; WT_{KO}: 0.4±0.04 n=25 cells / 4 animals; p<0.05 KO vs. WT_{KO}).

The fact that the amplitude of the systolic transient shows a smaller absolute numeric value than the diastolic Ca²⁺ level is a consequence of the calculation processing of absolute fluorescence emission signals.

Immunofluorescence stainings in OE vs. WT_{OE}

There were no obvious alterations in the distribution pattern of NCX in OE vs. WT_{OE} on a cellular level as investigated by immunocytochemistry (online supplement, Fig. 3).

Simultaneous recordings of caffeine-induced I_{NCX} and Ca²⁺ transient in OE vs. WT_{OE}

For OE and WT_{OE} simultaneous recordings of I_{NCX} and Ca²⁺ transient were conducted during caffeine-induced SR Ca²⁺ release. The I_{NCX} integral (area above the curve (AAC) was unaltered (AAC of I_{NCX}; in pA * s; OE: -139.1±21.3; n=16 cells / 3 animals; WT_{OE}:

-104.0 ± 19.1 ; $n=20$ cells / 3 animals; $p>0.05$; Fig. 7 A and B). The amplitude of the Ca^{2+} transient (F/F_0 ; OE: 4.02 ± 0.24 ; WT_{OE}: 3.94 ± 0.17 ; $p>0.05$) was also unaltered in OE vs. WT_{OE}. Both these parameters confirmed unaltered SR Ca^{2+} load in OE vs. WT_{OE}. Peak I_{NCX} (pA/pF; OE: -0.65 ± 0.08 ; WT_{OE}: -0.28 ± 0.02 ; $p<0.05$; Fig. 7A and C) and the decay velocity of the caffeine-induced Ca^{2+} transient (T_{50} in s; OE: 3.89 ± 0.5 ; WT_{OE}: 5.5 ± 0.39 ; $p<0.05$) were significantly increased in OE vs. WT_{OE} confirming increased NCX function in OE.

Recording of I_{K1} and I_{KACH} in OE vs. WT_{OE}

To test whether alterations of counterbalancing K^+ current populations may affect the resting membrane potential or the DAD amplitude in OE vs. WT_{OE}, I_{K1} and the composed current of I_{K1} and I_{KACH} ($I_{\text{K1}} + I_{\text{KACH}}$) were measured. Both I_{K1} (-20 mV; in pA/pF; OE: 1.32 ± 0.23 , $n=19$ cells / 3 animals; WT_{OE}: 1.06 ± 0.26 ; $n=18$ cells / 3 animals; $p>0.05$; Fig. 7 D and E) and $I_{\text{K1}} + I_{\text{KACH}}$ (-20 mV; in pA/pF; OE: 5.82 ± 0.47 , $n=18$ cells / 3 animals; WT_{OE}: 6.61 ± 0.61 ; $n=16$ cells / 3 animals; $p>0.05$; Fig. 7 F and G) were unaltered in OE vs. WT_{OE}.

Discussion

This is the first study to use genetically altered models to investigate whether an altered expression of NCX influences cellular triggered activity in atrial myocytes. By heterozygous knockout of NCX, this is also the first study to apply chronic and specific NCX inhibition in atrial tissue since pharmacological NCX inhibitors lack specificity [15, 16] and - to the best of our knowledge - have so far only been applied as single shot applications to investigate arrhythmogenesis.

NCX expression independently regulates triggered activity in atrial myocytes

There were no differences in atrial weight to body weight ratio, microscopic appearance or cell surface area, collagen deposition or electrical cell capacitance in atria of OE vs. WT_{OE} or KO vs. WT_{KO}. Previous studies have shown no evidence of whole-heart structural disease under baseline conditions in either heterozygous KO [8] or homozygous OE [10] at the age used in this study, although homozygous OE develop hypertrophy and heart failure at higher age [17]. Furthermore, in atrial myocytes from both OE vs. WT_{OE} and KO vs. WT_{KO} there were no differences in SR Ca^{2+} load or the number of spontaneous SR Ca^{2+} release events. Also, the expression of Ca^{2+} handling and structural proteins was unaltered with the only exception of increased junctin expression in OE. These findings point against the possibility that the cellular proarrhythmic effect in OE vs. WT_{OE} or - vice versa - the antiarrhythmic effect in KO vs. WT_{KO} are a consequence of adaptively altered cellular, anatomical or functional remodeling.

Junctin forms a functionally active complex with triadin and calsequestrin [18] regulating Ca^{2+} release via the ryanodine receptor (RyR) [19]. Thus, the observed increase in junctin could possibly result in altered Ca^{2+} handling, leading to increased spontaneous Ca^{2+} activity and thus proarrhythmia. However, in our OE model, diastolic and systolic Ca^{2+} level, SR Ca^{2+} load and the number of spontaneous Ca^{2+} release events were unaltered.

Also, the expression of triadin, calsequestrin and RyR was unaltered. Presumably, the entirety of these proteins are necessary to form the Ca^{2+} release regulating complex [19], which makes it less likely that the fraction of junctin molecules overexpressed in OE will be functionally active in regulating RyR. Other studies using genetic manipulation of junctin expression levels have demonstrated that apparently both, knockout [20] and overexpression [21] of junctin can produce a proarrhythmic phenotype in mice. Thus, presently, the definite role of junctin in proarrhythmia seems to be difficult to assess. Therefore, we cannot exclude, that the observed junctin overexpression may play a yet unidentified role in our OE model.

To unfold its exchanger function with all its physiological consequences, NCX must be overexpressed within the sarcolemmal membrane of the atrial myocyte. An important general caveat regarding the transgenic approach is that, potentially, NCX overexpression could have resulted in a relevant proportion of NCX protein not being overexpressed in the membrane but within the cytosol. Although immunocytochemistry did not reveal any obvious evidence to support this assumption, this method has some limitations in precisely quantifying the cellular domain location of NCX or other proteins within the cardiac myocyte [11, 22]. However, as demonstrated in this study, the removal of Ca^{2+} from the cytosol into the extracellular space is much faster and the transmembrane electrical inward current I_{NCX} is increased in amplitude in OE myocytes. The fact that these processes directly require transmembrane NCX confirms that NCX is overexpressed in OE membranes to a degree, that is sufficient to measurably and significantly enhance its function.

Mechanism of regulation of atrial triggered activity by NCX

If a DAD reaches a critical threshold amplitude, it may cause voltage-dependent Na^{+} channels to open, which then trigger a premature spontaneous AP that can initiate arrhythmia of the whole-heart. Evidence for this mechanism stems from ventricular myocytes of non-transgenic models of hypertrophy and heart failure (for review see [6, 23]) but has also been confirmed in ventricular myocytes from non-failing murine KO and OE models with genetically altered NCX expression [9, 10].

We here observed that the critical mechanism of NCX mediated atrial proarrhythmia in OE does not consist in an increase of the number of DADs but rather a facilitated translation of DADs into spontaneous APs. Vice versa, the translation of DADs into spontaneous APs is impaired in KO, resulting in the observed protective effect. One has to keep in mind that the spontaneous action potential and not the mere DAD is the final downstream “proarrhythmic effector” causing whole-heart triggered activity. The mean amplitude of DADs was significantly higher in OE vs. WT_{OE} while it was smaller in KO vs. WT_{KO} . This is plausible, since for any given spontaneous diastolic Ca^{2+} release, the underlying peak NCX carried inward current that produces a DAD is larger the more NCX protein is expressed in the membrane [7, 11] and - vice versa - is smaller when NCX expression is reduced [24, 25]. This hypothesis is supported by the finding of increased peak I_{NCX} despite an unaltered net amount of Ca^{2+} released from the SR during caffeine application (Fig. 7 A–C). Since a spontaneous AP is initiated by an “all or nothing” response, it is not the sheer number of

DADs occurring but rather the amplitude of a given DAD that determines whether a spontaneous AP occurs or not.

In addition to the altered DAD amplitude, we found a small but significant elevation of the resting membrane potential in OE. However, I_{K1} and I_{KACH} as repolarizing currents which also influence the resting membrane potential in atrial myocytes[26] were unaltered in OE. Although the differences in resting membrane potentials are minute, they may contribute to proarrhythmia in OE since the larger DAD amplitude rises on top of the already elevated baseline membrane potential. Conversely, the resting membrane hyperpolarization in KO would support the observed suppression of spontaneous APs. Although NCX inward mode is predominantly active during systolic SR Ca^{2+} release, there is evidence that a residual NCX inward current may also be active during diastole (for review see[27]) possibly explaining the altered resting membrane potential.

Spontaneous Ca^{2+} release as the trigger source of DADs

The initiating event of any DAD is a spontaneous release of Ca^{2+} from the SR and this concept also applies to the arrhythmic events observed in our study. Spontaneous Ca^{2+} activity leads to a sudden elevation of the cytosolic Ca^{2+} concentration driving NCX forward mode, resulting in a DAD. Among other factors, increased SR Ca^{2+} load[28], altered RyR expression or function[29, 30], altered Na^+ cycling[31, 32] and altered oxidation[33–35] increase spontaneous activity thus increasing the number of DADs which will result in proarrhythmia. In NCX KO and OE, SR Ca^{2+} load, RyR receptor expression and phosphorylation (Ser2808) were unaltered. Also the number of spontaneous Ca^{2+} releases were unaltered, which is in accordance with an unaltered number of DADs/cell as observed in our study. Thus, at least in our model and under the experimental conditions chosen, the mechanism by which NCX causes arrhythmia seems to occur downstream of spontaneous SR Ca^{2+} release by facilitating the translation of DADs into spontaneous APs.

However it has to be mentioned that studies using models of atrial fibrillation have demonstrated an increased number of spontaneous Ca^{2+} release events and have identified this as a proarrhythmic mechanism[5, 36, 37] (for review see[38, 39]). Most notably, some studies using atrial tissue from patients with early stage atrial fibrillation[37] or rapidly paced rabbit atrial myocytes[2] have demonstrated this effect in the absence of increased NCX protein levels. In this context, it has to be kept in mind that the transgenic mice used in our study are not models of atrial fibrillation and thus they do not encompass all pathophysiological mechanisms of this disease. Rather, this study aimed at investigating the potency of isolated altered NCX activity in promoting or inhibiting atrial cellular triggered activity in the absence of remodeling processes associated with atrial fibrillation. Whether this indeed influences the occurrence of atrial fibrillation or not has to be left open and has to be solved by future studies.

Also, it cannot be excluded that there are differences in Ca^{2+} dynamics not detected by our methodology. Future studies applying simultaneous recordings of spontaneous Ca^{2+} activity and membrane potential or confocal/line scan imaging may be necessary to further characterize the influence of NCX expression on detailed atrial spatiotemporal characteristics of spontaneous Ca^{2+} activity.

Other than in OE vs. WT_{OE}, we found an increased diastolic Ca²⁺ level and an increased Ca²⁺ transient in atrial myocytes from KO when compared to WT_{KO}. While at first glance, this seems plausible due to the reduced cytosolic Ca²⁺ extrusion in KO, this finding differs from ventricular myocytes with heterozygous and homozygous NCX knockout where no alterations in diastolic and systolic Ca²⁺ were observed[9, 24]. This may reflect differences in Ca²⁺ handling between atrial and ventricular myocytes. Nevertheless, it is unlikely that increased Ca²⁺ levels in KO are responsible for the observed suppression of arrhythmia, since elevated Ca²⁺ has been associated with increased spontaneous SR Ca²⁺ release which would rather promote than suppress proarrhythmia[5].

Limitations of the study

Limitations exist in using transgenic murine models to investigate mechanisms of proarrhythmia. First of all, cellular and whole-heart electrophysiology differ between mice and humans, which is depicted by the much faster repolarization of the action potential and the higher heart frequency[40, 41]. Secondly, the KO (mixed genetic background: C57BL/6 and CD-1 for WT_{KO} and KO) and the OE mouse lines (C57BL/6 in WT_{OE} and OE) are based on different genetic backgrounds. Differences in electrophysiological properties between different WT mouse lines have been described previously [13, 42] and we as well detected significant differences in some parameters of electrophysiology or Ca²⁺ handling between WT_{KO} and WT_{OE} (online supplement, table 1) This illustrates that direct comparisons between murine models are only valid within the same genetic background. Hence, in this study, we exclusively compared transgenic mice with their corresponding WT, i.e. OE vs. WT_{OE} or KO vs. WT_{KO}. The reasons for these background specific differences are not yet fully understood, although it has been suggested that different testosterone levels may underly [42].

The age of mice used in this study ranged between 7.5 – 12.5 weeks and the male / female ratio of (54.7 % vs. 45.3 %) was nearly balanced. Nevertheless, influences of age or sex[43] on the obtained results cannot be completely excluded.

Although we did not detect evidence for structural or functional remodeling in atria of OE or KO and I_{K1} and I_{KACH} were unaltered in OE vs. WT_{OE}, it cannot be excluded that other currents affecting repolarization or resting membrane potential such as I_{KATP}[44] affecting the translation of DAD into sAP are altered.

Additional proarrhythmic provocation in WT_{KO} and KO was necessary to increase the number of arrhythmic events in WT_{KO} to a level at which a valid quantitative comparison was possible. This is not surprising, since previous studies have also found a relatively low number of events in murine control WT myocytes under baseline conditions[45–47]. However, these studies investigated proarrhythmic and not protected models and therefore did not require a high number of arrhythmic events in their WT controls. Thus, to demonstrate a protective effect in KO, we were forced to identify conditions that increased the number of events in WT_{KO}, which only succeeded after applying more aggressive experimental conditions in the form of lowered K⁺ (2.6 mM), increased Ca²⁺ (3 mM) and isoprenaline (Fig. 3 E–G). The physiological free Ca²⁺ concentration in mice is ~1.15 mM[48] and thus the increase to 3 mM deviates from physiological conditions. The

aggressive nature of this protocol may pose a limitation to the study since these are non-physiological conditions. Nevertheless, we believe that these experiments may still deliver a mechanistic proof of principal – especially when interpreted together with the findings in OE. Thus, they demonstrate that while increased NCX expression enhances the translation of DADs into sAPs, the opposite mechanism may be at work in KO, thereby suppressing sAPs and possibly offering a protective effect.

Finally, this study exclusively investigated cellular mechanisms. Therefore, it remains unclear whether the enhanced number of spontaneous APs in OE will lead to atrial arrhythmia in the whole-heart or – vice versa – the lower number of spontaneous APs in KO will prove to be protective in whole-heart models. In the whole heart, mechanisms of proarrhythmia above the cellular level, not covered by this study, such as locoregional differences in extracellular matrix composition, fibrosis or localized micro- or macrodimensional scar tissue may become effective. This issue will have to be addressed in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

A: Confirmation of altered Na⁺/Ca²⁺-exchanger (NCX) protein expression by immunoblot analysis of atrial tissue homogenates. NCX protein level was increased in homozygous NCX-overexpressor mice (OE) vs. the corresponding wild-type (WT_{OE}) (upper panel) and reduced in heterozygous NCX-knockout mice (KO) vs. the corresponding wild-type (WT_{KO}) (lower panel). There was no alteration in the expression of calsequestrin (CsQ), sarco(-endo)plasmic reticulum Ca²⁺ ATPase (SERCA), triadin (Trd), phospholamban (Plb), ryanodine receptor (RyR), Ser2808 phosphorylated ryanodine receptor (RyRp), the alpha 1C subunit of the voltage-gated L-type Ca²⁺ channel (Ca_v1.2) and troponin I (Tni). Junctin (Jcn) expression was increased in OE vs. WT_{OE} but unaltered in KO vs. WT_{KO} (n=8 homogenates for each mouse line for each protein; quantification by densitometry). B: Confirmation of altered NCX function in isolated atrial myocytes during caffeine release experiments. The velocity of the decay of the caffeine-induced Ca²⁺ transient correlates to cellular Ca²⁺ extrusion capacity as a direct measure for NCX inward activity. C: Quantification of time to 50 % decay (T₅₀) of the caffeine-induced transient. NCX activity was reduced in KO vs. WT_{KO} (right) and increased in OE vs. WT_{OE} (left) (n=22–24 / 3–5 animals for each mouse line). D: Unaltered amplitude of the caffeine-induced Ca²⁺ transient indicates unaltered SR (sarcoplasmic reticulum) Ca²⁺ load in WT_{KO} vs. KO and OE vs. WT_{OE}. (*p<0.05; n.s.=non-significant).

Fig. 2.

Delayed afterdepolarizations (DADs) and spontaneous APs (sAPs) in OE (n=37 cells / 11 animals) vs. WT_{OE} (n=38 cells / 13 animals). Isolated atrial cardiomyocytes were patch-clamped in the current-clamp mode and subjected to a proarrhythmic pacing protocol containing sudden changes in cycle lengths and burst stimulation. Membrane potential was continuously recorded throughout the protocol. An sAP was defined as a full premature action potential not triggered by a current-clamp command but by a DAD. A: Representative tracings: The number of DADs was unaltered, however, a DAD triggered a spontaneous AP significantly more often in OE than in WT_{OE}. B–D: Quantification: Each sAP was also numerically counted as a DAD since it was triggered by one. B: Number of DADs per all investigated cells; C: Number of sAPs per all investigated cells; D: Ratio of sAPs to DADs. (*p<0.05; n.s.=non-significant).

Fig. 3.

The experiments presented in Fig. 2 were repeated in KO (n=39 cells / 10 animals) vs. WT_{KO} (n=34 cells / 10 animals) atrial myocytes: A: Representative tracings: The number of DADs was unaltered, however, a DAD triggered a spontaneous AP less often in KO than in WT_{KO} presenting the opposite finding as in OE vs. WT_{OE}. This finding reached borderline significance (p=0.06) using the same protocol as in Fig. 2 (quantification: B–D) but reached statistical significance (p=0.007) when the protocol was modified towards more aggressive proarrhythmic provocation by altering the stimulation sequence, adding isoprenaline, lowering extracellular K^+ concentration $[K^+]_o$ and increasing extracellular Ca^{2+} concentration $[Ca^{2+}]_o$ (WT_{KO}: n=14 cells / 5 animals; KO: n=17 cells / 6 animals) (quantification: E–G). (*p<0.05; n.s.=non-significant).

Fig. 4. Altered DAD amplitude in OE vs. WT_{OE} and KO vs. WT_{KO}. A: Representative tracings: The mean DAD amplitude was increased in OE vs. WT_{OE} (left panel) and reduced in KO vs. WT_{KO} (right panel). B: Quantification (n=34–39 cells / 10–13 animals for each mouse line; *p<0.05).

Fig. 5.

Altered resting membrane potential and repolarization in atrial myocytes from OE vs. WT_{OE} and KO vs. WT_{KO}. A: Representative tracings: Upper panel: Prolonged action potential (AP) duration in OE vs. WT_{OE}; lower panel: Shortened AP duration in KO vs. WT_{KO}. The resting membrane potential was slightly but significantly elevated in OE vs. WT_{OE} and hyperpolarized in KO vs. WT_{KO}. The AP amplitude was unaltered in OE vs. WT_{OE}, but slightly increased in KO vs. WT_{KO}. B–D: Quantification: B: Time to repolarization to 90% of AP amplitude (APD₉₀); C: Resting membrane potential; D: AP amplitude (n=34–39 / 10–13 animals for each mouse line; *p<0.05; n.s.=non-significant).

Fig. 6. Ca²⁺ handling and spontaneous sarcoplasmic Ca²⁺ release events (sCR) in atrial myocytes from OE vs. WT_{OE} and KO vs. WT_{KO}. Isolated atrial myocytes were subjected to a prolonged field stimulation protocol containing rapid changes in cycle lengths while continuously recording the Ca²⁺ fluorescence signal. A: Representative tracings from OE vs. WT_{OE} (upper panel) and KO vs. WT_{KO} (lower panel). Quantification (B–D): In OE vs. WT_{OE} and in KO vs. WT_{KO} there were no significant differences in the number of sCR per all investigated cells (B). The diastolic Ca²⁺ level (C) and the amplitude of the systolic Ca²⁺ transient (D) were unaltered in OE vs. WT_{OE} but were slightly increased in KO vs. WT_{KO} (n=17–27 cells / 3–4 animals for each mouse line; *p<0.05; n.s.=non-significant).

Fig. 7A–C.

Simultaneous measurement of the NCX inward current (I_{NCX}) (lower panel) and the Ca²⁺ transient (upper panel) during caffeine-induced Ca²⁺ release from the SR in OE vs. WT_{OE} (16–20 cells / 3 animals for each mouse line). A: Original tracings. B: Quantification of as area above the curve (AAC). C: Quantification of the peak amplitude of the integral of I_{NCX}. Peak I_{NCX} amplitude was increased while the integral as a measure of net Ca²⁺ flux over the membrane was unaltered. D–G: Measurement of the inward rectifier current (I_{K1}) and the G-protein-activated K⁺ current (I_{KACH}) in OE vs. WT_{OE} (16–19 cells / 3 animals for each mouse line). D: Original tracings of I_{K1}. E: Quantification of I_{K1} current-voltage curve. F: Original tracings of the composed current of I_{K1} and the carbachol-induced I_{KACH} (I_{K1} + I_{KACH}). G: Quantification of the I_{K1} + I_{KACH} current-voltage curve. No alterations of I_{K1} or I_{K1} + I_{KACH} could be detected between OE and WT_{OE} (p>0.05; n.s.=non-significant).