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

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Background—The Na⁺/Ca²⁺ exchanger (NCX) has been implied to cause arrhythmias. To date, information on the role of NCX in arrhythmogenesis derived from models with increased NCX expression, hypertrophy, and heart failure. Furthermore, the exact mechanism by which NCX exerts its potentially proarrhythmic effect, ie, by promoting early afterdepolarization (EAD) or delayed afterdepolarization (DAD) or both, is unknown.

Methods and Results—We investigated isolated cardiomyocytes from a murine model with heterozygous knockout of NCX (hetKO) using the patch clamp and Ca²⁺ imaging techniques. Action potential duration was shorter in hetKO with I_{Ktot} not being increased. The rate of spontaneous Ca²⁺ release events and the rate of DADs were unaltered; however, DADs had lower amplitude in hetKO. A DAD triggered a spontaneous action potential significantly less often in hetKO when compared with wild-type. The occurrence of EADs was also drastically reduced in hetKO. I_{Ca} activity was reduced in hetKO, an effect that was abolished in the presence of the Ca²⁺ buffer BAPTA.

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 Ca²⁺-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⁺/Ca²⁺ exchanger

The Na⁺/Ca²⁺ exchanger (NCX) works as the main Ca²⁺ removal mechanism of the cardiac myocyte, extruding 1 Ca²⁺ 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 Ca²⁺ 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 diastole.

A DAD is thought to be initiated by a spontaneous sarcoplasmic Ca²⁺ release (sCR) event that is translated via NCX inward mode into a depolarizing inward current forming a DAD.¹ 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 Ca²⁺ influx via L-type Ca²⁺ channels (I_{L}) during the repolarization phase may re-depolarize the membrane potential resulting in an EAD.²,³ However, others have suggested that DADs and EADs share a common mechanism that may be NCX dependent.⁴

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

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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.

Increased NCX expression\(^3\) and to animal models of heart failure or hypertrophy\(^6\) in which NCX is known to be overexpressed. Yet, in heart failure, a multitude of proarrhythmic alterations other than the upregulation of NCX has been identified,\(^11\) most importantly a reduction of K\(^+\) currents\(^12,13\) which promote a prolongation of AP duration and the occurrence of 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. If indeed genetic inhibition of NCX protects from afterdepolarizations, the question remains by which molecular mechanisms this protection is mediated. This could be a suppression of DADs, EADs, or even both, bearing the potential thereby possibly causing ventricular arrhythmia.

Reduced NCX Activity in hetKO

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.\(^14\) 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\(^4\) 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\(^5\) 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 I\(_{\text{Na}}\), the ruptured clamp method was used. For details, including intracellular and extracellular solutions, see Data Supplement.

Cellular Ca\(^{2+}\) 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 Ca\(^{2+}\) level. 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. 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 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 Ca\(^{2+}\) Handling and Structural Proteins

To confirm reduced NCX expression in heterozygous NCX knockout mice and to detect potential alterations of key Ca\(^{2+}\) 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), Ca\(_{\text{v}}\)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
The decay kinetics of the caffeine-induced Ca\textsuperscript{2+} transient serves as a direct measure of the Ca\textsuperscript{2+} extrusion capacity mediated via NCX. Ca\textsuperscript{2+} removal, obtained as time to half decay of the caffeine-induced Ca\textsuperscript{2+} transient (T\textsubscript{50}), was significantly slowed in hetKO compared with WT confirming functionally reduced NCX activity (T\textsubscript{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\textsuperscript{2+} (10 mmol/L; T\textsubscript{50} in s: WT, 4.0±0.6; n=12; P<0.05 versus basal; 1-way ANOVA test; 2-sided Dunnett’s method).

**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).

**Unaltered Amplitude of the Systolic Ca\textsuperscript{2+} Transient in hetKO**

The amplitude of the systolic Ca\textsuperscript{2+} transient was not altered between both genotypes (amplitude of the systolic Ca\textsuperscript{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\textsuperscript{2+} levels between both genotypes.

**Unaltered SR Ca\textsuperscript{2+} Content in hetKO**

The peak amplitude of the caffeine-induced Ca\textsuperscript{2+} transient is a measure for SR Ca\textsuperscript{2+} content.

The amplitude of the caffeine-induced Ca\textsuperscript{2+} transient was unaltered suggesting no difference in the SR Ca\textsuperscript{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).

**Figure 1.** Reduced Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX) expression and activity in heterozygous knockout of NCX (hetKO) vs wild-type (WT). **A,** Immunoblot analysis: NCX expression was reduced in hetKO, whereas there was no significant alteration in the expression of other key Ca\textsuperscript{2+} handling and structural proteins (CsQ indicates calsequestrin; RyR, ryanodine receptor; RyRp, phosphorylated ryanodine receptor; CaV1.2, alpha 1C subunit of the L-type calcium channel; SERCA, sarcoendoplasmic reticulum Ca\textsuperscript{2+} ATPase; Jcn, junctin; Trd, triadin; Plb, phospholamban; and Tni, troponin I [n=5–8 for hetKO and WT for each protein; quantification by densitometry]). **B,** Caffeine-induced Ca\textsuperscript{2+} transients. Ca\textsuperscript{2+} removal was slowed in hetKO (n=16) when compared with WT (n=14), confirming reduced NCX activity. The effect of genetic NCX inhibition was reproduced in WT by adding the NCX antagonist Ni\textsuperscript{2+} (n=12). **C,** Quantification of time to half decay of the caffeine-induced Ca\textsuperscript{2+} transient (T\textsubscript{50}) in hetKO and WT w/o Ni\textsuperscript{2+}. **D,** Quantification of the amplitude of the caffeine-induced Ca\textsuperscript{2+} transient which serves as a measure for sarcoplasmic reticular Ca\textsuperscript{2+} content (hetKO [n=15]; WT [n=16]). *P<0.05 hetKO vs WT; +P<0.05 WT+Ni\textsuperscript{2+} vs WT basal; n.s., non significant.

**Figure 2.** Shortened action potential (AP) duration in heterozygous knockout of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (hetKO). **A,** Representative AP recordings obtained in isolated cardiomyocytes using the patch clamp method. **B,** Quantification of the time to 50% repolarization of the AP (AP\textsubscript{D50}) in hetKO [n=20]; WT [n=20]; *P<0.05 hetKO vs wild-type (WT).
n=20; hetKO: 110.9±4.1, n=20; P=0.54 hetKO versus WT) were unaltered between hetKO and WT.

**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). 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: WT, 53.6±9.7; hetKO: 79.6±10.3; P=0.08 hetKO versus WT). Cell capacitance was unaltered between hetKO and WT (in pF: WT, 117.2±9.0; hetKO: 122.2±8.3; P=0.69 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 S₂-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; Figure 6E).

Reduced L-type Ca²⁺ 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 in mV to inhibit INa and then depolarized by square wave pulses ranging from −30 to +40 mV (Figure 7A). Peak current was normalized to cell capacitance. Pipette solution contained cesium chloride (120 mmol/L) and tetrathyamine monium 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 Ca²⁺ buffers.

I_{Ca} amplitude was found to be reduced in hetKO compared with WT at voltage steps ranging from −10 to +30 mV (+10 mV; in pA/pF; WT, −10.0±0.8; n=14; hetKO, −7.6±0.5; n=15; P<0.05 hetKO versus WT). Inactivation kinetics of I_{Ca} as time to 50% decay (T_{50}) were not different between WT and hetKO (T_{50} in ms: WT, 14.5±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).

Figure 4. Irregular Ca²⁺ release activity in isolated heterozygous knockout of Na⁺/Ca²⁺ 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 Ca²⁺ activity were distinguished. Spontaneous Ca²⁺ release (sCR) events, defined as spontaneously occurring isolated Ca²⁺ transients during diastole and Ca²⁺ oscillations (OSC), defined as singular or multiple spontaneous re-upstrokes occurring during the decay of a fully developed Ca²⁺ 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⁺/Ca²⁺ 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 triggered 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). 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\(_{\text{Ks}}\) in hetKO, however, this can neither explain the reduced AP duration nor the suppression of EADs, because reduced I\(_{\text{Ks}}\) 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 IC\(_{\text{a}}\) 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.

**Figure 6.** Early afterdepolarizations (EADs) in wild-type (WT) and heterozygous knockout of Na\(^+\)/Ca\(^{2+}\) exchanger (hetKO; n=20 each) during the pacing protocol used in Figure 5. The EAD shape varied between mild temporary membrane fluctuations (A), spike-like depolarizations (B), and steep upstrokes (C). D and E (quantification), Both the fraction of cells exhibiting EADs (D), and the number of EADs per all investigated cells (E) was significantly reduced in hetKO vs WT. *P<0.05.

**Figure 7.** Voltage-gated Ca\(^{2+}\) currents (I\(_{\text{Ca}}\)) in heterozygous knockout of Na\(^+\)/Ca\(^{2+}\) exchanger (hetKO; n=15) vs wild-type (WT; n=14). A, Exemplary tracings. Cells were held at −40 mV and subjected to square wave pulses of 400 ms ranging from −30 to +40 mV. B, Quantification of I\(_{\text{Ca}}\) amplitude in the absence of a Ca\(^{2+}\) buffer and (C) in the presence of BAPTA (WT: n=15; hetKO: n=17) normalized to cell capacitance and plotted against voltage commands. *P<0.05 in hetKO vs WT; 2-way repeated measures ANOVA; Bonferroni correction.
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 Ca\(^{2+}\) 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 Ca\(^{2+}\) is similar, NCX inactivation will be slower, that is, the period of NCX activity will be longer to extrude the same amount of Ca\(^{2+}\), 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 Ca\(^{2+}\) 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 populations,\(^{28}\) late Na\(^{+}\) current,\(^{29,30}\) and predominantly I\(_{\text{Ca}}\).\(^{5}\)

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 Ca\(^{2+}\) 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\(_{\text{Ca}}\) 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\(_{\text{Ca}}\) 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 Ca\(^{2+}\) induced SR Ca\(^{2+}\) release, is already fully developed at this early stage. I\(_{\text{Ca}}\) activation precedes SR Ca\(^{2+}\) 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\(_{\text{Ca}}\).

Since Ca\(_{V1.2}\) expression was not altered between both genotypes, we aimed at investigating whether the suppression of I\(_{\text{Ca}}\) in hetKO is caused by a functional interaction between NCX and I\(_{\text{Ca}}\). One of the strongest inhibitors of I\(_{\text{Ca}}\) is Ca\(^{2+}\)-dependent inactivation. We therefore repeated I\(_{\text{Ca}}\) measurements in the presence of the Ca\(^{2+}\) buffer BAPTA. BAPTA supresses Ca\(^{2+}\)-dependent inactivation of I\(_{\text{Ca}}\) because subsarcolemmal Ca\(^{2+}\) is buffered and therefore cannot function as an inhibitor of I\(_{\text{Ca}}\). In the presence of BAPTA, the differences of I\(_{\text{Ca}}\) amplitude between hetKO and WT were eliminated, suggesting enhanced Ca\(^{2+}\)-dependent inactivation as the cause for I\(_{\text{Ca}}\) 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 Ca\(^{2+}\) extrusion capacity by reduced NCX activity promotes Ca\(^{2+}\)-dependent I\(_{\text{Ca}}\) inactivation by accumulation of Ca\(^{2+}\) in the dyadic cleft. Thus, next to the genetic suppression of NCX inward current, this direct functional interplay between NCX and I\(_{\text{Ca}}\) will also contribute to the observed reduction of AP duration and the suppression of EAD in hetKO. The reduction of I\(_{\text{Ca}}\) is also essential for the cell to maintain Ca\(^{2+}\) homeostasis: because knockout of NCX reduces cellular Ca\(^{2+}\) extrusion, the cell has to limit Ca\(^{2+}\) uptake to avoid Ca\(^{2+}\) 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\(_{\text{Ca}}\) is increased,\(^{5,17}\) thereby preventing cellular Ca\(^{2+}\) loss because of increased Ca\(^{2+}\) removal by increased NCX activity.

The fact that in hetKO I\(_{\text{Ca}}\) is also reduced at higher potentials, where NCX inward mode is unlikely to be active suggests that altered NCX activity may also influence subsarcolemmal Ca\(^{2+}\) during the diastolic part of the excitation contraction cycle.\(^{32}\) Possibly, reduced Ca\(^{2+}\) extrusion during diastole leads to subsarcolemmal Ca\(^{2+}\) accumulation promoting I\(_{\text{Ca}}\) inactivation. This again could result in some Ca\(^{2+}\) channels being inactivated even before or very early during depolarization, which would explain a reduction of I\(_{\text{Ca}}\) 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 \(I_{\text{Ca}}\) 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 \(I_{\text{Ca}}\) 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 \(I_{\text{Ca}}\) inactivation.

Because altered NCX activity may also alter the dynamics of intracellular Na"^+ concentration and thereby the Na"^+ gradient, we cannot exclude that knockdown 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 an 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.\(^{5,20,35}\)

**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.

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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), sarco(-endo)plasmic reticulum Ca²⁺ ATPase (SERCA), triadin (Trd), alpha 1C subunit of L-type Ca²⁺ channel (CaV1.2): 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 Q (PA1-913 Thermo Scientific, Waltham, USA); RyRp (A010-30 Badrilla, Leeds, United Kingdom); CaV1.2 (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, NaCl 5.0, MgATP 2.5, EGTA 1.0, HEPES 5.0.  

**$I_{K_{tot}}$ 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 \( \text{Ca}^{2+} \) concentration. The fluorescence emission was plotted in arbitrary light units. Whenever the \( \text{Ca}^{2+} \) 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

