IJC Heart & Vasculature 44 (2023) 101168

Contents lists available at ScienceDirect

IJC Heart & Vasculature

journal homepage: www.sciencedirect.com/journal/ijc-heart-and-vasculature

Increased *in vivo* perpetuation of whole-heart ventricular arrhythmia in heterozygous Na^+/Ca^{2+} exchanger knockout mice

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ARTICLE INFO

Keywords:
Arrhythmia mechanisms
Antiarrhythmic strategies
Na^+/Ca^{2+} exchanger
Ventricular arrhythmia

ABSTRACT

Aims: Na^+/Ca^{2+} exchanger (NCX) upregulation in cardiac diseases like heart failure promotes as an independent proarrhythmic factor early and delayed afterdepolarizations (EADs/DADs) on the single cell level. Consequently, NCX inhibition protects against EADs and DADs in isolated cardiomyocytes. We here investigate, whether these promising cellular *in vitro* findings likewise apply to an *in vivo* setup.

Methods/Results: Programmed ventricular stimulation (PVS) and isoproterenol were applied to a murine heterozygous NCX-knockout model (KO) to investigate ventricular arrhythmia initiation and perpetuation compared to wild-type (WT). KO displayed a reduced susceptibility towards isoproterenol-induced premature ventricular complexes. During PVS, initiation of single or double ectopic beats was similar between KO and WT. But strikingly, perpetuation of ventricular tachycardia (VT) was significantly increased in KO (animals with VT - KO: 82 %; WT: 47 %; $p = 0.0122$ / median number of VTs - KO: 4.5 (1.0, 6.25); WT: 0.0 (0.0, 4.0); $p = 0.0039$). The median VT duration was prolonged in KO (in s; KO: 0.38 (0.19, 0.96); WT: 0.0 (0.0, 0.60); $p = 0.0239$). The ventricular refractory period (VRP) was shortened in KO (in ms; KO: 15.1 ± 0.7; WT: 18.7 ± 0.7; $p = 0.0013$).

Conclusions: Not the initiation, but the perpetuation of provoked whole-heart *in vivo* ventricular arrhythmia was increased in KO. As a potential mechanism, we found a significantly reduced VRP, which may promote perpetuation of reentrant ventricular arrhythmia. On a translational perspective, the antiarrhythmic concept of therapeutic NCX inhibition seems to be ambivalent by protecting from initiating afterdepolarizations but favoring arrhythmia perpetuation *in vivo* at least in a murine model.

1. Introduction

Evaluation of molecular proarrhythmic mechanisms and novel therapeutic concepts in translational arrhythmia research is commonly performed via an *in vitro* approach using single cells or less common multicellular preparations [1,2]. Despite frequent promising *in vitro* findings, these results may not translate to the whole-heart level and especially in human (patho-)physiology, due to complex intercellular interactions and interspecies differences [3–5].

The cardiac Na^+/Ca^{2+} exchanger (NCX) represents the main Ca^{2+} removal mechanism in cardiomyocytes, generating a net depolarizing inward current due to extrusion of 1 Ca^{2+} ion in exchange for 3 Na^+ ions entering the cell. NCX upregulation is observed in major cardiac diseases (e.g. non-paroxysmal atrial fibrillation [6] or heart failure [7]). In

Abbreviations: NCX, Na^+/Ca^{2+} exchanger; EAD, Early afterdepolarization; DAD, Delayed afterdepolarization; KO, Heterozygous Na^+/Ca^{2+} exchanger knockout mouse model; PCR, Polymerase chain reaction; EPS, Electrophysiological study; CorrSNRP, Corrected sinus node recovery period; WBP, Wenckebach periodicity; AV, Atrioventricular; AVNRP, AV-nodal refractory period; VT, Ventricular tachycardia; PVS, Programmed ventricular stimulation; CL, Cycle length; VRP, Ventricular refractory period; SEM, Standard error of the mean; IQR, Interquartile range; WT, Wild-type; PVC, Premature ventricular complex; I_{Ca}, voltage-dependent L-type Ca^{2+}-current.

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https://doi.org/10.1016/j.ijcha.2022.101168
Received 8 December 2022; Accepted 24 December 2022

previous single cell studies, we demonstrated, that NCX upregulation represents an independent proarrhythmic factor promoting cellular early and delayed afterdepolarizations (EADs/DADs) in ventricular cardiomyocytes [8]. In particular, we observed that genetic NCX inhibition via heterozygous NCX-knockout (KO) protects against EADs in ventricular cardiomyocytes [9] and the translation of DADs in spontaneous action potentials (AP) in ventricular [9] and atrial cardiomyocytes [10]. Furthermore, pharmacological inhibition of NCX in a

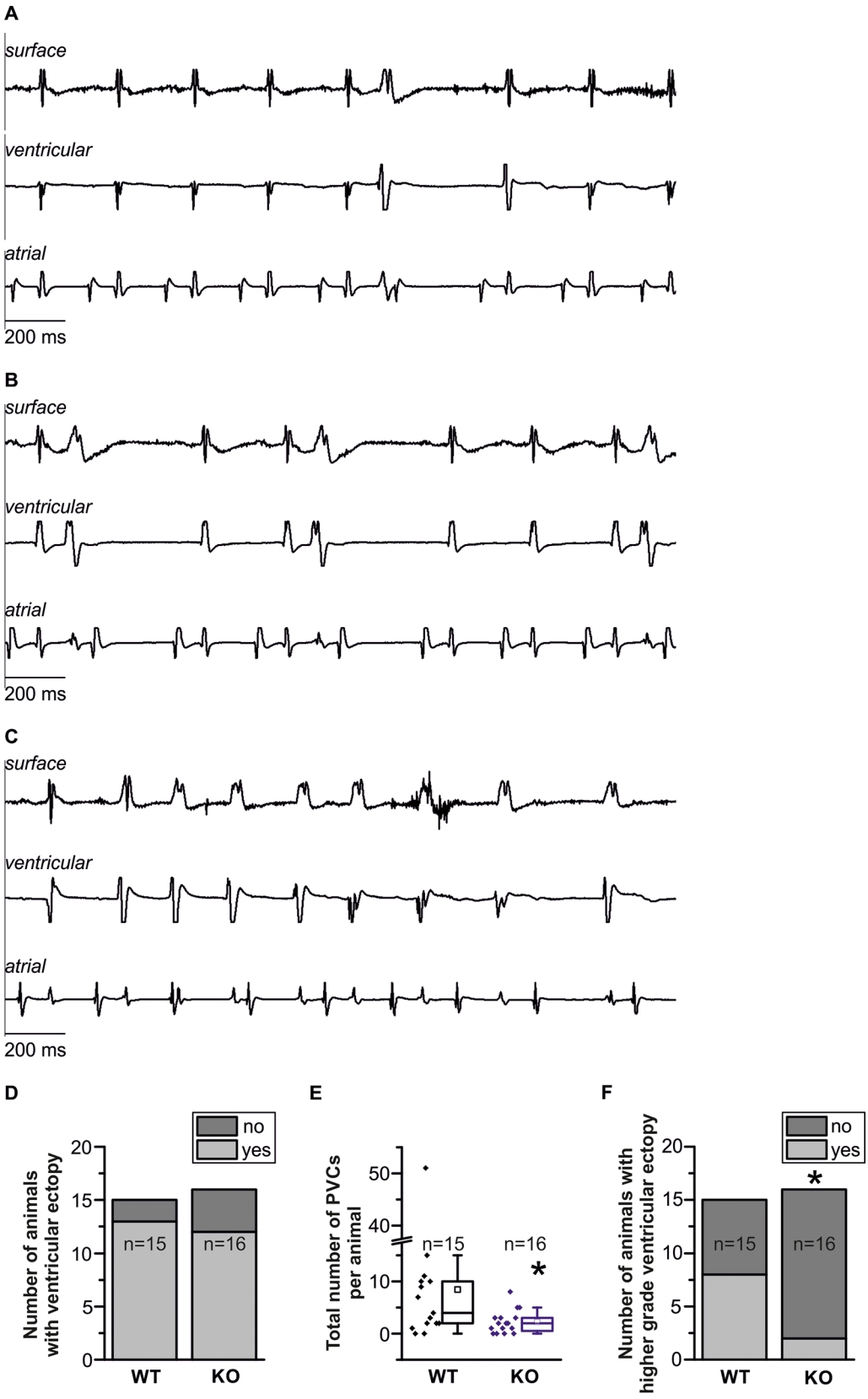

Fig. 1. Isoproterenol-mediated initiation of ventricular arrhythmia. (**A-C**) Representative examples of ventricular arrhythmia following isoproterenol application in a WT mouse (**A**) and an NCX-KO littermate (**B-C**). (**D**) Number of animals displaying $\geq$ 1 premature ventricular contraction (PVC) after intravenous administration of 10 mg/kg isoproterenol. (**E**) Total number of PVCs, occurring isolated or consecutively. (**F**) Number of animals with higher grade ectopic ventricular activity, defined as at least 10 isolated PVCs or a single episode with $\geq$ 3 consecutive PVCs.

transgenic NCX-overexpressor mouse model suppressed the number of proarrhythmic spontaneous Ca^{2+} release events [11]. Taken together, these cellular *in vitro* findings support the concept of NCX inhibition as a potential promising novel antiarrhythmic strategy.

But so far, it is unknown whether NCX inhibition can also protect from arrhythmia *in vivo* on the whole-heart level. Thus, we here investigate the *in vivo* initiation and perpetuation of whole-heart ventricular arrhythmia by electrophysiological studies using the previously investigated heterozygous NCX-KO mouse model that is protected against proarrhythmic afterdepolarizations at the single cell level.

2. Methods

2.1. Animals

Male and female, 9.0–12.5 weeks-old mice (mixed genetic background / CD1 and C57BL/6) were investigated. The animals were maintained at 22 °C with a 12 h light/dark cycle and received drinking water and chow ad libitum. The genotype of experimental animals was verified by polymerase chain reaction (PCR). The animal experiments confirmed to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and were approved by the National Office for Nature, Environment and Consumer Protection in Recklinghausen, Nordrhein-Westfalen (Permit Number: 81–02.04.2017.A476).

2.2. Electrophysiological study (EPS)

Following intraperitoneal injection of buprenorphine (0.1 mg/kg; 30 min prior to the procedure), electrophysiological studies were performed under inhalation anesthesia as described previously [12,13]. Briefly, a 2 French octapolar mouse electrophysiological catheter (Cib'er Mouse, NuMed Inc., New York, USA) was advanced into the right heart chambers via the right jugular vein with proximal bipolar electrograms showing atrial and distal electrograms ventricular signals (Fig. 2*A*). Electrical stimulation of the heart was performed via rectangular stimulus pulses of 1 mA for 1 ms.

Corrected sinus node recovery period (corrSNRP; interval between the last electrical stimulus and the first spontaneous atrial signal following 5 s of fixed rate atrial pacing subtracted by spontaneous sinus rate after stimulation), Wenckebach periodicity (WBP; cycle length (CL) at first appearance of cyclic atrio-ventricular (AV)-nodal conduction block) and AV-nodal refractory period (AVNRP; longest S1S2 CL with AV-nodal conduction block) were analyzed by performing fixed rate and extra stimulus pacing. Programmed stimulation maneuvers were used for evaluating the inducibility of ventricular tachycardia (VT), defined as $\geq$ 3 ectopic ventricular beats. VT duration was defined as duration from last stimulus spike until return to isoelectric baseline. Furthermore, induction of single or double (couplet) ectopic ventricular beats was quantified to study arrhythmia initiation. Of note, the last stimulus-induced ventricular complex was not counted as ectopic beat, since it was triggered by electrical stimulation.

Programmed ventricular stimulation (PVS): Extra stimulus pacing with one extra stimulus (seven times S1S1 CLs: 110 ms and 100 ms; S1S2 50 ms) with stepwise reduction of the final coupling interval S1S2 as follows 50; 40; 30; 25; 20; 15; 10 ms. After occurrence of first loss of capture, CL was increased by 6 ms with subsequent reduction in 2 ms steps until ventricular refractory period (VRP; longest S1S2 with missing ventricular capture) was reached.

For evaluation of non-PVS-triggered catecholamine-induced arrhythmia, 10 mg/kg isoproterenol was slowly injected via the inner lumen of the EP catheter into the right ventricle and the following 10 min period was analyzed for occurrence of ectopic ventricular activity [14]. Following EPS mice were euthanized by cervical dislocation.

2.3. Statistical analysis

All data were analyzed for normal distribution using the Kolmogorov Smirnov normality test and D'Agostino & Pearson omnibus normality test. Normally distributed continuous variables were analyzed via the unpaired student's *t*-test, whereas for non-normally distributed variables the Mann-Whitney *U* test was applied. Data are given as mean ± standard error of the mean (SEM) or as median value with interquartile range (IQR; between the 25th and 75th percentile), where appropriate. The bottom and top edges of the box plots represent the 25th and 75th percentiles respectively and the lower and upper whiskers give the 10th and 90th percentiles respectively. The line and the square within the boxes indicate the median and mean values respectively. Statistical analysis of categorical variables was performed using the Fisher's exact test. A p-value < 0.05 was considered statistically significant.

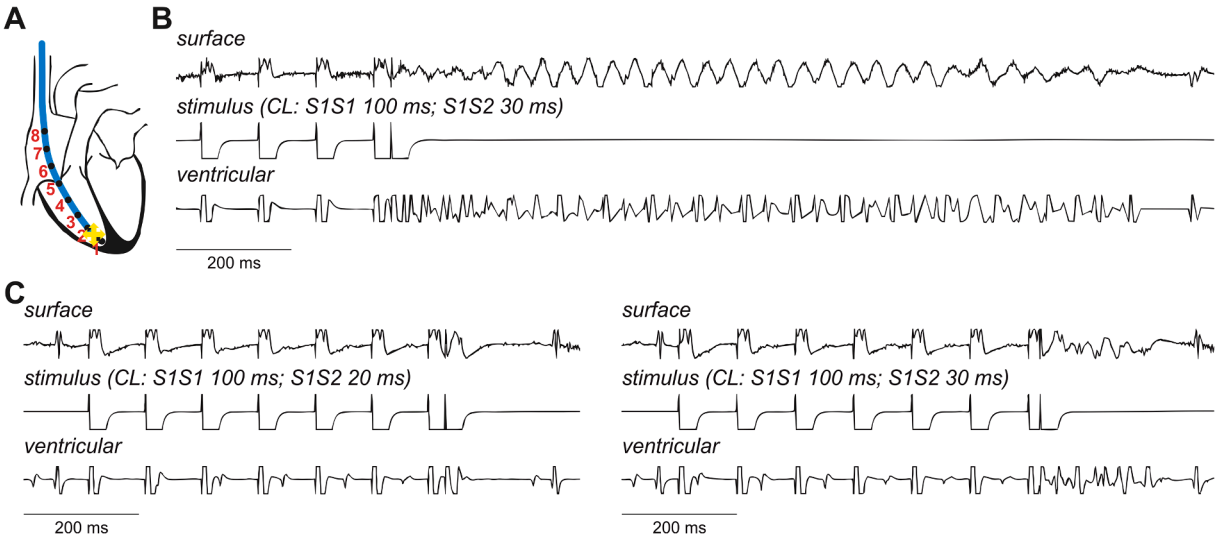

Fig. 2. PVS-mediated initiation of ventricular arrhythmia. (**A**) An octopolar mouse electrophysiology catheter was introduced via the right internal jugular vein and superior vena cava into the right sided heart chambers. Ventricular tachycardia (VT) inducibility was analyzed by standardized programmed electrical stimulation (PVS) between the two distal electrodes as indicated. (**B**) Representative example of PVS in a NCX-KO mouse. Note the induction of VT following a short-coupled extra stimulus (S1S2 30 ms). (**C**) Representative examples of PVS in a WT littermate. No (left) or only a short-lasting VT (right) was induced by PVS.

4. Results

4.1. Basal electrophysiological parameters in KO and wild-type mice

Wild-type (WT) and KO mice of comparable gender distribution, age and weight were analyzed (Table 1). Heart rate, P-wave duration and PQ interval were comparable between both genotypes (Table 1). KO mice revealed a shortened QRS duration (in ms; KO: 8.7 ± 0.1; WT: 9.4 ± 0.1; p = 0.0004) and QTc interval (in ms; KO: 46.5 ± 1.2; WT: 53.2 ± 1.0; p = 0.0001). Invasive electrophysiological parameters for intracardiac conduction, sinus-nodal and AV-nodal function were comparable between WT and KO mice (Table 1).

4.2. Isoproterenol-mediated initiation of ventricular arrhythmia

After administration of isoproterenol, without programmed ventricular stimulation, spontaneous single premature ventricular complexes (PVC) or runs of up to seven consecutive ectopic ventricular beats separated by a short isoelectric baseline (Fig. 1*A-C*) were observed in 87 % of WT und 75 % of KO mice (p = 0.6539; Fig. 1*D*). When counting the total number of PVCs, irrespective whether occurring isolated or sequentially, KO displayed a lower rate compared to WT (KO: 2.0 (0.25, 3.0); WT: 4.0 (2.0, 10.0); p = 0.0460; Fig. 1*E*). A high degree of ectopic ventricular activity, defined as at least 10 isolated PVCs or a single episode with ≥ 3 consecutive PVCs, was more frequently observed in WT (KO: 12.5 %; WT: 53 %; p = 0.0233; Fig. 1*F*).

4.3. Increased arrhythmia perpetuation in KO versus WT mice

During invasive EPS (Fig. 2*A*) mice were tested for inducibility of VT by PVS. Fig. 2*B* depicts a representative tracing consisting of surface ecg and intracardiac ventricular recording in a KO mouse with induction of sustained VT following PVS using a single extra stimulus. The same protocol in WT littermates resulted in no or only minor sustained ventricular arrhythmia as illustrated in the representative Fig. 2*C*. Initiation of single ectopic beats (KO: 55 %; WT: 68 %; p = 0.4009) or couplets (KO: 41 %; WT: 35 %; p = 0.7797) was similar between KO and WT. But occurrence of sustained VT (≥3 consecutive ectopic beats) was significantly increased in KO (82 %) compared to WT (47 %) mice (p = 0.0122). Induction of at least one single ectopic beat, a couplet or one episode of VT was similar between the groups (KO: 100 %; WT: 85 %; p = 0.1448).

To better differentiate between parameters of initiation and perpetuation of ventricular arrhythmia we distinguished between the number of single or double (couplet) ectopic beats (reflecting the susceptibility towards arrhythmia initiation) and sustained VTs (rather reflecting perpetuation), for each individual animal respectively, as well as median and cumulative VT duration. During PVS KO had comparable numbers of single ectopic beats (KO: 1.0 (0.0, 2.0); WT: 1.0 (0.0, 2.0); p = 0.3837; Fig. 3*A*) and couplets (KO: 0.0 (0.0, 2.25); WT: 0.0 (0.0, 1.0); p = 0.5048; Fig. 3*B*), but displayed a significant increase in the number of sustained VTs (KO: 4.5 (1.0, 6.25); WT: 0.0 (0.0, 4.0); p = 0.0039; Fig. 3*C*). Cumulative VT duration (in s; KO: 1.55 (0.33, 5.76); WT: 0.0 (0.0, 1.88); p = 0.0136; Fig. 3*D*) and median VT duration (in s; KO: 0.38 (0.19, 0.96); WT: 0.0 (0.0, 0.60); p = 0.0239; Fig. 3*E*) were prolonged in NCX-KO mice.

Table 1
Baseline parameters, surface ECG and invasive *EPI* parameters.

	WT (n = 34)	KO (n = 22)	p-value
baseline parameters			
male [%]	64.7	63.6	1.0000
age [weeks]	10.2 ± 0.1	10.1 ± 0.1	0.3184
weight [g]	23.2 ± 0.5	23.1 ± 0.5	0.8448
6-lead surface ECG			
heart rate [bpm]	495.9 ± 11.8	495.8 ± 13.4	0.9926
P [ms]	18.7 ± 0.2	18.2 ± 0.3	0.1734
PQ [ms]	38.8 ± 0.6	37.4 ± 0.7	0.1367
QRS [ms]	9.4 ± 0.1	8.7 ± 0.1	0.0004
QT [ms]	59.2 ± 1.7	51.6 ± 1.8	0.0042
QTc [ms]	53.2 ± 1.0	46.5 ± 1.2	0.0001
invasive electrophysiological parameters			
AV [ms]	44.0 ± 0.8	44.0 ± 1.4	1.0000
AH [ms]	35.8 ± 0.9	36.4 ± 1.2	0.6912
HV [ms]	8.2 ± 0.2	7.4 ± 0.4	0.0597
corrSNRP [ms] (S1S1 120 ms)	71.7 ± 6.7	58.4 ± 8.3	0.2228
AVNRP [ms] (S1S1 120 ms)	50.6 ± 1.1	52.1 ± 1.3	0.4066
WBP [ms]	85.0 (85.0, 90.0)	87.5 (80.0, 95.0)	0.4631

Summarized baseline parameters and results from 6-lead surface ECG and electrophysiological investigations *in vivo* under inhalation anesthesia.
All values are given in %, mean ± SEM or median with interquartile range (25th and 75th percentile). P: P-wave duration; PQ: PQ interval; QRS: Width of QRS complex; QTc: Rate-corrected QT interval according to Mitchell; AV: Interval from start of atrial to ventricular signal; AH: Interval from start of atrial to His signal; HV: Interval from beginning of His to ventricular signal; corrSNRP: Corrected sinus node recovery period; AVNRP: AV-nodal refractory period; WBP: Wenckebach periodicity.

4.4. Reduced ventricular refractory period potentially promotes arrhythmia perpetuation in KO

The VRP was assessed in each animal by PVS using a single extra stimulus. KO mice displayed a significantly shortened VRP compared to WT (in ms; KO: 15.1 ± 0.7; WT: 18.7 ± 0.7; p = 0.0013; Fig. 3*F*).

5. Discussion

This study is to the best of our knowledge the first to systematically evaluate the effect of genetically (i.e. chronic and specific) reduced NCX function on the initiation and perpetuation of ventricular arrhythmia *in vivo*. The susceptibility towards PVCs was reduced in KO during β-adrenergic stimulation with isoproterenol. But strikingly, arrhythmia perpetuation of at least three consecutive ectopic beats and the cumulative as well as median VT duration were significantly increased in KO during PVS. What is the mechanistic basis for this apparently ambivalent protection against arrhythmia initiation but proneness towards arrhythmia perpetuation on the whole-heart level in NCX-KO mice?

At first, initiation of PVCs results from translation of afterdepolarizations into spontaneous APs on the cellular level. Since KO is protected from the latter in conditions of β-adrenergic stimulation due to a reduced NCX-mediated membrane depolarization, the observed reduced susceptibility towards whole-heart PVC initiation under isoproterenol application in KO is in line with our previous *in vitro* findings [9,10].

As opposed to the effects of isoproterenol application on the spontaneous heart rhythm, the applied PVS protocol with a short-coupled extra stimulus mimics a PVC itself and evaluates, whether the latter can induce a sustained VT. Hence, this protocol rather investigates the proneness towards perpetuation of an already initiated arrhythmia, which may explain the observed distinct findings as compared to isoproterenol application. In other words, PVS evaluates a considerably diverse functional parameter (namely perpetuation) than isoproterenol application (namely initiation).

Obviously, the key factors to determine the susceptibility towards arrhythmia perpetuation differ from those that determine the arrhythmia initiation. The NCX-mediated depolarizing inward current is crucial for the initiation of PVC and thus arrhythmia. However, with regard to VT perpetuation on the whole-heart level, reduced NCX function can also mediate a significant AP shortening as observed previously [9]. AP shortening is most likely a result from both, a reduced NCX-mediated inward current and a reduced L-type Ca^{2+} current (I_{Ca}) on the AP plateau phase, whereas repolarizing voltage-dependent K^+ currents are not increased in the investigated heterozygous murine NCX-knockout model [9]. The reduced I_{Ca} may result from a reduced NCX-

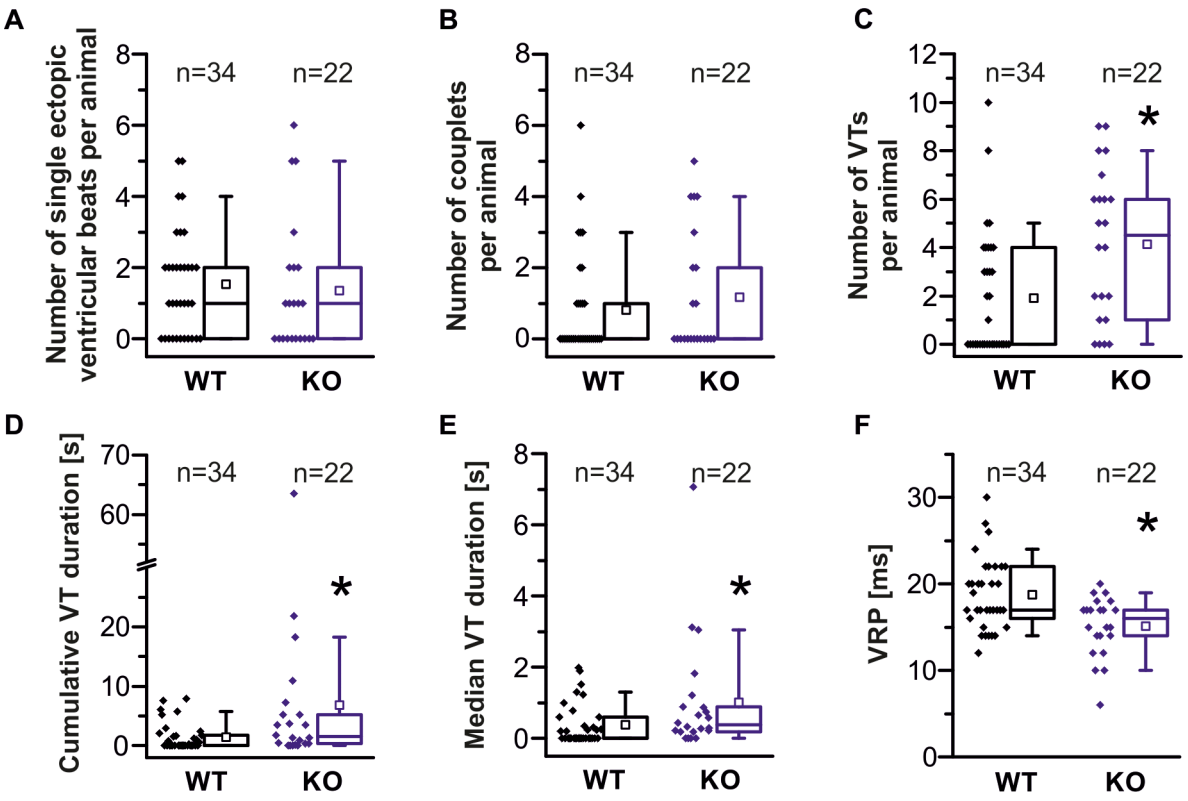

Fig. 3. Perpetuation of ventricular tachycardia initiated by PVS. (**A**) Number of single ectopic ventricular beats per animal during programmed ventricular stimulation (PVS). (**B**) Number of PVS-induced couplets. (**C**) Number of ventricular tachycardia (VT) episodes during PVS (≥3 ectopic ventricular beats). (**D**) Cumulative VT duration per individual mouse. (**E**) Median VT duration per animal. (**F**) Ventricular refractory period (VRP) as assessed by PVS.

mediated Ca^{2+} removal in the subsarcolemmal space, which promotes the Ca^{2+}-dependent inhibition of I_{Ca} [15]. AP shortening in single cells mediates the observed reduction of the VRP and QT-interval on the whole-heart level as observed in this study. In general, there are two key prerequisites for reentry arrhythmia, being unidirectional block of conduction (by scar tissue or functional inhomogeneities of cardiac conduction) and the critical length of the reentry circuit ≥ VT wavelength [16]. The critical VT wavelength represents the product of conduction velocity and VRP. Therefore, the observed VRP reduction in KO may increase the susceptibility towards arrhythmia perpetuation, since a faster repolarization to the resting membrane potential facilitates an earlier reactivation of voltage-dependent Na^{+} current, enabling quick re-excitability of myocardial tissue.

In contrast to our findings Jordan et al. observed no shortening of the QT interval in heterozygous NCX-KO mice [17]. However, the number of animals investigated was n = 5 per group in their study as compared to n = 22 KO mice and n = 34 wt littermates analyzed in this study. Since the observed numeric difference between the QTc intervals of KO and WT was small, the number of independent measurements may have been insufficient (i.e. underpowered) to detect this difference in the prior study. At the moment, we can not supply a mechanistic explanation for the observed shortening of QRS duration in KO.

As a potential alternative explanation for increased VT perpetuation in KO, increased $[Ca^{2+}]_i$ may apply in the face of a reduced NCX-mediated Ca^{2+} extrusion capacity in KO which could become more important at higher heart rates as observed during VT episodes. However, in previous single cell studies, we found no evidence for increased proarrhythmia due to intracellular Ca^{2+} accumulation in KO during increased pacing cycle lengths (e.g. 5 Hz), although these data can not be directly compared to the *in vivo* setup, since these experiments were conducted under room temperature [9]. As described above, Ca^{2+} overload in KO may be avoided by a reduced I_{Ca}, which possibly also applies for higher heart rates; however we cannot completely exclude this potential alternative explanation. Beyond that, alterations of the intracellular Ca^{2+} handling were described to promote reentrant arrhythmia by changes in Na^{+} channel function [18] or enhancing vulnerability to repolarization alternans [19]. Such effects might also contribute to the increased perpetuation of arrhythmia.

Another well-established factor that promotes arrhythmia perpetuation is myocardial scar tissue as a substrate to facilitate heterogeneous conduction properties and thus perpetuation of reentrant tachycardias independently from the cellular repolarization properties. However, KO mice exhibit no evidence for structural heart disease [9,17]. Therefore, this alternative explanation for the observed susceptibility towards arrhythmia perpetuation in KO seems less likely.

This study investigates the effect of chronic and specific (i.e. genetic) NCX inhibition on *in vivo* arrhythmia. Prior studies either applied pharmacological NCX inhibitors that lack selectivity and may thus enfold additional inhibiting effects for example on I_{Ca}, thus potentially mediating antiarrhythmic effects beyond mere NCX inhibition [20] or have only been investigated by acute application to counteract pharmacologically induced arrhythmia [21,22]. Promisingly, Kohajda et al. found that NCX inhibition protects against the initiation of ventricular arrhythmia *in vivo* [21]. Regarding perpetuation, the authors found a significantly decreased cumulative time at arrhythmia (arrhythmic period) when ouabain was applied in anaesthetized guinea pigs pretreated with ORM-10962 as a selective NCX inhibitor in comparison to the control group (without ORM-10962 pretreatment). However, the cumulative arrhythmic period was predominantly a result of frequent single PVCs, whereas VT or VF were rare [21]. Thus, this cumulative parameter may rather reflect the proneness towards arrhythmia initiation. Another study demonstrated in an *ex vivo* whole-heart Langendorff-approach with pharmacologically induced long-QT-syndrome, a reduced PVC burden, but no protective effect on the inducibility of torsade de pointes tachycardias by pharmacological NCX inhibition in both control and failing rabbit hearts [22]. Hence NCX inhibition does

not seem to protect against arrhythmia perpetuation.

The current study is limited to descriptive findings that are mechanistically interpreted based on previous single cell studies. Furthermore, there are considerable functional differences between the murine and human (electro-) physiology in health and disease, especially concerning intracellular calcium signaling. Consequently, these findings do not necessarily imply that NCX inhibition is futile in the clinical setting. Rather, anticipations on potential effects of NCX inhibition on arrhythmia initiation and perpetuation in human remain speculative. As a next step further investigations in higher mammals are necessary to delineate the value of NCX inhibition as a potential antiarrhythmic strategy. Nonetheless, this study reveals an important ambivalence of the influence of NCX inhibition on arrhythmia. Among the entirety of therapeutic drugs in cardiovascular medicine, ambivalent effects are peculiarly prominent among antiarrhythmic drugs that carry without exception proarrhythmic properties. In a broader perspective, these findings argue that a comprehensive preclinical evaluation of antiarrhythmic drugs should address both the effect on initiation and on perpetuation of arrhythmia.

Authors' contributions

NB and TB designed the study. VK and TB acquired the data. VK, PP and TB performed the statistical analysis. All authors contributed substantially to the interpretation of the data. NB and TB wrote the manuscript. NB, VK, PP, JIG, JSS, FUM, GN, LE, JWS and TB critically revised the manuscript. All authors approved the final version of the manuscript.

Sources of funding

This study was not supported by funding. Unrelated to this study, N. B. and L.E. are supported by the German Heart Foundation (Deutsche Stiftung für Herzforschung; F/11/17). The Deutsche Forschungsgemeinschaft supports L.E. (Sonderforschungsbereich SFB 656 (project C11)) and F.U.M. (Mu1376/11–3). J.I.G. is supported by the National Institutes of Health (NIHR01HL0147569). T.B. was supported by the BONFOR Program, Medical Faculty, University of Bonn (O-109.0063).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

None.

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