Conclusions—Genetic suppression of NCX reduces both EADs and DADs. The following molecular mechanisms apply: (1) Although the absolute number of DADs is unaffected, an impaired translation of DADs into spontaneous action potentials results from a reduced DAD amplitude. (2) EADs are reduced in absolute number of occurrence, which is presumably a consequence of shortened action potential duration because of reduced NCX activity but also reduced I_{Ca}, the latter possibly being caused by a direct modulation of Ca^{2+}-dependent I_{Ca} inhibition by reduced NCX activity. This is the first study to demonstrate that genetic inhibition of NCX protects against afterdepolarizations and to investigate the underlying mechanisms. (Circ Arrhythm Electrophysiol. 2015;8:1210-1218. DOI: 10.1161/CIRCEP.115.002927.)

Key words: action potentials, arrhythmias, cardiac, heart failure, Na+/Ca^{2+} exchanger

The Na+/Ca^{2+} exchanger (NCX) works as the main Ca^{2+} removal mechanism of the cardiac myocyte, extruding 1 Ca^{2+} ion to the extracellular compartment in exchange for 3 Na^{+} ions. Because of this electrogenic stoichiometry, NCX generates an electric inward current during elevation of the cytosolic Ca^{2+} concentration. This results in a prolongation of the action potential (AP) and may furthermore cause destabilization of the membrane potential resulting in cardiac arrhythmia by generation of early and late afterdepolarizations. Early afterdepolarizations (EADs) are defined as re-depolarizations occurring during the decay of the AP, whereas delayed afterdepolarizations (DADs) arise after full repolarization during electric diastole.

A DAD is thought to be initiated by a spontaneous sarcoplasmic Ca^{2+} release (sCR) event that is translated via NCX inward mode into a depolarizing inward current forming a DAD. If the amplitude of the DAD exceeds the threshold for voltage gated Na^{+} channels (I_{Na}), a spontaneous AP (sAP) may occur, which may propagate and thus cause whole heart arrhythmia in the form of triggered activity.

It is unknown whether the same mechanism may be responsible for the occurrence of EADs. Some studies have suggested an NCX-independent mechanism: Thus, reactivation of Ca^{2+} influx via L-type Ca^{2+} channels (I_{Ca,L}) during the repolarization phase may re-depolarize the membrane potentially resulting in an EAD. However, others have suggested that DADs and EADs share a common mechanism that may be NCX dependent.

To date, the role of chronically altered NCX expression in the generation of arrhythmia has been limited to models with...
WHAT IS KNOWN

- The cardiac Na⁺/Ca²⁺ exchanger (NCX) may promote afterdepolarizations of the cellular action potential thereby possibly causing ventricular arrhythmia, which may be especially crucial in patients with heart failure, where NCX expression is increased.
- So far it is unknown whether genetic reduction of NCX expression may indeed suppress afterdepolarizations and - if yes - by which mechanism a potentially protective effect would work, ie, by suppression of early or late afterdepolarizations or both.

WHAT THE STUDY ADDS

- This is the first study demonstrating that genetic knockout of NCX protects from cellular afterdepolarizations.
- Knockout of NCX leads to a reduced amplitude of delayed afterdepolarizations and also reduced the likelihood of early afterdepolarizations.
- This confirms that chronic and specific NCX inhibition by pharmacological or genetic therapy could be a promising future strategy to treat ventricular arrhythmia.

It is therefore unknown whether genetic inhibition of NCX activity will suppress arrhythmia. Just as well, genetic inhibition may promote arrhythmia by mechanisms yet to be identified. Most importantly a reduction of K⁺ currents which promote a prolongation of AP duration and the occurrence of arrhythmia.

In a second step, this study aimed at investigating the underlying molecular mechanisms this protection is mediated. This could be a suppression of DADs, EADs, or even both, bearing the potential answer to the question of whether DADs and EADs share a common or a distinctly different molecular mechanism.

We here investigated a non-failing murine model with heterozygous global knockout of NCX (hetKO). The aim of this study was to determine whether genetic NCX suppression affects the occurrence of afterdepolarizations and, if yes, whether this effect is limited to DADs or also includes EADs. In a second step, this study aimed at investigating the underlying molecular mechanisms that lead from reduced NCX activity to a potential antiarrhythmic effect by suppressing afterdepolarizations.

Methods

Generation of Heterozygous NCX Knockout Mice
Generation of heterozygous global NCX knockout mice has been reported previously. The genotype of every animal entering experimentation was confirmed by polymerase chain reaction. Breeding, housing, and experimentation were performed in accordance to the guidelines of the Westfälische Wilhelms-University Münster and have been approved by the Landesamt für Natur, Umwelt, und Verbraucherschutz NRW. Animals used for experimentation were between 8 and 12.5 weeks of age.

Immunoblot Analysis
Immunoblot analysis on ventricular homogenates was performed as previously reported with minor modifications. Signal intensity was normalized to the housekeeping protein Csq.

Isolation of Ventricular Cardiomyocytes
Mice were sedated and euthanized with carbon dioxide inhalation. Single ventricular cardiomyocytes were isolated as reported previously with modifications as reported in the Data Supplement.

Cellular Electrophysiology
AP measurements and the potassium carried outward current were conducted using the perforated patch clamp technique. When recording I₉, the ruptured clamp method was used. For details, including intracellular and extracellular solutions, see Data Supplement.

Cellular Ca²⁺ Imaging
Fluorescence emission at 522 nm (fluo-4-AM) or 405 and 495 nm wavelength (indo-1-AM) was taken as an indicator for the cytosolic Ca²⁺ level. The fluorescence emission was plotted in arbitrary light units. Whenever the Ca²⁺ signal was directly compared between separate cells, the ratio of the fluorescence signal of indo-1 was used for this subset of experiments. Details are available in the Data Supplement.

Statistics
Statistical analyses and database management were performed using SigmaPlot 11.2.0.5 (Systat Software Inc.). Data are expressed as mean±SEM. Student unpaired 2-tailed t test was used for direct comparison between both genotypes and between different cell populations of the same genotype. We performed 1-way or 2-way ANOVA test where applicable and accounted for multiple comparisons with 2-sided Dunnett’s method or Bonferroni correction, respectively. Rates and proportions were quantified using the z test. Unless not otherwise quoted, P values were calculated using Student t test. A P value ≤0.05 was considered to be statistically significant.

Results

Expression of Ca²⁺ Handling and Structural Proteins
To confirm reduced NCX expression in heterozygous NCX knockout mice and to detect potential alterations of key Ca²⁺ handling and structural proteins, we performed immunoblot analyses (Figure 1A).

NCX expression was significantly reduced to 46.6±9.2% in hetKO versus wild-type (WT: n=8 each; P<0.05). There was no significant difference in the expression of ryanodine receptor (hetKO: n=5; WT: n=5), Ca₁,1.2 (hetKO: n=8; WT: n=8), phosphorylated ryanodine receptor (here and for all following proteins hetKO: n=6; WT: n=6), Csq, Tni, Plb, Trd, Jcn, and SERCA.

Reduced NCX Activity in hetKO
To validate, whether the reduced NCX expression leads to a reduced NCX activity in hetKO, caffeine (10 mmol/L) was rapidly applied to fluo-4-AM loaded isolated cardiomyocytes. Caffeine leads to a sudden and complete sarcoplasmic
reticular (SR) Ca\(^2+\) release (Figure 1B). The decay kinetics of the caffeine-induced Ca\(^2+\) transient serves as a direct measure of the Ca\(^2+\) extrusion capacity mediated via NCX. Ca\(^2+\) removal, obtained as time to half decay of the caffeine-induced Ca\(^2+\) transient (T\(_{50}\)), was significantly slowed in hetKO compared with WT confirming functionally reduced NCX activity (T\(_{50}\) in s: WT, 1.7±0.2; n=14; hetKO, 3.1±0.2; n=16; \(P<0.05\) hetKO versus WT; 1-way ANOVA test; 2-sided Dunnett’s method; Figure 1C). For control purposes, the effect of reduced NCX activity was reproduced in WT by adding the NCX inhibitor Ni\(^2+\) (10 mmol/L; T\(_{50}\) in s: WT, 4.0±0.6; n=12; \(P<0.05\) versus basal; 1-way ANOVA test; 2-sided Dunnett’s method).

Unaltered Amplitude of the Systolic Ca\(^2+\) Transient in hetKO

The amplitude of the systolic Ca\(^2+\) transient was not altered between both genotypes (amplitude of the systolic Ca\(^2+\) transient; WT: 0.40±0.03, n=16; hetKO: 0.42±0.04, n=16; \(P=0.71\) hetKO versus WT). There was no evidence for altered diastolic Ca\(^2+\) levels between both genotypes.

Unaltered SR Ca\(^2+\) Content in hetKO

The peak amplitude of the caffeine-induced Ca\(^2+\) transient is a measure for SR Ca\(^2+\) content. The amplitude of the caffeine-induced Ca\(^2+\) transient was unaltered suggesting no difference in the SR Ca\(^2+\) content between hetKO and WT (WT: 0.65±0.06, n=16; hetKO: 0.70±0.05, n=15; \(P=0.49\) hetKO versus WT; Figure 1D).

AP Kinetics

To assess the effects of chronically reduced NCX activity on AP kinetics, APs were recorded using the perforated patch clamp method in current clamp mode (Figure 2A).

AP duration obtained as time to half decay of the AP (APD\(_{50}\)) was significantly reduced in hetKO compared with WT (WT: 7.9±1.5 ms, n=20; hetKO: 4.1±0.5 ms, n=20; \(P<0.05\) hetKO versus WT; Figure 2B). Resting membrane potential (WT: −68.4±1.2, n=20; hetKO: −68.7±1.1, n=20; \(P=0.84\) hetKO versus WT) and AP amplitude (WT: 114.4±3.7,
Potassium Carried Outward Current in hetKO Versus WT

To exclude that the shortening of the AP is caused by an increase of voltage gated K+ currents, we measured the total K+ carried peak outward current ($I_{\text{Ktot}}$). $I_{\text{Ktot}}$ was recorded in voltage clamped cardiomyocytes using perforated patch configuration in the presence of tetrodotoxin (10 µmol/L) and nifedipine (2 µmol/L) to avoid contamination of the recorded current by $I_{\text{Na}}$ or $I_{\text{Ca}}$. Cells were clamped at −80 mV and then depolarized by square wave pulses (duration 1 s) ranging from −40 to +60 mV (Figure 3A). $I_{\text{Ktot}}$ peak current was normalized to cell capacitance. Inactivation kinetics of $I_{\text{Ktot}}$ was measured taking the time to 25% decay ($T_{25}$).

$I_{\text{Ktot}}$ peak current was slightly but significantly reduced in hetKO versus WT at potentials ranging from +40 to +60 mV (+60 mV; in pA/pF: WT, 29.2±2.8; n=10; hetKO, 23.7±2.4; n=10; *P<0.05 hetKO versus WT; 2-way repeated measures ANOVA; Bonferroni correction). This finding was not statistically significant ($T_{25}$ in ms: WT, 53.6±9.7; hetKO: 79.6±10.3; P=0.08 hetKO versus WT). There was a trend toward a slowed inactivation of $I_{\text{Ktot}}$ in hetKO; however, this finding was not statistically significant ($T_{25}$ in ms: hetKO versus WT).

Unaltered Spontaneous SR Ca²⁺ Release in hetKO Versus WT

Ca²⁺ transients of field stimulated fluo-4-AM-loaded cardiomyocytes were recorded during a stimulation protocol that was comprised of abrupt changes in pacing cycle length (20 sweeps each in Hz: 2, 1, 0.5, 0.25, 0.125, 0.5, 0.125, 1, and 3 minutes of rest). This protocol was repeated in the presence of 1 µmol/L isoproterenol.

Throughout the protocol irregular Ca²⁺ activity was observed as (1) sCR, defined as a non–field-stimulated rise of the fluorescence signal during diastole; and (2) Ca²⁺ oscillations, defined as repeated-fluorescence upstrokes during the decay phase of a field stimulated fully developed Ca²⁺ transient (Figure 4A). The number of sCR events per all investigated cells was not significantly different between hetKO and WT (sCR/cell: WT, 70.4±10.1; n=30; hetKO, 54.8±9.3; n=27; P=0.26 WT versus hetKO; Figure 4B). The number of oscillations per all investigated cells was significantly smaller in hetKO than in WT (oscillations/cell: WT, 58.4±10.5; n=30; hetKO, 15.5±4.7; n=27; *P<0.05 WT versus hetKO; Figure 4C).

Reduced DAD Amplitude and Reduced Occurrence of sAPs in hetKO Versus WT

Occurrence of DADs and sAPs was investigated in isolated amphotericin-perforated cardiomyocytes subjected to a proarrhythmic stimulation protocol (20 sweeps each in Hz: 5, 2, 1, 0.5, 0.25, 0.125, and 3 minutes of rest) that was repeated in the presence of isoproterenol (1 µmol/L) followed by 50 repetitions of an S₃-beat-protocol containing 4 sweeps with 5 Hz followed by 1 short coupled sweep after 50 ms and a subsequent period of rest for 4 s.

DADs were defined as temporary depolarizations of low amplitude during electric diastole, whereas sAPs were defined as full APs exceeding −10mV threshold triggered by a DAD and not by a current clamp command (Figure 5A). Each sAP was also numerically counted as a DAD because each sAP was triggered by a DAD. The number of cells showing DADs was not different between WT (19 of 20 [95%]) and hetKO (17 of 20 [85%]; P=0.60 WT versus hetKO; z test; Figure 5C), however, the number of cells exhibiting sAPs was significantly smaller in hetKO (hetKO: 6 of 20 [30%]; WT: 15 of 20 [75%]; P<0.05 WT versus hetKO; z test; Figure 5C).

The number of DADs averaged to all investigated cells was also not significantly different between WT and hetKO (DAD/ cell: WT, 40.3±12.2; n=20; hetKO, 47.9±12.0; n=20; P=0.66 hetKO versus WT; Figure 5D), whereas the number of sAPs averaged to all investigated cells was significantly smaller in hetKO (sAPs/cell: WT, 10.6±3.0; n=20; hetKO, 2.1±1.1; n=20; P<0.05 hetKO versus WT; Figure 5E). Furthermore, in those cells that exhibited DADs (event-positive cells), the ratio of sAP/DAD was significantly smaller in hetKO than in WT. The mean amplitude of DADs was significantly reduced in hetKO versus WT (mean DAD amplitude in mV: WT, 4.52±0.09; hetKO, 2.89±0.05; P<0.05 hetKO versus WT; Figure 5B). At the same time, the mean duration of DADs was significantly prolonged in hetKO versus WT (mean DAD duration in s: WT, 0.58±0.02; hetKO, 0.67±0.01; P<0.05 hetKO versus WT).

Reduced Occurrence of EADs in hetKO

EADs were defined as re-depolarizations during repolarization of the AP that exceeded −40 mV after the membrane potential had dropped to below −40 mV (Figure 6A–6C). The shape of the EADs varied between temporary membrane fluctuations up to multiple steep upstrokes. The number of cells exhibiting EADs was significantly smaller in hetKO (2 of 20 [10%]) than in WT (17 of 20 [85%]; P<0.05 WT versus hetKO; z test; Figure 6D). The number of
EADs per all investigated cells was also significantly reduced in hetKO compared with WT (EADs/cell: WT, 66.2±20.8; n=20; hetKO, 0.2±0.1; n=20; *P<0.05 hetKO versus WT). Reduced L-type Ca\(^{2+}\) Current in hetKO

Altered NCX activity has been described to regulate I\(_{Ca}\).\(^{16-18}\) We therefore measured I\(_{Ca}\) in voltage clamped cardiomyocytes in ruptured patch configuration. Cells were clamped at −40 mV to inhibit I\(_{Na}\) and then depolarized by square wave pulses (400 ms) ranging from −30 to +40 mV (Figure 7A). Peak current was normalized to cell capacitance. Pipette solution contained cesium chloride (120 mmol/L) and tetraethylammonium chloride (10 mmol/L) to inhibit voltage gated K\(^{+}\) currents. The baseline measurements of I\(_{Ca}\) (Figure 7A and 7B) were performed in the absence of specific Ca\(^{2+}\) buffers.

I\(_{Ca}\) amplitude was found to be reduced in hetKO compared with WT at voltage steps ranging from −10 to +30 mV(449,1079),(482,1101)(482,1077),(515,1100)(515,1079),(549,1101)(549,1079),(582,1102)(582,1080),(615,1103)(615,1082),(648,1105)(648,1082),(681,1105)(681,1082),(714,1106)(714,1082),(747,1106)(747,1082),(780,1106)(780,1082),(813,1106)(813,1082),(846,1106)(846,1082),(879,1106)(879,1082),(912,1106)(912,1082),(945,1106)(945,1082),(978,1106)(978,1082),(1010,1106)(1010,1082),(1043,1106)(1043,1082),(1076,1106)(1076,1082),(1109,1106)(1109,1082),(1142,1106) (pA/pF; WT, −10.0±0.8; n=14; hetKO, −7.6±0.5; n=15; *P<0.05 hetKO versus WT). The parameters I\(_{Ca}\) decayed with a mono-exponential function (Figure 7B, Inset). The time to 50% decay (T\(_{50}\)) was not different between WT and hetKO (T\(_{50}\) in ms, WT, 14.9±1.2; hetKO, 12.7±0.6; *P=0.10 hetKO versus WT). Cell capacitance was unaltered between hetKO and WT (in pF: WT, 132.0±10.5; hetKO, 125.9±5.6; *P=0.60 hetKO versus WT).

To test, whether enhanced Ca\(^{2+}\)-dependent inactivation of I\(_{Ca}\) is responsible for the observed reduction of I\(_{Ca}\) in hetKO, we repeated the measurements of I\(_{Ca}\) in the presence of the Ca\(^{2+}\) buffer 1,2-Bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA; Figure 7C). The cells (WT: n=15; hetKO: n=17) were dialyzed via the pipette solution with BAPTA (10 mmol/L) similar to previously reported studies.\(^{16,17}\) Under these conditions, there was no significant difference in I\(_{Ca}\) amplitude (maximum I\(_{Ca}\) amplitude; in pA/pF: WT, 9.7±0.6; hetKO, 9.1±0.8; *P=0.57 hetKO versus WT).

Figure 4. Irregular Ca\(^{2+}\) release activity in isolated heterozygous knockout of Na+/Ca\(^{2+}\) exchanger (hetKO; n=27) and wild-type (WT; n=30) myocytes during a defined prolonged field stimulation protocol containing rapid changes in pacing cycle lengths and isoproterenol exposure. A, Representative tracings of WT (top) and hetKO (bottom). Two types of irregular Ca\(^{2+}\) activity were distinguished. Spontaneous Ca\(^{2+}\) release (sCR) events, defined as spontaneously occurring isolated Ca\(^{2+}\) transients during diastole and Ca\(^{2+}\) oscillations (OSC), defined as singular or multiple spontaneous re-upstrokes occurring during the decay of a fully developed Ca\(^{2+}\) transient. B and C, Quantification: although OSC occurred more often in WT (C), there was no difference in the rate of occurrence of sCR (B). *P<0.05 hetKO vs WT; n.s., non significant.

Figure 5. Impaired translation of delayed afterdepolarizations (DADs) into spontaneous action potentials (APs) in heterozygous knockout of Na+/Ca\(^{2+}\) exchanger (hetKO) vs wild-type (WT, n=20 each). Membrane potential was recorded in isolated cells in the current clamp mode during a proarrhythmic pacing protocol containing sudden changes in pacing cycle lengths, burst stimulation, and isoproterenol exposure. Spontaneous APs were defined as full APs triggered by a DAD and not by a current clamp command. A, Exemplary tracings: in WT (top) the first AP is stimulated by a voltage clamp command. This is followed by a DAD triggering a spontaneous AP. In hetKO (bottom), the stimulated AP is followed by a DAD that does not trigger a spontaneous AP. B, Exemplary tracings of DADs with enlarged time and voltage scales. C–E (quantification). C There was no difference in the number of cells exhibiting DADs, but the number of cells exhibiting spontaneous APs was significantly smaller in hetKO. The average number of DADs per all investigated cells was also similar between both genotypes (D), whereas the average number of spontaneous APs per all investigated cells was significantly reduced in hetKO (E). *P<0.05 in hetKO vs WT; n.s., non significant.
versus WT; 2-way repeated measures ANOVA; Bonferroni correction).

**Discussion**

This study is the first to evaluate the effect of genetically suppressed NCX activity on the generation of arrhythmia and the underlying cellular mechanisms. It is also the first study to investigate the effects of chronic and specific NCX inhibition on the generation of arrhythmia because synthetic NCX inhibitors lack specificity and—to our knowledge—have only been applied acutely as single shot applications and not chronically in the investigation of arrhythmia.

**Knockout of NCX Suppresses Cellular Proarrhythmia**

Heterozygous KO mice did not show changes in the expression pattern of key Ca\(^{2+}\) handling and structural proteins (Figure 1A) or evidence of cellular hypertrophy as assessed by electric capacitance. Also, SR Ca\(^{2+}\) load (Figure 1D), the diastolic and the systolic Ca\(^{2+}\) levels were unaltered. A previous study has demonstrated normal whole heart cardiac function and morphology and no signs of structural heart disease or clinical cardiovascular pathology in hetKO. We observed a slight but significant reduction of \(I_{\text{tot}}\) in hetKO, however, this can neither explain the reduced AP duration nor the suppression of EADs, because reduced \(I_{\text{tot}}\) would tend to prolong AP duration instead of shortening it.

The observed suppression of afterdepolarizations thus seems to be a genuine consequence of reduced NCX activity—and potentially its direct influence on \(I_{\text{Ca}}\)—activity—and not of long-term remodeling of cellular electrophysiology or Ca\(^{2+}\) homeostasis as an adaption to NCX knockout.

Although in our model both EADs and DADs are suppressed, the molecular mechanisms by which reduction of NCX activity achieves this suppression may differ in both cases and are thus discussed separately in the following.

**NCX Knockout Suppresses Translation of DADs into sAPs**

DADs are triggered by sCR events. DADs are temporary depolarizations of low amplitude that occur during electric diastole and may or may not trigger a spontaneous AP. Thus, in mechanistic terms, the spontaneous AP occurs downstream
of the DAD and therefore the spontaneous AP has to be regarded as the final proarrhythmic substrate or proarrhythmic effector leading to triggered activity and arrhythmia of the whole heart.

The most significant finding of this study is that NCX knockout does not reduce the absolute number of sCR events or DADs, but instead, it drastically reduces the number of spontaneous APs. How is this made possible? Any sCR from the SR will lead to a temporary increase of subsarcolemmal Ca2+ concentration which will drive NCX inward mode. If there are less NCX proteins expressed in the membrane, the peak amplitude of the NCX current will be smaller. At the same time, if the amount of spontaneously released SR Ca2+ is similar, NCX inactivation will be slower, that is, the period of NCX activity will be longer to extrude the same amount of Ca2+, a finding that has been demonstrated in previous studies.5,25-27 This would result in a DAD of smaller amplitude but longer duration as observed in this study (Figure 5). Because the triggering of a spontaneous AP is an all or nothing response, it is the peak amplitude of a DAD and not the absolute number of DADs or the duration of the individual DAD that is crucial for the generation of sAPs. In other words, a small number of high amplitude DADs are more likely to trigger a spontaneous AP than large numbers of low amplitude DADs. In our model, this is the mechanism by which NCX knockout impairs the translation of DADs into spontaneous APs.

**Suppression of EADs: Reduced AP Duration and Interaction of NCX With L-type Ca2+ Current**

It is currently not fully understood whether EADs and DADs share an identical mechanism, ie, whether NCX is involved only in the generation of DADs or also plays a role in the generation of EADs.

The classic concept of NCX-mediated arrhythmia is that during diastole an sCR event is translated via NCX forward mode into a DAD, which subsequently triggers a spontaneous AP, and a similar mechanism has also been proposed for the role of NCX in the generation of EADs. However, the potential mechanisms underlying EADs seem to be more complex and ambiguous. EADs occur during the AP plateau or repolarization. During this phase, a multitude of ion channels are active, and ionic gradients are fluctuating. Thus, several alternative mechanisms have been suggested to contribute to the generation of EADs, including K+ current,28 late Na+ current,29,30 and predominantly I_{Ca,L}.31

Therefore, one of the key findings of this study is that a selective suppression of NCX activity via knockout not only suppresses DADs but also drastically reduces the occurrence of EADs and Ca2+ oscillations (Figures 4 and 6). One obvious mechanism of this phenomenon is that the reduction of the AP duration (Figure 2) may reduce the occurrence of EADs in hetKO because EADs are more likely to occur when there is a prolongation of the AP.31 The reduction of NCX inward current alone may reduce AP duration to a degree where a suppression of EADs is achieved. However, the reduction of I_{Ca,L} activity (Figure 7A and 7B) as observed in this study may well also contribute to the shortening of the AP and thereby to the suppression of EADs because I_{Ca,L} is an inward current and its reduction would thus accelerate repolarization.

If the shortening of the AP was exclusively caused by reduced inward NCX current, one would not expect a reduction of the APD during the early and positive potentials of the AP because under these conditions, NCX reverse mode might be favored. Indeed, the most obvious absolute shortening of the AP was observed during later stages of the AP; however, the acceleration of the repolarization already reached significance at 25% repolarization, which in the spike-like murine AP is still relatively early. It is debatable whether NCX inward current, which is substantially driven by Ca2+ induced SR Ca2+ release, is already fully developed at this early stage. I_{Ca,L} activation precedes SR Ca2+ release and thus maximum activation of NCX inward mode may influence APD earlier than NCX inward mode. Thus, the accelerated repolarization during the early stages of the AP observed in hetKO are most likely caused by the combination of reduced NCX inward current and reduced inward I_{Ca,L}.

Since Ca_{1,2} expression was not altered between both genotypes, we aimed at investigating whether the suppression of I_{Ca,L} in hetKO is caused by a functional interaction between NCX and I_{Ca,L}. One of the strongest inhibitors of I_{Ca,L} is Ca2+-dependent inactivation. We therefore repeated I_{Ca,L} measurements in the presence of the Ca2+ buffer BAPTA. BAPTA suppresses Ca2+-dependent inactivation of I_{Ca,L} because subsarcolemmal Ca2+ is buffered and therefore cannot function as an inhibitor of I_{Ca,L}. In the presence of BAPTA, the differences of I_{Ca,L} amplitude between hetKO and WT were eliminated, suggesting enhanced Ca2+-dependent inactivation as the cause for I_{Ca,L} suppression in hetKO (Figure 7C). These findings are in line with previous studies in other murine models with altered NCX expression.16,17 These observations support the hypothesis that reduced Ca2+ extrusion capacity by reduced NCX activity promotes Ca2+-dependent I_{Ca,L} inactivation by accumulation of Ca2+ in the dyadic cleft. Thus, next to the genetic suppression of NCX inward current, this direct functional interplay between NCX and I_{Ca,L} will also contribute to the observed reduction of AP duration and the suppression of EAD in hetKO. The reduction of I_{Ca,L} is also essential for the cell to maintain Ca2+ homeostasis: because knockout of NCX reduces cellular Ca2+ extrusion, the cell has to limit Ca2+ uptake to avoid Ca2+ overload. This observation is supported by previous findings in a model with homozygous (complete) knockout of NCX.16,22 Further evidence for this adaptive mechanism has been obtained in a model with increased NCX expression in which—complementary to NCX knockout mice—I_{Ca,L} is increased,5,17 thereby preventing cellular Ca2+ loss because of increased Ca2+ removal by increased NCX activity.

The fact that in hetKO I_{Ca,L} is also reduced at higher potentials, where NCX inward mode is unlikely to be active suggests that altered NCX activity may also influence subsarcolemmal Ca2+ during the diastolic part of the excitation contraction cycle.32 Possibly, reduced Ca2+ extrusion during diastole leads to subsarcolemmal Ca2+ accumulation promoting I_{Ca,L} inactivation. This again could result in some Ca2+ channels being inactivated even before or very early during depolarization, which would explain a reduction of I_{Ca,L} even at higher potentials. As
an alternative explanation, intracellular Ca\(^{2+}\) might also affect and modulate the voltage sensor of the L-type-Ca\(^{2+}\) channel, a mechanism that has been suggested by a previous study.\(^{33}\) Future studies are necessary to resolve this issue.

If Ca\(^{2+}\)-dependent inhibition of I\(_{\text{Ca}}\) is more pronounced in the face of reduced NCX expression, one would not only expect a reduced I\(_{\text{Na}}\) amplitude but also an accelerated inactivation of I\(_{\text{Ca}}\). Indeed, this has been observed in homozygous knockout mice\(^{6,25}\) and—conversely—a slowed inactivation was observed in homozygous overexpressor mice.\(^{5,17}\) In our study, we also observed a trend toward accelerated I\(_{\text{Na}}\) inactivation in heterozygous KO mice, however, other than in homozygous KO mice, this trend did not reach statistical significance. One explanation may be that in homozygous KO mice, there is a complete NCX ablation, whereas in the heterozygous KO mice used in this study, NCX is only reduced to about half the normal expression level, thus resulting in a milder effect of NCX suppression on I\(_{\text{Na}}\) inactivation.

Because altered NCX activity may also alter the dynamics of intracellular Na\(^+\) concentration and thereby the Na\(^+\) gradient, we cannot exclude that knock-out of NCX affects the voltage-dependent Na\(^+\) current (I\(_{\text{Na}}\)) or—vice versa—that altered I\(_{\text{Na}}\) will affect NCX activity. This may also influence AP duration and proarrhythmia in the face of altered NCX activity. Future studies are necessary to address this issue.

In contrast to DADs, which exhibited a characteristic all or nothing response that allowed easy separation of DADs from spontaneous APs (Figure 5), this was different in the case of EADs that appeared in several shapes and forms. To apply standard criteria, we defined a EAD as a repolarization to more than ~40 mV during the declining phase of the AP (after the membrane potential had first dropped to less than ~40 mV). Applying these criteria, EADs ranged in shapes from mild temporary membrane fluctuations to steep upstrokes reaching up to potentials of >30 mV (Figure 6A–6C). Because the shapes of EADs were randomly distributed between these extremes, we were unable to mechanistically distinguish EADs from spontaneous APs. This may be a specific problem of murine models, where the plateau of the AP is at a low voltage when compared with higher mammals.\(^{34}\)

As a limitation of this study, a direct transfer of our results to human pathophysiology should only be undertaken with caution, because in humans, myocardial cellular electrophysiology significantly differs from murine electrophysiology as reflected by the much longer action potential. Thus, the pathophysiology of proarrhythmia may also differ between species. Also, as with other genetically modified murine models of arrhythmia, direct comparisons between different murine models may not be always be possible because different pacing protocols and different genetic backgrounds are used.\(^{5,29,35}\)

**Conclusions**

This is the first work to investigate the effect of genetic NCX knockout on arrhythmogenesis and on the underlying molecular and electrophysiological mechanisms. This study demonstrates that chronic and specific NCX knockout directly reduces cellular proarrhythmia mediated by both late and early afterdepolarizations, although the underlying mechanisms downstream of NCX inhibition may differ. NCX knockout mice are thereby one of the few genetic models in which genetic manipulation does not result in a proarrhythmic phenotype but rather a phenotype that is protected against afterdepolarizations. Because cardiac diseases like heart failure are accompanied by NCX overexpression, the findings of this study should encourage further research on NCX suppression as a promising target for clinical pharmacological or genetic therapy to counter arrhythmia.

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**Disclosures**

None.

**References**


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Suppression of Early and Late Afterdepolarizations by Heterozygous Knockout of the Na⁺/Ca²⁺ Exchanger in a Murine Model

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SUPPLEMENTAL MATERIAL

Methods

Generation of heterozygous NCX knockout mice
Heterozygous global NCX knockout mice were generated in the lab of K.D. Philipson (UCLA, California, USA) and have been described previously. These mice were on C57BL/6 background. After import, we performed embryonic transfer for health care and hygienic reasons on CD-1 background. Thus hetKO mice as used in this study were on mixed genetic background. Littermates were used as wildtype (WT) controls. The genotype of every animal entering experimentation was confirmed by polymerase chain reaction (PCR). Breeding, housing and experimentation were performed in accordance to the regulations of the Westfälische Wilhelms-University Münster. Animals used for experimentation were between 8 and 17 weeks of age.

Immunoblot analysis
Immunoblot analysis on ventricular homogenates was performed with minor modifications as previously reported. The following amounts of homogenate protein were applied for immunoblot analysis: NCX: 200 µg; troponin I (Tni), junctin (Jcn), phospholamban (Plb), calsequestrin (Csq), sarcoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA), triadin (Trd), alpha 1C subunit of L-type Ca\(^{2+}\) channel (Ca\(\text{V}1.2\)): 100 µg; ryanodine receptor (RyR) and phosphorylated ryanodine receptor (RyRp): 250 µg. The following specific antibodies were used: NCX (R3F1 SWANT, Bellinzona, Switzerland); Tni (4002 Cell Signaling Technology, Inc. Danvers, USA); Plb (05-205 Merck Millipore, Billerica, USA); CsQ (PA1-913 Thermo Scientific, Waltham, USA); RyRp (A010-30 Badrilla, Leeds, United Kingdom); Ca\(\text{V}1.2\) (ACC-003, Alomone labs, Jerusalem, Israel). Dr. L. R. Jones (Indianapolis, USA) kindly provided the antibodies used for detection of RyR, SERCA, Trd and Jcn. Signal intensity was normalized to the housekeeping protein Csq.

Isolation of ventricular cardiomyocytes
Mice were sedated and euthanized with carbon dioxide inhalation. After thoracotomy the beating heart was explanted and the aorta was cannulized immediately. The heart was connected to a Langendorff apparatus and perfused with a buffer containing heparin (14.3 IE/ml). Single ventricular cardiomyocytes were isolated as reported previously.

Cellular electrophysiology
200 µl of the cell suspension was added to 2 ml of extracellular solution into an experimental chamber mounted on a Leika DMIL inverted microscope. Measured cells were continuously exposed to a laminar flow using a perfusion pencil (AutoMate Scientific, Berkeley, USA). An EPC 800 amplifier and an InstruTECH ITC-18 (both HEKA, Bellmore, USA) data acquisition system were controlled by PatchMaster v2x53 software (HEKA, Bellmore, USA). Patch pipettes GB150TF-8P (Science Products, Hofheim, Germany) were pulled with a P-97 Micropipette Puller (Sutter Instruments, Novato, USA) to a tip resistance of 2.5-3.5 MΩ. AP measurements and the potassium carried outward current were conducted using the perforated patch clamp technique. Therefore, the tip was filled with intracellular solution and intracellular solution containing amphotericin B was added via backfill technique to a final concentration of 260 µg/L. When recording $I_{Ca}$, the ruptured patch clamp method was used in absence of amphotericin B.

**Cellular electrophysiology solutions (in mM):**

*AP recordings: Extracellular:* NaCl 136.0, KCl 5.4, NaH$_2$PO$_4$ 0.33, CaCl$_2$ 1.0, MgCl$_2$ 1.0, HEPES 10.0, Glucose 10.0. *Intracellular:* NaCl 90.0, KCl 5.0, KOH 35.0, MgATP 2.5, EGTA 1.0, HEPES 5.0.

$I_{Ktot}$ recordings: Extracellular: NaCl 136.0, KCl 5.4, NaH$_2$PO$_4$ 0.33, CaCl$_2$ 1.0, MgCl$_2$ 1.0, HEPES 10.0, Glucose 10.0, nifedipine 2 µM, tetrodotoxin 10 µM.

*Intracellular:* NaCl 5.0, KCl 90.0, KOH 35.0, MgATP 2.5, EGTA 1.0, HEPES 5.0.

$I_{Ca}$ recordings: Extracellular: NaCl 136.0, KCl 5.4, HEPES 10.0, MgCl$_2$ 1.0, NaH$_2$PO$_4$ 0.33, CaCl$_2$ 1.0, Glucose 10.0. *Intracellular:* CsCl 120.0, TEA-Cl 10.0, NaCl 10.0, HEPES 20.0, MgATP 5.0, cAMP 0.05.

$I_{Ca}$ recordings with BAPTA: Extracellular: NaCl 136.0, KCl 5.4, HEPES 10.0, MgCl$_2$ 1.0, NaH$_2$PO$_4$ 0.33, CaCl$_2$ 1.0, Glucose 10.0. *Intracellular:* CsCl 120.0, TEA-Cl 10.0, NaCl 10.0, HEPES 20.0, MgATP 5.0, cAMP 0.05, BAPTA 10. pH was adjusted to 7.4 in all solutions.

**Cellular Ca$^{2+}$ imaging**

100 µl of the cell suspension was incubated with the Ca$^{2+}$ dye using either 100 µl of fluo-4-AM (4 µM) (Invitrogen, Life Technologies, Darmstadt, Germany) and 2 µl of the non-ionic surfactant pluronic (Invitrogen, Life Technologies, Darmstadt, Germany) or 100 µl of indo-1-AM (23.3 µM) (Invitrogen, Life Technologies, Darmstadt, Germany) and 4 µl pluronic for 10 minutes at room temperature. The incubated cells were placed into an experimental chamber (1 ml) and perfused with the following bath solution (in mM) NaCl 140.0, CaCl$_2$ 2.0, KCl 5.8, KH$_2$PO$_4$ 0.5, Na$_2$HPO$_4$ 0.4, MgSO$_4$ 0.9, HEPES 10.0, Glucose 11.1, pH 7.4 was adjusted with NaOH. For rapid caffeine (10 mM) and isoproterenol (1 µM) application a SF-77B stimulus solution delivery system (Warner Instruments, Hamden, USA) was used. Fluorescence emission at 522 nm (fluo-4-AM) or 405 nm and 495 nm wavelength (indo-1-
AM) was taken as an indicator for the cytosolic Ca\(^2+\) concentration. The fluorescence emission was plotted in arbitrary light units. Whenever the Ca\(^2+\) signal was directly compared between separate cells, the ratio of the fluorescence signal of indo-1 was used for this subset of experiments. We used a dual-emission microfluorescence system (Photon Technologies Inc., South Brunswick, USA) connected to an Olympus IX50 microscope and Felix 1.42 data processing software (Photon Technologies Inc., South Brunswick, USA).

References

