



# CNP Promotes Antiarrhythmic Effects via Phosphodiesterase 2

Eleder Cachorro\*, Mario Günscht<sup>1</sup>, Mario Schubert\*, Mirna S. Sadek, Johanna Siegert, Fabian Dutt, Carla Bauermeister<sup>1</sup>, Susann Quickert, Henrik Berning<sup>1</sup>, Felix Nowakowski, Simon Lämmle, Rebecca Firneburg, Xiaojing Luo, Stephan R. Künzle, Erik Klapproth, Peter Mirtschink, Manuel Mayr<sup>1</sup>, Matthias Dewenter, Christiane Vettel, Jordi Heijman<sup>1</sup>, Kristina Lorenz, Kaomei Guan<sup>1</sup>, Ali El-Armouche<sup>1</sup>, Michael Wagner\*, Susanne Kämmerer\*

**BACKGROUND:** Ventricular arrhythmia and sudden cardiac death are the most common lethal complications after myocardial infarction. Antiarrhythmic pharmacotherapy remains a clinical challenge and novel concepts are highly desired. Here, we focus on the cardioprotective CNP (C-type natriuretic peptide) as a novel antiarrhythmic principle. We hypothesize that antiarrhythmic effects of CNP are mediated by PDE2 (phosphodiesterase 2), which has the unique property to be stimulated by cGMP to primarily hydrolyze cAMP. Thus, CNP might promote beneficial effects of PDE2-mediated negative crosstalk between cAMP and cGMP signaling pathways.

**METHODS:** To determine antiarrhythmic effects of cGMP-mediated PDE2 stimulation by CNP, we analyzed arrhythmic events and intracellular trigger mechanisms in mice in vivo, at organ level and in isolated cardiomyocytes as well as in human-induced pluripotent stem cell-derived cardiomyocytes.

**RESULTS:** In ex vivo perfused mouse hearts, CNP abrogated arrhythmia after ischemia/reperfusion injury. Upon high-dose catecholamine injections in mice, PDE2 inhibition prevented the antiarrhythmic effect of CNP. In mouse ventricular cardiomyocytes, CNP blunted the catecholamine-mediated increase in arrhythmogenic events as well as in  $I_{CaL}$ ,  $I_{NaL}$ , and  $Ca^{2+}$  spark frequency. Mechanistically, this was driven by reduced cellular cAMP levels and decreased phosphorylation of  $Ca^{2+}$  handling proteins. Key experiments were confirmed in human iPSC-derived cardiomyocytes. Accordingly, the protective CNP effects were reversed by either specific pharmacological PDE2 inhibition or cardiomyocyte-specific PDE2 deletion.

**CONCLUSIONS:** CNP shows strong PDE2-dependent antiarrhythmic effects. Consequently, the CNP-PDE2 axis represents a novel and attractive target for future antiarrhythmic strategies.

**GRAPHIC ABSTRACT:** A [graphic abstract](#) is available for this article.

**Key Words** cardiomyocytes ■ infarction ■ mice ■ myocytes, cardiac ■ sudden cardiac death ■ reperfusion

**In This Issue, see p 397 | Meet the First Author, see p 398**

Ventricular arrhythmia and sudden cardiac death (SCD) are main causes for mortality in patients suffering from congestive heart failure (HF) as well as from acute myocardial infarction.<sup>1</sup> Despite their potential negative inotropic effects,  $\beta$ -adrenoceptor blockers have been the mainstay of antiarrhythmic therapy in these

patients, while classical ion channel blockers have proved to be relatively ineffective or even harmful.<sup>2</sup> Implantable cardioverter defibrillator therapy is highly successful in preventing SCD but cannot prevent arrhythmia.<sup>3</sup> Consequently, novel antiarrhythmic therapeutic principles are highly desired.

Correspondence to: Ali El-Armouche, MD, Institut für Pharmakologie und Toxikologie, Technische Universität Dresden, Fetscherstraße 74, Dresden 01307, Germany. Email [ali.el-armouche@tu-dresden.de](mailto:ali.el-armouche@tu-dresden.de)

\*E. Cachorro, M. Günscht, M. Schubert, A. El-Armouche, M. Wagner, and S. Kämmerer.

Supplemental Material is available at <https://www.ahajournals.org/doi/suppl/10.1161/CIRCRESAHA.122.322031>.

For Sources of Funding and Disclosures, see pages 412–413

© 2023 The Authors. *Circulation Research* is published on behalf of the American Heart Association, Inc., by Wolters Kluwer Health, Inc. This is an open access article under the terms of the [Creative Commons Attribution Non-Commercial-NoDerivs](#) License, which permits use, distribution, and reproduction in any medium, provided that the original work is properly cited, the use is noncommercial, and no modifications or adaptations are made.

*Circulation Research* is available at [www.ahajournals.org/journal/res](http://www.ahajournals.org/journal/res)

## Novelty and Significance

### What Is known?

- Ventricular arrhythmia and sudden cardiac death are the most common lethal complications after myocardial infarction.
- Current antiarrhythmic pharmacotherapy is limited by low efficiency and potentially harmful side effects.
- Myocardial PDE2 (phosphodiesterase 2) is upregulated in patients with heart failure and shows cardioprotective and antiarrhythmic effects in preclinical models.

### What New Information Does This Article Contribute?

- CNP (C-type natriuretic peptide) shows antiarrhythmic effects in murine cardiomyocytes and Langendorff-perfused mouse hearts early after ischemia/reperfusion as well as in human iPSC cardiomyocytes.
- Upon high-dose catecholamine injections in mice, PDE2 inhibition prevents the anti-arrhythmic effect of CNP.
- Pharmacological inhibition as well as genetic PDE2 depletion abrogates the anti-arrhythmic effect of CNP, indicating that PDE2 activation by the CNP pathway is a promising novel antiarrhythmic principle.

Patients suffering from myocardial infarction are at high risk for ventricular arrhythmia and sudden cardiac death. Anti-arrhythmic pharmacotherapy remains a clinical challenge and novel concepts are highly desired. We focus on the cardioprotective CNP as a novel anti-arrhythmic principle. PDE2 is a phosphodiesterase, which has the unique property to be stimulated by cGMP to primarily hydrolyze cAMP. Thus, CNP might promote beneficial effects of PDE2-mediated negative crosstalk between cAMP and cGMP signaling pathways. In ex vivo perfused mouse hearts, CNP abrogated arrhythmia after ischemia/reperfusion injury. Upon high-dose catecholamine injections in mice, PDE2 inhibition prevented the antiarrhythmic effect of CNP. In mouse ventricular cardiomyocytes, CNP blunted the catecholamine-mediated increase in arrhythmogenic events as well as in cellular arrhythmogenic triggers. Mechanistically, this was driven by reduced cellular cAMP levels and decreased phosphorylation of Ca<sup>2+</sup> handling proteins. Key experiments were confirmed in human iPSC-derived cardiomyocytes. The protective CNP effects were reversed by either specific pharmacological PDE2 inhibition or cardiomyocyte-specific PDE2 deletion. CNP shows strong PDE2-dependent antiarrhythmic effects. Consequently, the CNP-PDE2 axis represents a novel and attractive target for future antiarrhythmic strategies.

## Nonstandard Abbreviations and Acronyms

<b>ANP</b>	atrial natriuretic peptide
<b>AP</b>	action potential
<b>BNP</b>	brain natriuretic peptide
<b>CaSp</b>	Ca <sup>2+</sup> spark
<b>CaSpF</b>	Ca <sup>2+</sup> spark frequency
<b>CNP</b>	C-type natriuretic peptide
<b>HF</b>	heart failure
<b>I/R</b>	ischemia reperfusion
<b>NP</b>	natriuretic peptide
<b>OE</b>	overexpression
<b>PDE2</b>	phosphodiesterase 2
<b>sAP</b>	spontaneous action potential
<b>SCD</b>	sudden cardiac death
<b>SCW</b>	spontaneous Ca <sup>2+</sup> waves
<b>SR</b>	sarcoplasmic reticulum
<b>WT</b>	wildtype

The NP (natriuretic peptide) system comprises ANP (atrial natriuretic peptide), BNP (brain natriuretic peptide), and CNP (C-type natriuretic peptide). They exert their

cardiovascular actions predominantly via the guanylyl-cyclase A and B receptors (GC-A, GC-B) in the plasma membrane, which produce the second messenger cGMP. While ANP and BNP display high affinity for GC-A, GC-B represents the main receptor for CNP.<sup>4</sup> ANP and BNP are released from the atria and ventricles.<sup>4</sup> Cardiomyocytes can release CNP, but it is also secreted by chondrocytes, fibroblasts, blood and endothelial cells.<sup>5</sup> CNP acts mainly in a paracrine way. Consequently, its plasma levels are low and only moderately increased in patients with HF.<sup>6,7</sup> Moreover, myocardial CNP expression is significantly reduced in advanced human HF.<sup>8,9</sup> Of note, the activity of its receptor GC-B is increased in HF, while the activity of the ANP/BNP receptor GC-A is decreased.<sup>10</sup> ARNIs (angiotensin receptor-neprilysin inhibitors) are clinically highly effective and target the scavenging of the NPs by the protease neprilysin, thus augmenting their action.<sup>11</sup> Recent data indicate that ARNIs predominantly elevate ANP levels and have little influence on CNP although it is a neprilysin substrate.<sup>12,13</sup> Therefore, it has been hypothesized that patients with HF exhibit a relative CNP deficiency that is not sufficiently corrected by ARNIs.<sup>12</sup>

PDE2 (phosphodiesterase 2) is a dual-specific phosphodiesterase contributing to confine cAMP as well as cGMP responses in specific subcellular cardiomyocyte

microdomains. PDE2 hydrolyzes both second messengers with similar affinities for cGMP and cAMP.<sup>14</sup> PDE2 is allosterically activated by cGMP via its GAF-B domain, increasing its cAMP hydrolytic activity.<sup>14,15</sup> Thus, PDE2 is uniquely suited to mediate a negative cGMP-cAMP-crosstalk.<sup>16</sup> PDE2 is upregulated in human as well as experimental HF.<sup>17,18</sup> In previous studies, we demonstrated that cardiomyocyte-specific PDE2 overexpression in mice protects from death and arrhythmia after MI.<sup>19,20</sup>

In the present study, we show that CNP-induced PDE2 activation exerts a strong antiarrhythmic effect at the cellular, organ and animal level. We suggest that activating PDE2 via the CNP pathway is a novel, feasible, and promising antiarrhythmic approach.

## METHODS

### Data Availability

The authors declare that all supporting data are available within the article and [Supplemental Material](#).

### Study Approval

All patients participating in this study gave written informed consent according to the Declaration of Helsinki and the study was approved by the institutional review committee (Official file numbers: EK 114082202) and the Albert Szent-Gyorgyi Medical University Ethical Review Board (Szeged). The animal experiments comply with the ARRIVE guidelines, conform to the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and were approved by the Dresden University Committee on the Use and Care of Animals (TVV 04/2016, TVA 25/2017, TVV 55/2017, TVT 08/2019).

### Human Cardiac Tissue Samples

Human heart tissue was derived from healthy nonfailing (NF) donor hearts, which could not be used for transplantation due to technical reasons, or from explanted hearts of end-stage NYHA IV HF patients. Patient characteristics are summarized in [Tables S1 and S2](#). Biopsies were excised from the left ventricle (LV).

### Generation of the PDE2-Knockout Mice

Mice with cardiomyocyte-specific deletion of PDE2 (PDE2 knockout) were generated using the Cre/loxP system.

### Echocardiography and ECG Monitoring

Anesthetized mice were analyzed by echocardiography using a Vevo 3100 System (Visual Sonics, Inc) as previously described.<sup>19,21</sup> ECGs were recorded in freely moving mice using implanted telemetry devices (Data Sciences International) as previously reported.<sup>19</sup> Arrhythmia provocation was performed by double injections of isoprenaline (ISO, 2 mg/kg, IP) separated by an interval of 30 minutes; analyses were performed for 90 minutes after the first injection.<sup>19</sup>

Arrhythmia in ex vivo perfused Langendorff hearts were quantified during a 30-minute reperfusion phase after ligation of the left anterior descending coronary artery for 30 min as previously published (Powerlab, ADInstruments).<sup>20</sup>

## Electrophysiology

Whole-cell voltage-clamp was used to measure L-type Ca<sup>2+</sup> current (I<sub>CaL</sub>) and late Na<sup>+</sup> current (I<sub>NaL</sub>) as described previously.<sup>20</sup> To assess action potential (AP) properties, cellular AP were evoked by brief current pulses at 1 Hz and 37°C. Arrhythmia provocation was evaluated by eliciting AP at 4 Hz (5 s) followed by 0.125 Hz (160 s) to assess spontaneous AP (sAP) generation.

## Ca<sup>2+</sup> Handling, Contractility, and cAMP Analysis

Ca<sup>2+</sup> spark (CaSp) measurements were performed in Fluo-4 AM (10 μM, Invitrogen) loaded ventricular cardiomyocytes after 1 Hz pacing using the line-scan mode of the laser scanning confocal microscope (LSM 880 Pascal, Zeiss) at the Core Facility Cellular Imaging (CFCI, Dresden, Germany) as previously described.<sup>20,22,23</sup>

Intracellular Ca<sup>2+</sup> transients were detected at 1 Hz in Fura-2 AM-loaded (3 μM, Invitrogen) ventricular cardiomyocytes using an IonOptix system as previously reported.<sup>19,24</sup> Spontaneous Ca<sup>2+</sup> waves (SCW) were quantified after 2 Hz pacing in Ca<sup>2+</sup> and Na<sup>+</sup>-free bath solution and tetracaine washout. Sarcomere shortening was recorded as described previously.<sup>19</sup> Intracellular cAMP levels were assessed in isolated cardiomyocytes (Direct cAMP ELISA kit Enzo Life Sciences, Inc).

## mRNA and Protein Quantification

Total RNA was isolated with Trizol and purified using the RN-Easy mini Kit (Quiagen). Reverse transcription reaction was conducted with iScript cDNA synthesis kit (Bio-Rad). qPCR was performed using a CFX96 C1000 Touch thermal cycler Real-Time System (Bio-Rad) using the SsoAdvanced Universal SYBR Green and gene-specific primers as previously published.<sup>9</sup> Protein lysates of either human heart tissue or isolated murine cardiomyocytes were run on 10% SDS-PAGE and blotted onto nitrocellulose membranes. For protein quantification the following specific primary antibodies were used: CaMKIIδ total (R&D Systems, MAB4176), CaMKII pThr286 (Thermo, MA1-047), cTNT (Abcam, ab45932), PLB (Badrilla, Ltd, A010-14), PLB pSer16 (Badrilla, Ltd, A010-12), PLB pThr17 (Badrilla, Ltd, A010-13), RYR2 (Sigma-Aldrich, HPA020028), RyR2 pSer2808 (Badrilla Ltd., A010-30), Tnl (Abcam, ab47003), Tnl pSer23/24 (HyTest, Cat4T45), PDE2 (Santa cruz, sc 17228), PDE3A (FabGennix, PD3A-101AP), PDE4D (FabGennix, PD4-401AP), NPR-A (Abcam, ab14356), NPR-B (FabGennix, PGCB-201AP), NPR-C (Novus Biologicals, NBP1-31365), EEF2 (Abcam, ab40812), GAPDH (Santa-Cruz, sc-365062), CSQ (Thermo, #PA1-913; see [Supplemental Material](#)), Antimouse (Sigma Aldrich, A0545), Anti-rabbit (Sigma Aldrich, A3682).

## Statistics

Animals of both sexes were randomly assigned by the supervisor of the experiments from the animal database stratified by genotype, age and sex. Echocardiography was performed and evaluated in a blinded manner. Analysis of ECG telemetry was evaluated in a blinded manner. Results are presented as mean±SD or box plots with whiskers showing minimum to maximum values, median and interquartile range. Statistical analysis was performed with GraphPad Prism software (V7;

Dotmatics). For  $n \geq 6$ , normal distribution of the experimental data was tested with Kolmogorov-Smirnov normality test. For comparisons between  $> 2$  groups, 1-way ANOVA followed by Sidak multiple comparison test (normal distribution) or Kruskal-Wallis test followed by a Dunn's multiple comparison (non-normal distribution with non-paired groups or  $n < 6$ ) or Friedman test followed by a Dunn's multiple comparison (non-normal distribution or  $n < 6$  with paired groups) were performed. Ion currents,  $\text{Ca}^{2+}$  imaging data, and  $\text{Ca}^{2+}$  sparks were analyzed using a hierarchical model in R using the lme4 package with clustering for individual mice as described previously.<sup>25</sup> Non-normally distributed clustered data were log-transformed before hierarchical statistical testing. Two-sided  $P$  values of  $< 0.05$  were considered statistically significant. Number of experimental animals and cells per group was chosen based on our experience in previous experiments.<sup>17,19,20</sup> Representative images were chosen to closely match the average values of the respective groups.

## RESULTS

### CNP Protects From Arrhythmia in Ex Vivo I/R Injury

To evaluate the potential antiarrhythmic effect of CNP, we quantified ischemia/reperfusion (I/R)-induced arrhythmia in ex vivo perfused mouse hearts. Figure 1A shows representative ECG recordings of ex vivo perfused hearts after ligation of the left anterior descending coronary artery for 30 minutes and subsequent reperfusion for 30 minutes. Perfusion with CNP (6 nM) blunted I/R-induced arrhythmia. The number of ventricular extrasystoles and bigeminy was significantly reduced by CNP (Figure 1B and 1C). PDE2 inhibition with BAY 60-7550 (BAY, 100 nM) prevented the antiarrhythmic effect of CNP and increased the total number of arrhythmic events to similar levels as observed in control hearts after I/R (Figure 1D). Mean heart rates during reperfusion were not affected by the substances (Figure 1E). We have shown previously that BAY alone does not significantly increase heart rate in Langendorff-perfused hearts.<sup>20</sup> Together with CNP, PDE3 inhibition with milrinone as well as unspecific PDE inhibition with IBMX (100  $\mu\text{M}$ ) led to excessive arrhythmia beyond baseline in ex vivo perfused hearts after I/R (Figure S1).

### CNP Reduces Cellular Arrhythmogenic Events

To assess the antiarrhythmic effects of CNP on the cellular level, isoprenaline-induced sAP were quantified in isolated mouse ventricular cardiomyocytes after 10 min incubation. Representative current-clamp recordings at 0.125 Hz (following stimulation at 4 Hz to fill the sarcoplasmic reticulum [SR]) are shown in Figure 1F. CNP (1  $\mu\text{M}$ ) significantly decreased the isoprenaline (10 nM)-induced number of sAP. PDE2 inhibition with BAY (100 nM) prevented this effect (Figure 1G). Incubation with CNP alone did not affect the number of sAP.

PDE2 inhibition with BAY did not affect the number of isoprenaline-induced sAP in the absence of CNP (Figure S2A). Importantly, drug incubation only slightly affected AP morphology. AP amplitude, AP duration at 90% repolarization ( $\text{APD}_{90}$ ) (Figure 1H and 1I) as well as maximal upstroke velocity ( $dV/dt_{\text{max}}$ ) (Figure S2B) were similar at all conditions. Isoprenaline increased  $\text{APD}_{50}$ , which was reversed by CNP (Figure S2B).

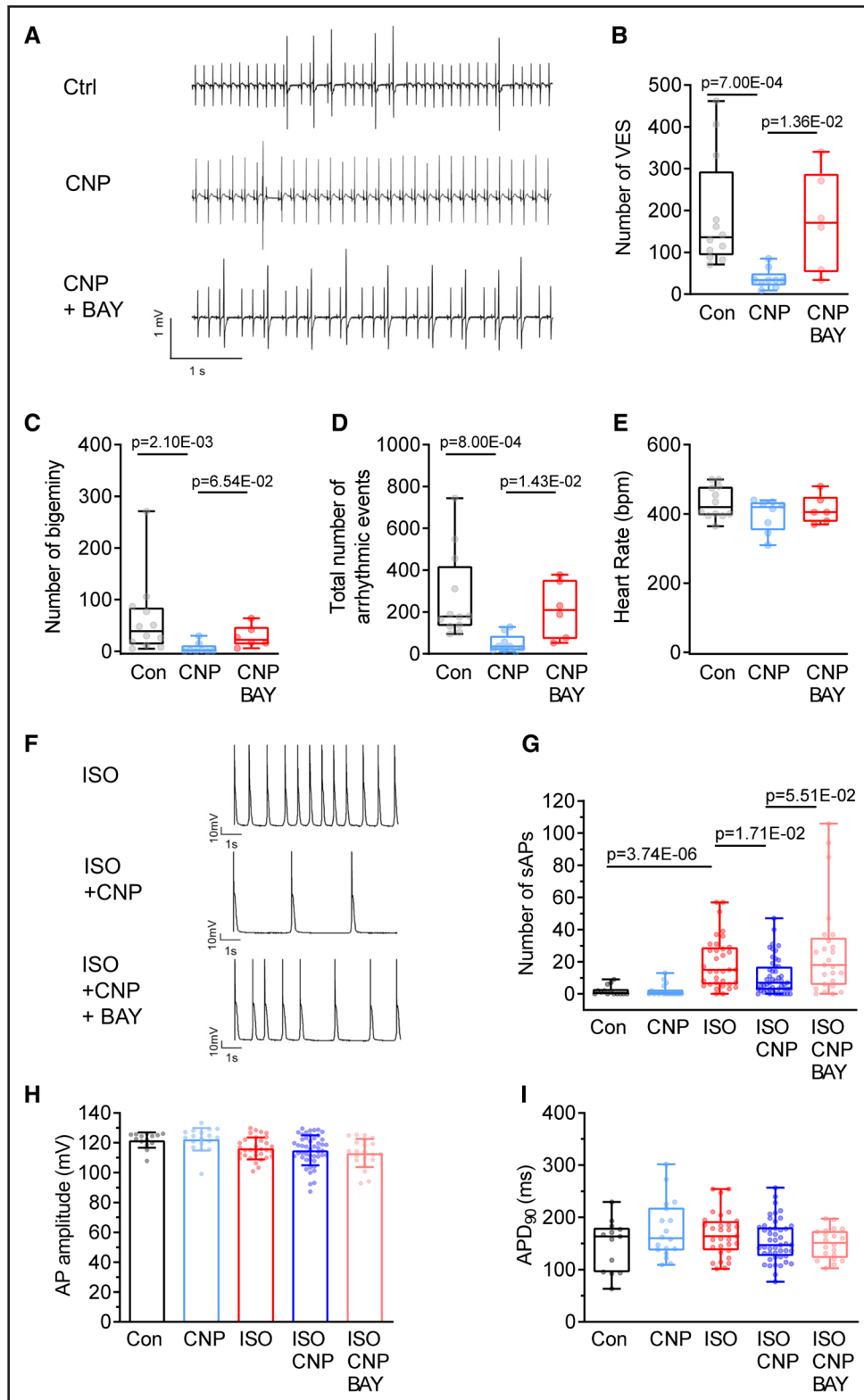
Next, we assessed the effect of CNP on the isoprenaline-induced increase of the L-type calcium ( $I_{\text{CaL}}$ ) and the late sodium currents ( $I_{\text{NaL}}$ ) in isolated mouse ventricular cardiomyocytes (Figure 2). Representative  $I_{\text{CaL}}$  recordings at various membrane potentials and the average current-voltage relations are depicted in Figure 2A and 2B. Comparing the maximum current at 0 mV, simultaneous CNP incubation significantly reduced the isoprenaline-induced increase of  $I_{\text{CaL}}$  (Figure 2C). The effect of CNP was abrogated upon PDE2 inhibition. BAY alone neither affected  $I_{\text{CaL}}$  density nor the isoprenaline-induced elevation in  $I_{\text{CaL}}$  (Figure S2C). Moreover, CNP blunted the isoprenaline-mediated increase in  $I_{\text{NaL}}$ , which could not be observed upon simultaneous PDE2 inhibition (Figure 2D and 2E).

### CNP Decreases $\text{Ca}^{2+}$ -Mediated Arrhythmogenic Triggers

We further evaluated the effect of CNP on CaSp frequency (CaSpF) (Figure 3A). CNP alone did not affect CaSpF, whereas isoprenaline significantly increased the number of CaSp over time. Concomitant CNP application significantly lowered the isoprenaline-mediated increase of CaSp occurrence. This effect was prevented by PDE2 inhibition with BAY (Figure 3B). BAY alone did not alter basal CaSpF or the isoprenaline-induced increase in CaSp number (Figure S3A and S3B). The inhibition of the cGMP-activated protein kinase G (PKG) did not affect the CNP-induced reduction of CaSpF (Figure S3C and S3D). SCW were not different between our experimental groups (Figure S4A and S4B).

We next studied the influence of isoprenaline and CNP on intracellular  $\text{Ca}^{2+}$  transient properties at 1 Hz (Figure S4). As expected, isoprenaline led to a significant increase in  $\text{Ca}^{2+}$  transient amplitude and an acceleration of the transient decay that were not or only slightly affected by CNP alone (Figure S4C through S4F). Simultaneous isoprenaline stimulation with CNP or CNP plus BAY did not influence the isoprenaline-mediated effects.

To investigate the effect of isoprenaline and CNP on cellular contractile function, sarcomere shortening was recorded in isolated ventricular cardiomyocytes at 1 Hz (Figure S5A). As expected, isoprenaline significantly increased fractional sarcomere shortening, whereas CNP had no effect (Figure S5B and S5C). In addition, isoprenaline enhanced contraction and relaxation velocities and reduced relaxation half-time. CNP alone



**Figure 1. CNP (C-type natriuretic peptide) reduces the number of arrhythmias after ischemia/reperfusion (I/R) via PDE2 (phosphodiesterase 2) in ex vivo perfused mouse hearts and on the cellular level.**

**A**, ECG was recorded in ex vivo perfused mouse hearts during reperfusion for 30 minutes with Krebs-Henseleit buffer with physiological catecholamine concentrations of 10 nM norepinephrine and 3.5 nM epinephrine following ischemia induction for 30 minutes. Representative registrations from hearts perfused with Krebs-Henseleit buffer (Con, N=12), CNP (6 nM, N=9) or CNP plus BAY 60-7550 (BAY, 100 nM, N=6). **B**, Quantification of the number of ventricular extrasystoles (VES), **(C)** bigeminy, **(D)** total arrhythmic events, and **(E)** heart rate (data are presented as box plots with whiskers showing minimum to maximum values, median and interquartile range; *P* were determined (*Continued*))

significantly enhanced contraction velocity and reduced time to 50 % relaxation decay but did not affect contraction amplitude (Figure S5D through S5F). Concurrent incubation of isoprenaline with CNP and CNP plus BAY did not affect the isoprenaline-induced effects on cellular contraction and relaxation parameters.

To directly assess the intracellular crosstalk mechanism of CNP-induced PDE2 stimulation, we quantified intracellular cAMP levels by ELISA in isolated cardiomyocytes. CNP treatment significantly reduced cAMP levels upon isoprenaline stimulation, which were in turn restored by simultaneous PDE2 inhibition with BAY (Figure 4A). Next, we studied the molecular consequences of reduced cAMP levels upon isoprenaline and CNP on down-stream targets and quantified the expression and phosphorylation levels of CaMKII, PLB, TnI and RYR2 (Figure 4B through 4D). Representative western blots are depicted in Figure 4B and 4C. Upon coincubation with isoprenaline and CNP, the CaMKII phosphorylation levels at Thr286 were significantly reduced compared to isoprenaline alone. Concomitant PDE2 inhibition with BAY significantly increased CaMKII phosphorylation (Figure 4B). In addition, CNP similarly reduced phosphorylation of PLB (at Ser16 and Thr17), TnI (at Ser23/24) and RYR2 (at Ser2808) under  $\beta$ -adrenergic stimulation with isoprenaline, indicating reduced activity of PKA and CaMKII (Figure 4C and 4D). Simultaneous PDE2 inhibition significantly increased phosphorylation levels of the respective proteins.

### Cardiac PDE2 Deletion Blunts the CNP Effect on $\beta$ -AR-Mediated $\text{Ca}^{2+}$ Parameters

To further study the impact of PDE2 on arrhythmia susceptibility, we generated a cardiac-specific PDE2 knockout mouse line (PDE2 knockout) by crossing mice with inserted lox P sites in PDE2 exon 4 (PDE2<sup>fl/fl</sup>) and transgenic mice expressing the Cre recombinase under the control of the  $\alpha$ -myosin heavy chain promoter ( $\alpha$ MHC-Cre). Echocardiography revealed normal cardiac function in 3 months old PDE2 knockout compared with the control lines (Figure S6). Heart rate, ejection fraction (EF), fractional area shortening (FAS) and relative left ventricular heart weight were similar between the 3 genotypes (Figure S6B through S6E).

Next, we performed *in vitro* experiments on isolated ventricular cardiomyocytes from PDE2 knockout and PDE2<sup>fl/fl</sup> mice assessing CaSpF, L-type  $\text{Ca}^{2+}$  current ( $I_{\text{CaL}}$ ) and SCW. Like in wild-type (WT) mice, CNP significantly

reduced the isoprenaline-mediated increase in CaSpF as well as  $I_{\text{CaL}}$  amplitude at 0 mV in the control line (PDE2<sup>fl/fl</sup>). Moreover, CNP blunted the isoprenaline mediated increase in SCW. These CNP effects were prevented by PDE2 inhibition with BAY (Figure 5A, 5C, and 5E). In contrast, cardiomyocytes from PDE2 knockout mice did not show an attenuation of isoprenaline-induced CaSp, SCW and  $I_{\text{CaL}}$  by CNP: the CaSpF was not significantly reduced in PDE2 knockout upon coincubation with isoprenaline and CNP (Figure 5B). In addition, the isoprenaline-induced increase in  $I_{\text{CaL}}$  current density was similar in PDE2 knockout upon isoprenaline and isoprenaline plus CNP (Figure 5D). Also, the number of SCW was not altered by CNP in cardiomyocytes from PDE2 knockout mice (Figure 5F). The effect of isoprenaline and isoprenaline plus CNP on  $\text{Ca}^{2+}$  transient amplitude as well as contraction and relaxation time courses did not differ between PDE2 knockout and control mice (Figure S7).

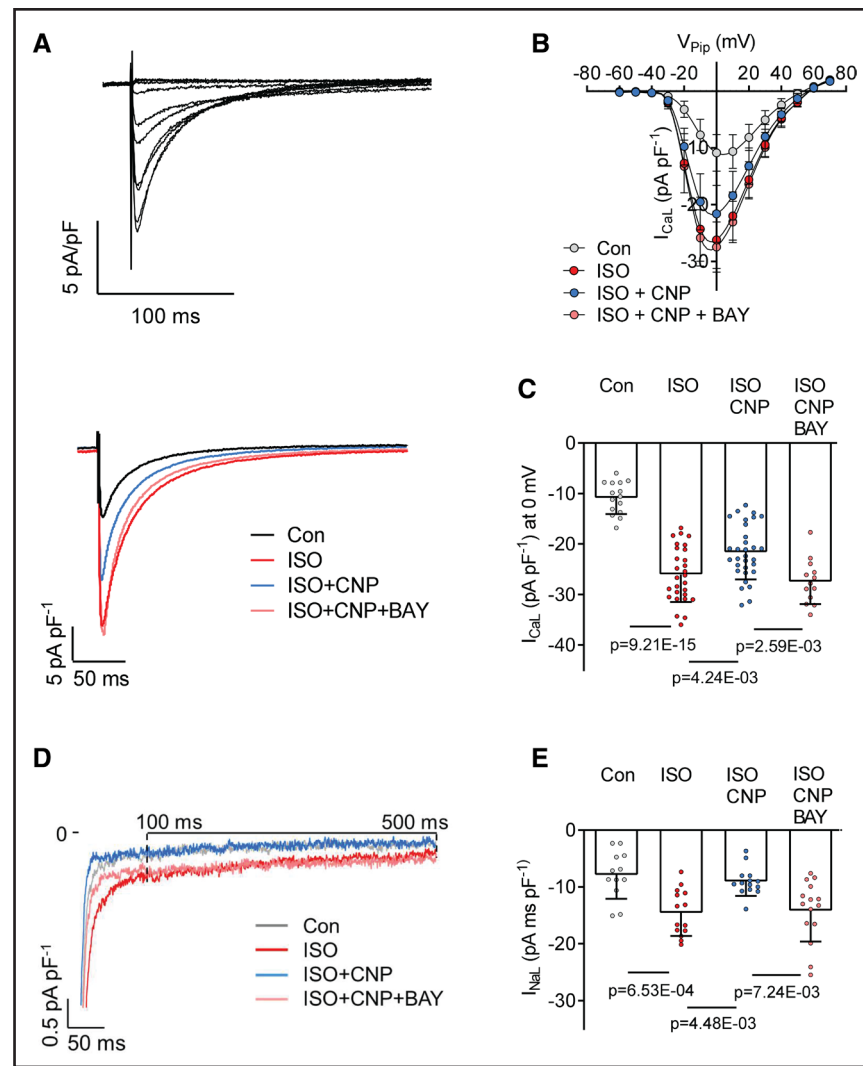
### CNP Reduces Arrhythmia After $\beta$ -Adrenergic Stress In Vivo

To evaluate whether CNP protects from isoprenaline-induced arrhythmia *in vivo*, WT mice were implanted with ECG telemeters and subjected to  $\beta$ -adrenergic stress by double isoprenaline injection (2 mg/kg, ip, repeated after 30 min) to provoke arrhythmia.<sup>19</sup> The number of arrhythmic events was quantified from ECG recordings for 90 min after the first injection (Figure 6A). After isoprenaline injection, various arrhythmic events were observed, mostly ventricular ectopic beats. Coinjection with CNP (33 mg/kg, IP) significantly reduced the number of arrhythmic events. The simultaneous injection with BAY (3 mg/kg, IP) increased the number of arrhythmias that was not significantly different from isoprenaline (Figure 6B), thus indicating a dependence on PDE2 activity.

### CNP Protects From Arrhythmia in I/R Injury via PDE2

Next, we quantified I/R-induced arrhythmia in CNP-perfused hearts from PDE2 knockout and PDE2<sup>fl/fl</sup> mice (Figure 6C). Heart rate was not affected by CNP in *ex vivo* perfused hearts from both genotypes (Figure 6D). PDE2<sup>fl/fl</sup> hearts displayed about 22 arrhythmic events per 30 min upon CNP after I/R (Figure 6E). In contrast, PDE2 knockout hearts still exhibited about 150 arrhythmic events per 30 min after I/R upon CNP ( $P=2.81 \times 10^{-6}$  for interaction by 2-way-ANOVA). Of

**Figure 1 Continued.** by Dunn's multiple comparison test after Kruskal-Wallis test ( $P=9.00 \times 10^{-4}$  [B],  $P=4.00 \times 10^{-3}$  [C],  $P=1.00 \times 10^{-3}$  [D],  $P=4.89 \times 10^{-1}$  [E]). In isolated ventricular mouse cardiomyocytes, cellular arrhythmia was provoked by ISO (10 nM, 10 min) and pacing at 4 Hz followed by 0.125 Hz during which arrhythmic events were analyzed. F, Representative recordings in presence of ISO, ISO plus CNP (1  $\mu$ M, 10 minutes) or ISO plus CNP plus BAY 60-7550 (BAY, 100 nM). G, Quantification of the number of sAP. AP morphology at 1 Hz; (H) AP amplitude (mV), (I) AP duration at 90% repolarization (APD<sub>90</sub>, ms; n=cells/N=animals: Con [14/8], CNP [18/10], ISO [35/20], ISO+CNP [49/22], ISO+CNP+BAY [25/13]); stated as mean $\pm$ SD (H) or box plots with whiskers showing minimum to maximum values, median and interquartile range (G and I); P were determined by Bonferroni test after a hierarchical model using log-transformed data ( $P=2.49 \times 10^{-7}$  [G],  $P=5.13 \times 10^{-1}$  [H],  $P=6.06 \times 10^{-2}$  [I]).



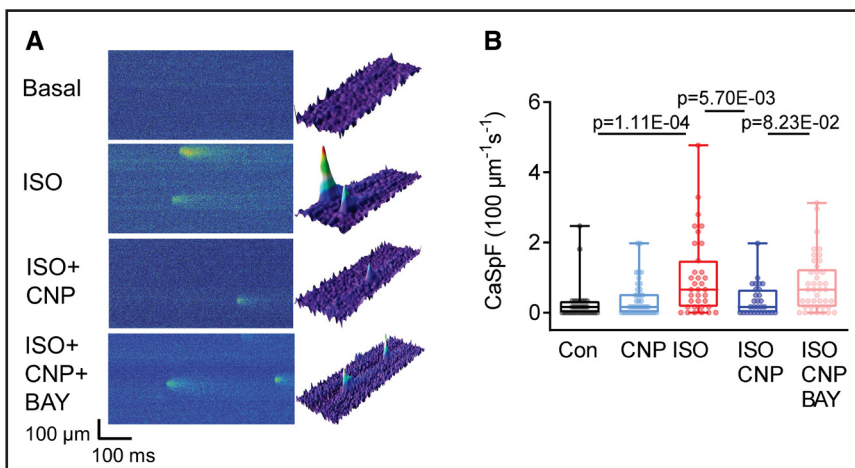
**Figure 2. CNP (C-type natriuretic peptide) attenuates the ISO-induced increase of L-type calcium current ( $I_{CaL}$ ) and late sodium current ( $I_{NaL}$ ) via PDE2 (phosphodiesterase 2) in mouse ventricular cardiomyocytes.**

**A**, Representative original traces of  $I_{CaL}$  in cardiomyocytes under control conditions at different membrane potentials (upper panel) or at 0 mV after incubation with ISO (10 nM), ISO+CNP (1  $\mu$ M), or ISO plus CNP plus BAY 60-7550 (BAY, 100 nM) for 10 min (lower panel). **B**,  $I_{CaL}$  current density-voltage relation and **(C)**  $I_{CaL}$  current density at 0 mV ( $n$ =cells/ $N$ =animals: Con [15/3], ISO ([28/5]), ISO+CNP ([30/5], ISO+CNP+BAY [13/2]) stated as mean $\pm$ SD;  $P$  were determined by Bonferroni test after a hierarchical model ( $P=1.54\times 10^{-15}$ ). **D**, Representative  $I_{NaL}$  recordings measured under basal conditions and after 10 min stimulation with ISO (10 nM), CNP (1  $\mu$ M) and BAY 60-7550 (100 nM). **E**,  $I_{NaL}$  was quantified as the TTX-sensitive current integral ( $n$ =cells/ $N$ =animals: Con [12/6], ISO [14/5], ISO+CNP [14/5], ISO+CNP+BAY [14/6]) stated as mean $\pm$ SD;  $P$  were determined by Bonferroni test after a hierarchical model ( $P=1.09\times 10^{-4}$ ).

note, a CNP effect was still present in the cardiomyocyte-specific PDE2 knockout hearts, but not in WT hearts treated with BAY (Figure 6B). This hints to the possibility that other cardiac cell types or other signaling mechanisms contribute to the PDE2-dependent antiarrhythmic effect of CNP.

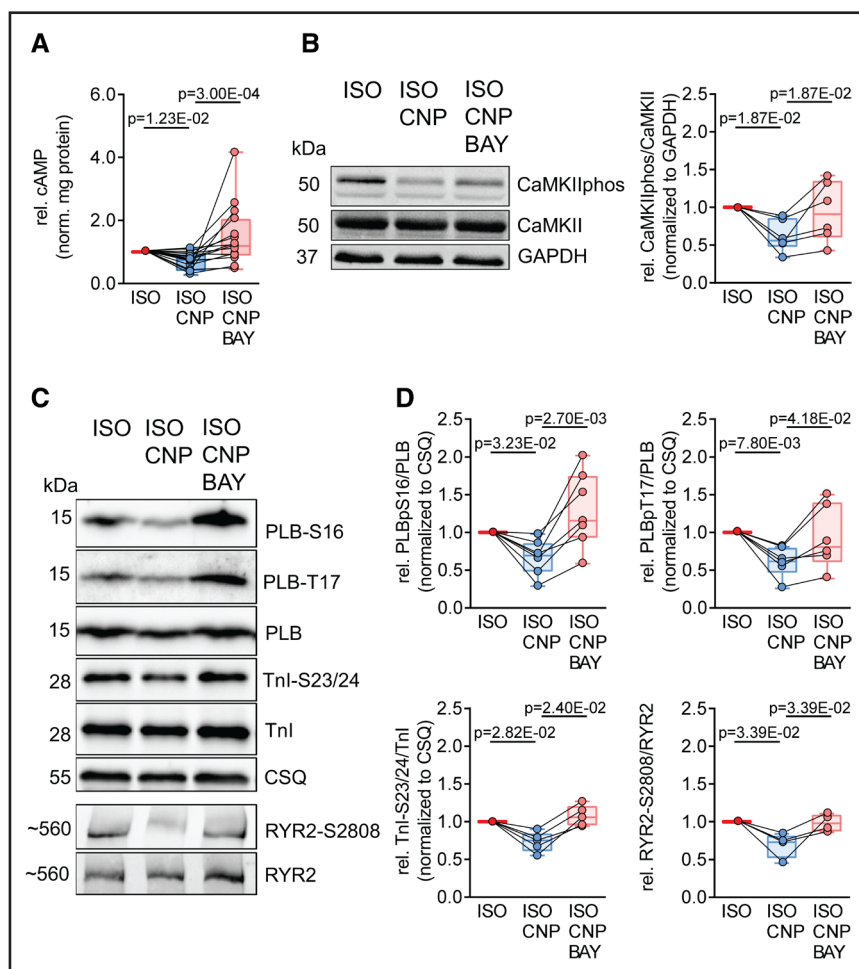
### CNP Reduces CaSpF in Human iPSC-Derived Cardiomyocytes

Our data demonstrate that PDE2 stimulation by CNP mediates antiarrhythmic effects in the mouse heart. We next evaluated the effect of CNP on arrhythmia



**Figure 3. CNP (C-type natriuretic peptide) reduces the ISO-induced spontaneous Ca<sup>2+</sup> spark (CaSp) via PDE2 (phosphodiesterase 2).**

**A**, Representative recordings of CaSp in mouse ventricular cardiomyocytes under basal conditions and stimulated with ISO (10 nM), ISO plus CNP (1  $\mu$ M), or ISO plus CNP plus BAY 60-7550 (BAY, 100 nM) for 10 minutes. **B**, Quantification of Ca<sup>2+</sup> spark frequency (CaSpF;  $n$ =cells/ $N$ =animals: Con [31/6], CNP [42/5], ISO [35/5], ISO+CNP [29/5], ISO+CNP+BAY [41/3]) stated as box plots with whiskers showing minimum to maximum values, median and interquartile range;  $P$  were determined by Bonferroni test after a hierarchical model using log-transformed data ( $P=1.11\times 10^{-5}$ ).



**Figure 4. CNP (C-type natriuretic peptide) reduces ISO-induced intracellular cAMP levels and downstream signals.**

**A**, Intracellular cAMP content evaluated via direct cAMP ELISA upon 10-minute treatment with ISO (10 nM), CNP (1  $\mu$ M), and BAY60-7550 (100 nM) in isolated cardiomyocytes (N=15). Relative cAMP concentrations were normalized to corresponding sample protein content and ISO. **B**, Representative western blots and quantification of relative phosphorylation levels of CaMKII at Thr286 in isolated cardiomyocytes upon 10-minute treatment with ISO (10 nM), CNP (1  $\mu$ M), and BAY60-7550 (100 nM; N=6). **C**, Representative blot and **(D)** quantification of relative phosphorylation levels of phospholamban (PLB) at Ser16 (N=7) and Thr17 (N=6), troponin (TnI) at Ser23 and Ser24 (N=5), and RYR2 (ryanodine receptor 2) at Ser2808 (N=4) in perfused hearts with ISO (10 nM), CNP (1  $\mu$ M), and BAY60-7550 (100 nM) for 10 minutes. All data were normalized to control in each set of experiments. Data are presented as box plots with whiskers showing minimum to maximum values, median and interquartile range. *P* were determined by Dunn's multiple comparison test after Friedman test ( $P=4.00 \times 10^{-4}$  in **A**;  $P=8.10 \times 10^{-3}$  in **B**; **D**:  $P=1.20 \times 10^{-3}$  for PLBpSer16/PLB;  $P=5.50 \times 10^{-3}$  for PLBpThr17/PLB;  $P=2.39 \times 10^{-2}$  for TnI-Ser23/24/TnI,  $P=4.96 \times 10^{-2}$  for RYR2pSer2808/RYR2).

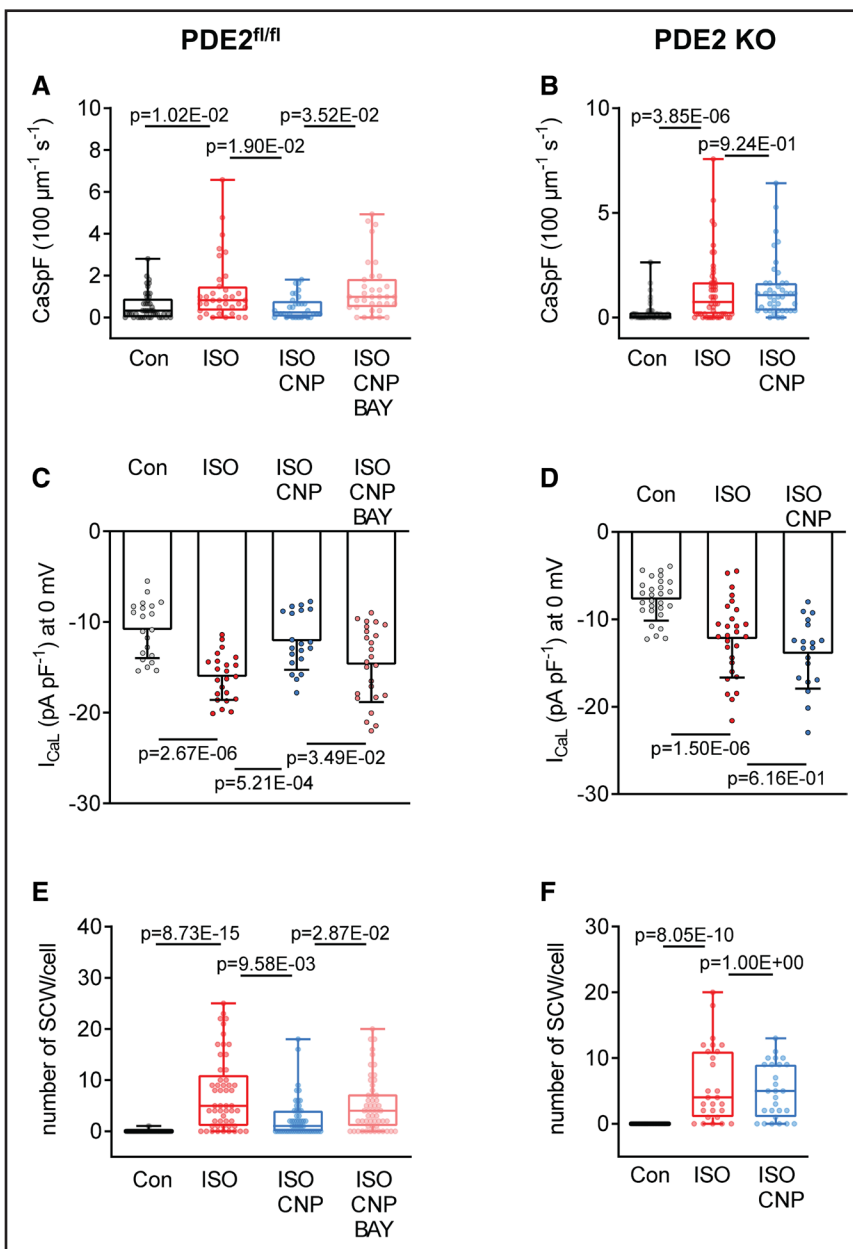
in human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). Differentiated hiPSC-CMs were obtained in high purity (Figure S8A through S8C) and cultured for 75–90 days to ensure maturation. Western blot analysis revealed the expression of GC-B receptors and different PDEs including PDE2 (Figure 7A). CaSp were examined in hiPSC-CMs, differentiated from iPSC of 2 healthy donors, under control conditions and after 10 min incubation with isoprenaline, isoprenaline plus CNP and with additional PDE2 inhibitor (BAY). Isoprenaline stimulation caused a significant increase in CaSpF in hiPSC-CM (Figure 7B and 7C). Consistent with results obtained in adult mouse cardiomyocytes, CNP blunted the occurrence of CaSp in hiPSC-CM, which was prevented by PDE2 inhibition with BAY. Importantly, BAY alone did not affect CaSpF in human cardiomyocytes (Figure 7C). In contrast, PDE3 inhibition with milrinone (Mil) or PDE4 inhibition with rolipram (Rol) alone significantly increased CaSpF in hiPSC-CM similar to isoprenaline (Figure S8D). As expected from these results, both inhibitors, Mil and Rol, also led to increased CaSpF upon coincubation with isoprenaline and CNP, without indication of a specific interaction.

Finally, we extrapolate our results to large animal cardiomyocytes by computer modeling to selectively evaluate the effects of CNP-mediated PDE2 upregulation or PDE3 inhibition.<sup>20,26</sup> Simulated inhibition of PDE2 did not have a major effect on any of the outcome parameters at baseline or upon isoprenaline stimulation (Figure S9), consistent with our experimental data (Figure S2). Selective upregulation of PDE2 reproduced the experimentally observed effects of CNP (Figure S10). By contrast, inhibition of PDE3 exacerbated proarrhythmic indices such as CaMKII activation and RyR2 phosphorylation (Figure S10), and this increase was blunted by concomitant stimulation of CNP administration. Taken together, these results suggest antiarrhythmic effects of CNP-induced PDE2 stimulation also in cardiomyocytes of large mammals, including humans.

### Cardiac Expression of CNP Is Downregulated in Human HF

The CNP-induced PDE2 stimulation might mediate antiarrhythmic effects not only after I/R injury but also in human HF. To investigate the regulation of NPs and their receptors in human HF, we quantified mRNA expression levels in





**Figure 5. Cardiac-specific PDE2 (phosphodiesterase 2) KO prevents CNP (C-type natriuretic peptide)-mediated inhibition of proarrhythmic effects of ISO on spontaneous Ca<sup>2+</sup> spark (CaSp), spontaneous Ca<sup>2+</sup> waves (SCW), and I<sub>CaL</sub>.**

Ca<sup>2+</sup> parameters were detected in mouse ventricular cardiomyocytes from floxed WT mice (WT<sup>fl/fl</sup>, left) and PDE2 KO mice (right) under basal conditions and stimulated with ISO (10 nM), ISO plus CNP (1 μM) or (only WT<sup>fl/fl</sup>) ISO plus CNP plus BAY 60-7550 (BAY, 100 nM) for 10 minutes. **A** and **B**, Quantification of CaSpF (n=cells/N=animals: **A**) Con [45/5], ISO [35/6], ISO+CNP [32/5], ISO+CNP+BAY [34/4]; **B**) Con [49/4], ISO [50/4], ISO+CNP [42/4]) stated as box plots with whiskers showing minimum to maximum values, median and interquartile range; *P* were determined by Bonferroni test after a hierarchical model using log-transformed data (**A**: *P*=1.70×10<sup>-3</sup>; **B**: *P*=1.28×10<sup>-6</sup>). **C** and **D**, L-type calcium current (I<sub>CaL</sub>) density at 0 mV (n=cells/N=animals: **C**) Con [21/11], ISO [23/9], ISO+CNP [20/10], ISO+CNP+BAY [25/10]; **D**) Con [28/9], ISO [28/10], ISO+CNP [19/6]) stated as mean±SD; *P* were determined by Bonferroni test after a hierarchical model (**C**: *P*=4.46×10<sup>-7</sup>; **D**: *P*=4.99×10<sup>-7</sup>). **E** and **F**, Quantification of the total number of SCW after arrhythmia provocation (2 Hz pacing, 30 s). (n=cells/N=animals: **E**) Con [42/8], ISO [53/10], ISO+CNP [51/10], ISO+CNP+BAY [54/10]; **F**) Con [25/6], ISO [27/6], ISO+CNP [27/6]) stated as box plots with whiskers showing minimum to maximum values, median and interquartile range; *P* were determined by Bonferroni test after a hierarchical model using log-transformed data (**E**: *P*=1.46×10<sup>-15</sup>; **F**: *P*=2.68×10<sup>-10</sup>).

ventricular tissues from failing and non-failing hearts (NF). In HF, the NPs *NPPA* and *NPPB* were significantly upregulated. In contrast, CNP expression was substantially downregulated in failing ventricles compared with healthy donor tissue (Figure 8A). Western blot analysis of the 3 NP receptors GC-A, GC-B and NPR-C revealed significant upregulation of GC-B and NPR-C, but not GC-A (Figure 8B and 8C). These findings are consistent with a relative CNP deficiency in patients suffering from end-stage HF,<sup>12</sup> offering a unique opportunity for therapeutic intervention.

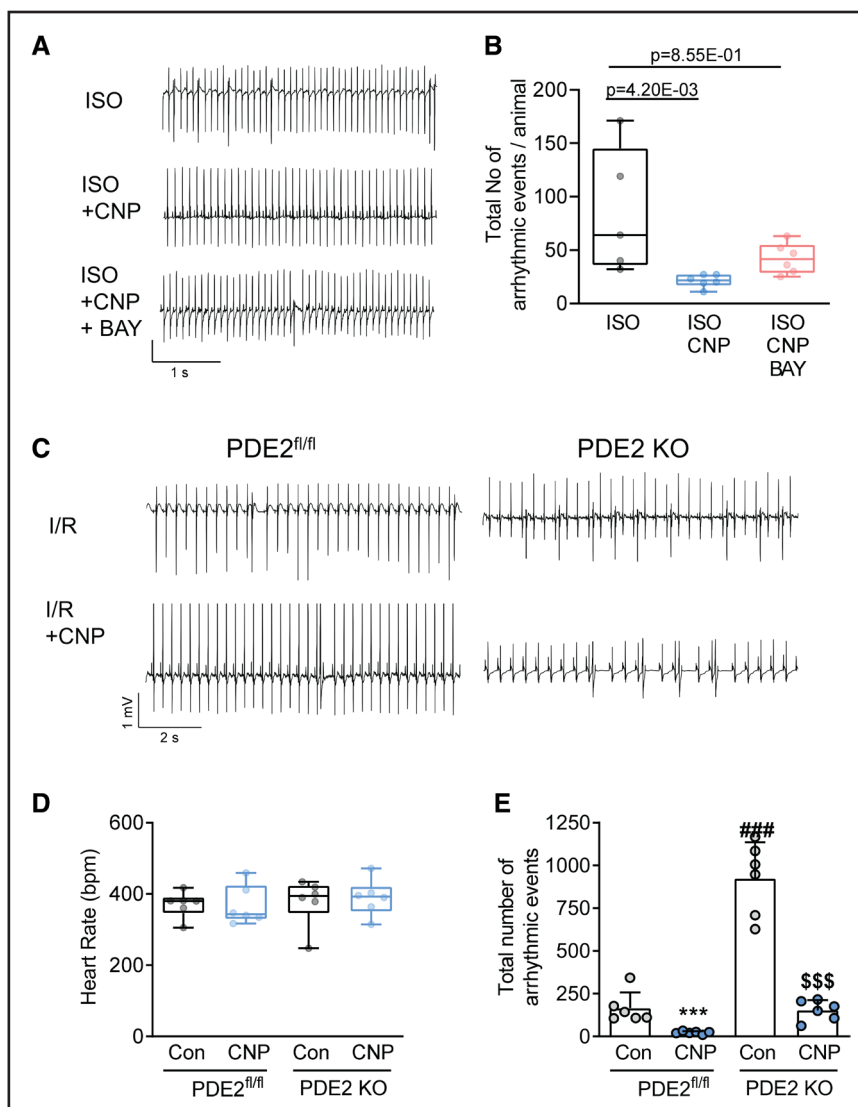
## DISCUSSION

In the current work, we demonstrate that activating the CNP-PDE2 axis exerts antiarrhythmic effects in perfused mouse hearts as well as in isolated mouse and

hiPSC-derived cardiomyocytes. The underlying mechanisms include reduced Ca<sup>2+</sup> sparks, I<sub>CaL</sub> and I<sub>NaL</sub> as well as reduced phosphorylation of CaMKII and PKA target proteins. Moreover, CNP prevents β-adrenergic-induced arrhythmia in vivo. Thus, activating the CNP-PDE2 axis may serve as a novel, highly effective antiarrhythmic therapeutic strategy.

## Cardiac-Specific PDE2 Knockout Mice

Since global PDE2 knockout is perinatally lethal,<sup>27</sup> we have created a novel cardiomyocyte-specific PDE2 knockout mouse line and for the first time describe the phenotype of cardiac PDE2 knockout. Under basal conditions, the contribution of PDE2 to total PDE activity in ventricular cardiomyocytes is relatively small; however,



### Figure 6. CNP (C-type natriuretic peptide) blunts the proarrhythmic effect of ISO in vivo.

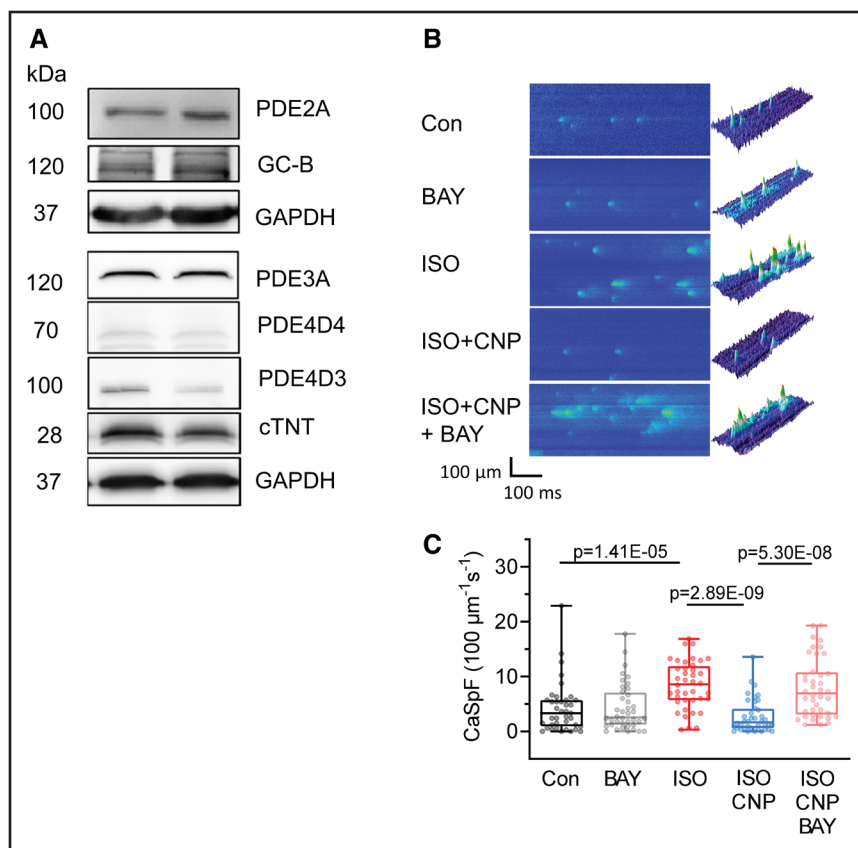
Two weeks after telemetry implantation WT animals were monitored by ECG-telemetry for a period of 90 minutes after double ISO injection (2 mg/kg, IP; 30-minute time interval, N=5) or simultaneous injection of CNP (33 mg/kg IP, N=6) or CNP+BAY 60-7550 (3 mg/kg IP, N=6) with the second injection. **A**, Representative ECG traces after the second injection. **B**, Average number of arrhythmogenic events per mouse, within 90 minutes after the second injection. Data are presented as box plots with whiskers showing minimum to maximum values, median and interquartile range. *P* were determined by Dunn's multiple comparison test after Kruskal-Wallis test ( $P=7.00 \times 10^{-4}$ ). **C**, Representative ECG recordings from ex vivo perfused PDE2<sup>fl/fl</sup> and PDE2 KO mouse hearts during reperfusion for 30 minutes with Krebs-Henseleit buffer with physiological catecholamine concentrations of 10 nM norepinephrine and 3.5 nM epinephrine following ischemia induction for 30 minutes. **D**, Average heart rates after I/R. **E**, Number of arrhythmic events within 30 minutes of reperfusion (N=6 per group). Data are presented as box plots with whiskers showing minimum to maximum values, median, and interquartile range (**D**) or mean $\pm$ SD (**E**). *P* were determined by 2-way-ANOVA \*\*\* $P=9.60 \times 10^{-9}$  for CNP, ### $P=1.49 \times 10^{-8}$  for PDE2 KO, \$\$\$ $P=2.81 \times 10^{-6}$  for interaction.

PDE2 is augmented in HF<sup>17</sup> and, importantly, its activity is increased by intracellular cGMP levels. Consequently, we see normal ventricular function, similar Ca<sup>2+</sup> transients and contractile activity in PDE2 knockout myocytes at rest and in response to isoprenaline as in controls. As indicated by the proarrhythmic effect of BAY in WT mice treated with isoprenaline+CNP, the CNP effects on isoprenaline-induced arrhythmias were blunted in PDE2 knockout myocytes. In I/R, BAY mitigated the antiarrhythmic effect of CNP to a larger extent than cardiomyocyte-specific PDE2 knockout. This indicates that in the intact heart, also other cell types or other signaling mechanisms may contribute to the antiarrhythmic effect of CNP.

### Effects of CNP-Mediated PDE2 Stimulation on Cardiac Arrhythmia

In cardiomyocytes, CNP activates GC-B to produce cGMP.<sup>4</sup> This stimulates PDE2, leading to increased cAMP

hydrolysis and mediating a negative crosstalk between the cGMP and cAMP pathways.<sup>28</sup> In the present study, CNP significantly reduced the isoprenaline-stimulated arrhythmogenic ion currents  $I_{CaL}$  and  $I_{NaL}$  in mouse ventricular cardiomyocytes, which was not observed in PDE2-deficient cells. In human atrial myocytes, PDE2 inhibition was reported to enhance  $I_{CaL}$  under basal conditions.<sup>29,30</sup> In our hands, the PDE2 inhibitor BAY alone did not influence  $I_{CaL}$  in ventricular mouse cardiomyocytes. These findings correspond well to our previous report that cardiac-specific PDE2 OE blunts the  $\beta$ -adrenergic increase in  $I_{NaL}$  and  $I_{CaL}$ .<sup>20</sup> The CNP-mediated reduction in arrhythmogenic ion channels is likely to contribute to the reduced occurrence of sAP. Concordantly, enhanced PDE2 activity in over-expressing cardiomyocytes significantly decreased the incidence of delayed afterdepolarizations and sAP.<sup>20</sup> In accordance with our previous findings in PDE2 OE, CNP-mediated PDE2 stimulation did not affect AP morphology. CNP infusion was recently shown to suppress ventricular arrhythmia in a rat model of acute MI.<sup>31</sup>



**Figure 7. CNP (C-type natriuretic peptide) reduces ISO-induced arrhythmogenic Ca<sup>2+</sup> spark (CaSp) in human iPSC-derived cardiomyocytes.**

**A**, Representative Western blots demonstrating the expression of GC-B and different PDEs (phosphodiesterases) in hiPSC-CMs (iBM76.3 hiPSC line). **B**, Representative measurements of CaSp in iPSC-CMs during 0.25 Hz pacing in an unstimulated state (Con) and after stimulation with BAY 60-7550 (BAY, 100 nM), ISO (10 nM), ISO plus CNP (1 μM), ISO plus CNP plus BAY for 10 minutes. **C**, Quantification of Ca<sup>2+</sup> spark frequency (CaSpF). Human iPSC-CMs were differentiated from hiPSC-lines created from 2 healthy donors (iBM76.1, N=2; iWTD2.1, N=2). Pooled data from N=4 independent experiments with 10 cells measured per condition (n=40). Data are presented as box plots with whiskers showing minimum to maximum values, median and interquartile range; *P* were determined by Bonferroni test after a hierarchical model using log-transformed data (*P*=3.90×10<sup>-3</sup>).

### Influence of CNP-Induced PDE2 Activation on Intracellular Ca<sup>2+</sup> Handling and Cellular Contractile Activity

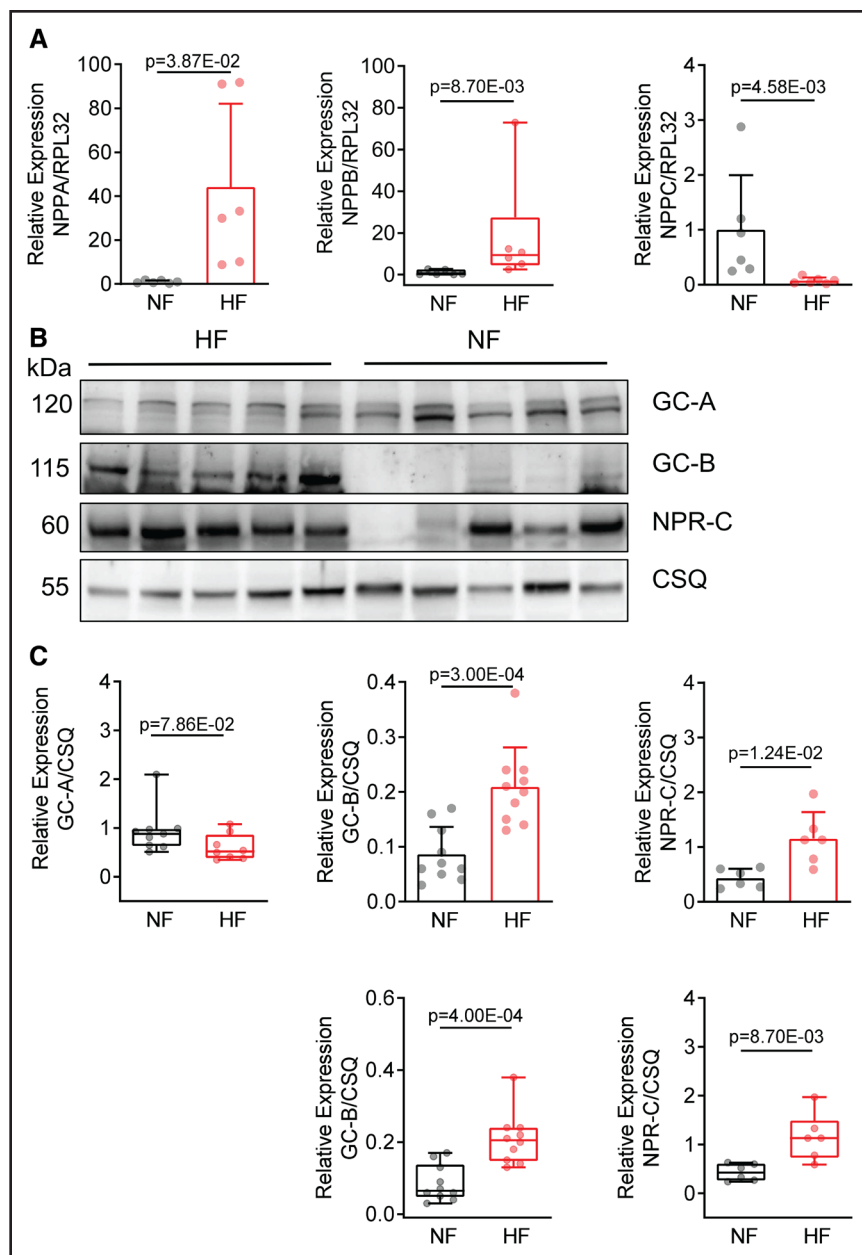
Both, cAMP and cGMP signaling pathways modulate intracellular Ca<sup>2+</sup> cycling via the activity of the downstream kinases PKA, CaMKII and PKG and thereby regulate cardiac contractile activity. Stimulated by cGMP, PDE2 contributes to negative regulation of PKA and CaMKII activity and their phosphorylation of Ca<sup>2+</sup> handling proteins. In line, PDE2 OE in cardiomyocytes resulted in reduced phosphorylation of CaMKII and RYR2 (ryanodine receptor 2) at the CaMKII site,<sup>17,20</sup> which promotes HF and arrhythmia.<sup>32</sup> The increases in CaSpF and Ca<sup>2+</sup> transient amplitude after β-adrenergic stimulation were blunted in PDE2 OE<sup>19,20</sup> and in the present study were markedly reduced upon CNP stimulation. CNP was previously shown to reduce intracellular Ca<sup>2+</sup> transients in mouse cardiomyocytes.<sup>33,34</sup> This effect of CNP was diminished upon PDE2 inhibition with erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) or BAY.<sup>34</sup> However, in our hands, CNP rather tended to a non-significant increase of Ca<sup>2+</sup> transient amplitudes. This fits to other studies demonstrating that CNP does not affect Ca<sup>2+</sup> transient amplitudes in failing hearts from rat or mice.<sup>35,36</sup> As reported previously, CNP enhanced the decay of the Ca<sup>2+</sup> transient, possibly by activation of PKG.<sup>33</sup>

CNP effects on contraction reported in the literature differ widely, showing either negative inotropic

effects<sup>33,36-38</sup> or positive inotropic responses in canine, murine, and rat cardiomyocytes, preparations or working hearts.<sup>39-43</sup> Differences between species, tissues (atria versus ventricles) or experimental conditions might contribute to these inconsistent effects. In addition, it has been suggested that the crosstalk between cAMP and cGMP pathways may contribute to these discrepancies.<sup>36</sup> Thus, simultaneous stimulation of the cAMP pathway activates PKA-induced phosphorylation of downstream targets, like LTCC, PLB, and TnI, counteracting cGMP and mediating positive inotropic effects.<sup>44</sup> Under certain circumstances, CNP can also enhance cAMP-induced signaling via cGMP-mediated inhibition of PDE3.<sup>42,45</sup> In the present study, CNP alone significantly increased cellular contraction rates but did not affect maximal contraction amplitude. Upon simultaneous β-adrenergic stimulation, CNP did not alter isoprenaline-mediated effects on cellular contraction. Furthermore, CNP was shown in several studies to mediate positive lusitropic effects via PKG-dependent phosphorylation of PLB (Ser16) and TnI (Ser23/24).<sup>36-39,41</sup> Consistently, CNP displayed a lusitropic effect similar to isoprenaline in this study. Stimulation with isoprenaline plus CNP did not further increase the lusitropic effect.

### Limitations

Cyclic nucleotide crosstalk is complex. Increasing levels of cGMP could inhibit PDE3, counteracting cAMP



**Figure 8. Reduced CNP (C-type natriuretic peptide) expression and upregulation of the GC-B receptor in human failing hearts.**

**A**, Quantification of mRNA expression from natriuretic peptides NPPA (ANP [atrial natriuretic peptide]), NPPB (BNP [brain natriuretic peptide]), NPPC (CNP [C-type natriuretic peptide]) in ventricular tissue from patients with HF or healthy donors relative to the housekeeping gene 60S ribosomal protein L32 (RPL32;  $N=6$ ). Data are presented as mean $\pm$ SD or box plots with whiskers showing minimum to maximum values, median and interquartile range;  $P$  were determined by unpaired  $t$  test or Mann-Whitney test. **B**, Representative western blots and **(C)** quantification of protein expression levels of natriuretic peptide receptors (GC-A [ $N=9$ ], GC-B [ $N=10$ ], NPR-C [ $N=6$ ]) in ventricular tissue from patients with HF or healthy donors relative to the housekeeping protein calsequestrin (CSQ). Data are presented as box plots with whiskers showing minimum to maximum values, median and interquartile range or mean $\pm$ SD;  $P$  were determined by unpaired  $t$  test or Mann-Whitney test.

hydrolysis by PDE2<sup>46</sup> and limiting its effect. PDE2 may itself degrade cGMP under specific circumstances, albeit to a smaller extent than cAMP, limiting its own activation. PDE3 and PDE4 are activated by PKA phosphorylation, thus increased PDE2 activity may in turn reduce their activation by degrading cAMP.<sup>28</sup> In this study, we focused on the role of PDE2 as the unique cGMP-stimulated phosphodiesterase. Despite the aforementioned complexity, inhibition of PDE2 prevented the CNP-mediated antiarrhythmic effects in the whole heart as well as in isolated cardiomyocytes and selective PDE2 upregulation was sufficient to reproduce the experimentally observed effects of CNP in a computer model. Moreover, antiarrhythmic effects of CNP were absent in cardiomyocytes of PDE2 knockout mice. Furthermore, PDE2 inhibition alone did not increase subcellular trigger mechanisms

like  $I_{CaL}$ , CaSp and waves in the absence of isoprenaline. In contrast, combined PDE inhibition with IBMX significantly increased CaSpF, promoted arrhythmia in ex vivo CNP-perfused hearts after I/R, and triggered potentially arrhythmogenic CaSp in human iPSC-derived cardiomyocytes.

Increasing cGMP levels also activate the PKG-dependent pathway that partially overlaps with the cAMP-PKA pathway. PKG has been shown to phosphorylate PLB, TnI and the L-type  $Ca^{2+}$  channel, contributing to negative inotropic and positive lusitropic responses.<sup>47</sup> In our hands, CNP alone did not affect  $Ca^{2+}$  transient amplitude and CaSpF. In addition, PKG inhibition did not alter the CNP-induced reduction of CaSp occurrence, indicating that cGMP-induced PKG activation might have only minor effects on this

arrhythmogenic trigger mechanism. However, we cannot completely exclude a contribution of PKG to the CNP-induced effects.

The effects of CNP may differ in humans compared with mice due to differences in PDE expression patterns and their remodeling during HF. In the human heart, PDE1, 2, and 3 are the most prominent isoforms, whereas PDE3 and PDE4 dominate in murine hearts.<sup>48,49</sup> Our experiments in human iPSC-derived cardiomyocytes indicate that the effect of CNP is in fact comparable in human and mouse cardiomyocytes. Furthermore, we demonstrate that in end-stage human HF, cardiac CNP expression is downregulated and not upregulated like ANP and BNP, which may contribute to ventricular arrhythmia and SCD in affected patients. The upregulation of PDE2 as well as the GC-B receptor in human HF may compensate this in part. This constellation is especially promising for therapeutic CNP supplementation in HF. The fact that nanomolar concentrations of CNP are effective in arrhythmia suppression in our preclinical studies is important, since this opens the possibility for CNP to be clinically effective and economically affordable when used as a drug in humans. However, the mechanisms of CNP actions may vary depending on the concentrations used, and the therapeutic potential of CNP clearly has to be further explored in forthcoming studies. Correction for multiple testing was performed for each individual test as indicated. We did however not correct for multiple testing across the entire work. This should be considered in interpreting our statistical analyses and constitutes a potential weakness of the study.

## Clinical Implications

In this study, we demonstrate that CNP-mediated PDE2 stimulation exerts a strong antiarrhythmic effect without negative inotropy, making it an interesting drug candidate. In human HF, CNP expression is reduced in ventricular tissues, leading to the hypothesis of a relative CNP deficit that is not sufficiently corrected by ARNIs.<sup>12</sup> Consequently, CNP could be useful to further attenuate intracellular  $\beta$ -adrenergic overdrive in patients suffering from acute ventricular arrhythmia even in addition to  $\beta$ -receptor blockers. Due to rapid degradation, CNP has a plasma half-life of only 2 to 3 minutes.<sup>5</sup> This means that CNP should be given by syringe pump in the acute clinical setting, such as MI or VT storm. For long-term therapy, for example, in patients suffering from congestive HF, a long-lasting CNP derivate or even an orally bioavailable small molecule GC-B agonist would be preferable. Recently, such long-lasting CNP derivates have been developed and show positive cardiac effects in experimental HF.<sup>50</sup> Of note, the long-acting CNP derivate vasoritide has already been approved for achondroplasia and could be available for repurposing.<sup>51</sup> Furthermore, PDE2 is activated by cGMP via its GAF domains.<sup>15</sup> Its

activation by small molecule compounds may provide a promising direction for future development.

## CONCLUSIONS

The present study provides evidence for strong antiarrhythmic effects of CNP by stimulation of PDE2. Abrogated increase of arrhythmogenic ion currents and SR CaSpF upon  $\beta$ -adrenergic stimulation were identified as underlying mechanisms. Of note, the positive inotropic effect of isoprenaline was not affected by CNP. Consequently, activating the CNP-PDE2 axis is a worthwhile path for further preclinical and clinical development to treat cardiac arrhythmia.

## ARTICLE INFORMATION

Received September 23, 2022; accepted January 16, 2023.

### Affiliations

Institut für Pharmakologie und Toxikologie, Technische Universität Dresden, Germany (E.C., M.G., M.S., M.S.S., J.S., F.D., C.B., S.Q., H.B., F.N., S.L., R.F., X.L., S.R.K., E.K., K.G., A.E.-A., M.W., S.K.). Institute of Clinical Chemistry and Laboratory Medicine, Department of Clinical Pathobiochemistry, University Hospital Dresden, Germany (P.M.). The James Black Centre, King's College, University of London, United Kingdom (M.M.). Internal Medicine III, University Hospital Carl Gustav Carus, Technische Universität Dresden, Germany (M.M.). Department of Molecular Cardiology and Epigenetics, Heidelberg University, Germany (M.D.). DZHK (German Center for Cardiovascular Research), Partner Site, Heidelberg-Mannheim, Germany (M.D., C.V.). Institute of Experimental and Clinical Pharmacology and Toxicology, University Medical Center Mannheim, Germany (C.V.). Department of Cardiology, CARIM School for Cardiovascular Diseases, Faculty of Health, Medicine, and Life Sciences, Maastricht University, The Netherlands (J.H.). Institut für Pharmakologie und Toxikologie, Julius-Maximilians-Universität Würzburg, Germany (K.L.). Leibniz-Institut für Analytische Wissenschaften-ISAAS-e.V., Dortmund, Germany (K.L.). Bereich Rhythmologie, Klinik für Innere Medizin und Kardiologie, Herzzentrum Dresden, Dresden University of Technology, Germany (M.W.).

### Acknowledgments

We are grateful to Annett Opitz, Romy Kempe, Manja Neue, Konstanze Fischer, and Marcel Hasse for their excellent technical assistance.

### Author Contributions

E. Cachorro and M. Günscht performed the in vivo and in vitro experiments, analyzed the data, prepared the figures and corrected the article. M. Schubert designed and supervised experiments with human-induced pluripotent stem cell-derived cardiomyocyte (hiPSC-CM). M.S. Sadek, J. Siegert, F. Dutt, C. Bauermeister, S. Quicker, F. Nowakowski, H. Berning, S. Lämmle, R. Firneberg performed experiments with mouse cardiomyocytes and analyzed the data. X. Luo performed experiments with hiPSC-CM. S.R. Künzel and E. Klapproth contributed to the article preparation. K. Lorenz performed experiments and contributed to article preparation. J. Heijman performed the computer simulations and contributed to article preparation. M. Dewenter, C. Vettel, P. Mirschink, M. Mayr, K. Guan, and A. El-Armouche supervised the experiments and critically reviewed the article. M. Wagner and S. Kämmerer supervised the work and prepared the article.

### Sources of Funding

This work was supported by the German Research Foundation (DFG) grants EL 270/7-3, Transregio-SFB CRC/TRR 205 to A. El-Armouche, grant KA 4194/3-3 to S. Kämmerer, project no. 288034826—international research training group (IRTG) 2251 to A. El-Armouche and S. Kämmerer, grant WA 2586/4-1 to M. Wagner, the Netherlands Organization for Scientific Research NWO/ZonMW Vidi 09150171910029 to J. Heijman, Grant No. 236177352-SFB1116 grant to K. Lorenz, SFB1525 project no. 453989101 to K. Lorenz, DFG-TR 296 Projektnummer 424957847; TRR 296/1 2020 to K. Lorenz and by research stipend of the German Heart Foundation/German Foundation of Heart Research K/26/18 to S. Quicker, F/45/19 to S. Kämmerer, and F/34/19 to E. Klapproth. F. Nowakowski received a doctoral student scholarship from the Carus Promotionskolleg from the Medical Faculty of the TU Dresden. M. Günscht was financially supported by the Graduiertenakademie of TU Dresden.

## Disclosures

All authors concur with the submission of the article, and none of the data have been previously reported or are under consideration for publication elsewhere. Parts of the doctoral theses of M.S. Sadek, J. Siegert, and F. Dutt are included in this article. The authors have declared that no conflict of interest exists related to this work.

## Supplemental Material

Figures S1–S11

Tables S1 and S2

Supplemental Methods

References 52–62

Major Resources Table

## REFERENCES

- Lip GY, Heinzel FR, Gaita F, Juanatey JR, Le Heuzey JY, Potpara T, Svendsen JH, Vos MA, Anker SD, Coats AJ, et al. European heart rhythm association/heart failure association joint consensus document on arrhythmias in heart failure, endorsed by the heart rhythm society and the Asia pacific heart rhythm society. *Europace*. 2016;18:12–36. DOI: 10.1093/eurheartj/ehv191
- Priori SG, Blomstrom-Lundqvist C, Mazzanti A, Blom N, Borggrefe M, Camm J, Elliott PM, Fitzsimons D, Hatala R, Hindricks G, et al; Group ESCSD. 2015 ESC Guidelines for the management of patients with ventricular arrhythmias and the prevention of sudden cardiac death: the task force for the management of patients with ventricular arrhythmias and the prevention of sudden cardiac death of the European Society of Cardiology (ESC). Endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC). *Eur Heart J*. 2015;36:2793–2867. Doi: 10.1093/eurheartj/ehv316
- Biton Y, Baman JR, Polonsky B. Roles and indications for use of implantable defibrillator and resynchronization therapy in the prevention of sudden cardiac death in heart failure. *Heart Fail Rev*. 2016;21:433–446. doi: 10.1007/s10741-016-9542-y
- Kuhn M. Molecular physiology of membrane guanylyl cyclase receptors. *Physiol Rev*. 2016;96:751–804. doi: 10.1152/physrev.00022.2015
- Moyes AJ, Hobbs AJ. C-type natriuretic peptide: a multifaceted paracrine regulator in the heart and vasculature. *Int J Mol Sci*. 2019;20:2281. doi: 10.3390/ijms20092281
- Wei CM, Heublein DM, Perrella MA, Lerman A, Rodeheffer RJ, McGregor CG, Edwards WD, Schaff HV, Burnett JC Jr. Natriuretic peptide system in human heart failure. *Circulation*. 1993;88:1004–1009. doi: 10.1161/01.cir.88.3.1004
- Palmer SC, Prickett TC, Espiner EA, Yandle TG, Richards AM. Regional release and clearance of C-type natriuretic peptides in the human circulation and relation to cardiac function. *Hypertension (Dallas, Tex.: 1979)*. 2009;54:612–618. doi: 10.1161/HYPERTENSIONAHA.109.135608
- Moyes AJ, Chu SM, Aubdool AA, Dukinfield MS, Margulies KB, Bedi KC, Hodivala-Dilke K, Baliga RS, Hobbs AJ. C-type natriuretic peptide co-ordinates cardiac structure and function. *Eur Heart J*. 2020;41:1006–1020. doi: 10.1093/eurheartj/ehz093
- Ichiki T, Schirger JA, Huntley BK, Brozovich FV, Maleszewski JJ, Sandberg SM, Sangaralingham SJ, Park SJ, Burnett JC Jr. Cardiac fibrosis in end-stage human heart failure and the cardiac natriuretic peptide guanylyl cyclase system: regulation and therapeutic implications. *J Mol Cell Cardiol*. 2014;75:199–205. doi: 10.1016/j.jmcc.2014.08.001
- Dickey DM, Dries DL, Margulies KB, Potter LR. Guanylyl cyclase (GC)-A and GC-B activities in ventricles and cardiomyocytes from failed and non-failed human hearts: GC-A is inactive in the failed cardiomyocyte. *J Mol Cell Cardiol*. 2012;52:727–732. doi: 10.1016/j.jmcc.2011.11.007
- McMurray JJ, Packer M, Desai AS, Gong J, Lefkowitz MP, Rizkala AR, Rouleau JL, Shi VC, Solomon SD, Swedberg K, et al. Angiotensin-nephrilysin inhibition versus enalapril in heart failure. *N Engl J Med*. 2014;371:993–1004. doi: 10.1056/NEJMoa1409077
- Ibrahim NE, McCarthy CP, Shrestha S, Gaggini HK, Mukai R, Szymonifka J, Apple FS, Burnett JC Jr, Iyer S, Januzzi JL Jr. Effect of neprilysin inhibition on various natriuretic peptide assays. *J Am Coll Cardiol*. 2019;73:1273–1284. doi: 10.1016/j.jacc.2018.12.063
- Murphy SP, Prescott MF, Camacho A, Iyer SR, Maisel AS, Felker GM, Butler J, Pina IL, Ibrahim NE, Abbas C, et al. Atrial natriuretic peptide and treatment with sacubitril/valsartan in heart failure with reduced ejection fraction. *JACC Heart Fail*. 2021;9:127–136. doi: 10.1016/j.jchf.2020.09.013
- Martins TJ, Mumby MC, Beavo JA. Purification and characterization of a cyclic GMP-stimulated cyclic nucleotide phosphodiesterase from bovine tissues. *J Biol Chem*. 1982;257:1973–1979. doi: 10.1016/s0021-9258(19)68134-2
- Martinez SE, Wu AY, Glavas NA, Tang XB, Turley S, Hol WG, Beavo JA. The two GAF domains in phosphodiesterase 2A have distinct roles in dimerization and in cGMP binding. *Proc Natl Acad Sci U S A*. 2002;99:13260–13265. doi: 10.1073/pnas.192374899
- Sadek MS, Cachorro E, El-Armouche A, Kammerer S. Therapeutic implications for PDE2 and cGMP/cAMP mediated crosstalk in cardiovascular diseases. *Int J Mol Sci*. 2020;21:7462. doi: 10.3390/ijms21207462
- Mehel H, Emons J, Vettel C, Wittkopper K, Seppelt D, Dewenter M, Lutz S, Sossalla S, Maier LS, Lechene P, et al. Phosphodiesterase-2 is up-regulated in human failing hearts and blunts beta-adrenergic responses in cardiomyocytes. *J Am Coll Cardiol*. 2013;62:1596–1606. doi: 10.1016/j.jacc.2013.05.057
- Yanaka N, Kurosawa Y, Minami K, Kawai E, Omori K. cGMP-phosphodiesterase activity is up-regulated in response to pressure overload of rat ventricles. *Biosci Biotechnol Biochem*. 2003;67:973–979. doi: 10.1271/bbb.67.973
- Vettel C, Lindner M, Dewenter M, Lorenz K, Schanbacher C, Riedel M, Lammler S, Meinecke S, Mason FE, Sossalla S, et al. Phosphodiesterase 2 protects against catecholamine-induced arrhythmia and preserves contractile function after myocardial infarction. *Circ Res*. 2017;120:120–132. doi: 10.1161/circresaha.116.310069
- Wagner M, Sadek MS, Dybkova N, Mason FE, Klehr J, Firneburg R, Cachorro E, Richter K, Klapproth E, Kuenzel SR, et al. Cellular mechanisms of the anti-arrhythmic effect of cardiac PDE2 Overexpression. *Int J Mol Sci*. 2021;129:804–820. doi: 10.3390/ijms22094816
- Kunzel SR, Hoffmann M, Weber S, Kunzel K, Kammerer S, Gunscht M, Klapproth E, Rausch JSE, Sadek MS, Kolanowski T, et al. Diminished PLK2 induces cardiac fibrosis and promotes atrial fibrillation. *Circ Res*. 2021;129:804–820. doi: 10.1161/CIRCRESAHA.121.319425
- Lopez-Lopez JR, Shacklock PS, Balke CW, Wier WG. Local, stochastic release of Ca<sup>2+</sup> in voltage-clamped rat heart cells: visualization with confocal microscopy. *J Physiol*. 1994;480:21–29. doi: 10.1113/jphysiol.1994.sp020337
- Trafford AW, Diaz ME, O'Neill SC, Eisner DA. Comparison of subsarcolemmal and bulk calcium concentration during spontaneous calcium release in rat ventricular myocytes. *J Physiol*. 1995;488:577–586. doi: 10.1113/jphysiol.1995.sp020991
- Bassani RA, Bers DM. Rate of diastolic Ca release from the sarcoplasmic reticulum of intact rabbit and rat ventricular myocytes. *Biophys J*. 1995;68:2015–2022. doi: 10.1016/s0006-3495(95)80378-4
- Sikkel MB, Francis DP, Howard J, Gordon F, Rowlands C, Peters NS, Lyon AR, Harding SE, MacLeod KT. Hierarchical statistical techniques are necessary to draw reliable conclusions from analysis of isolated cardiomyocyte studies. *Cardiovasc Res*. 2017;113:1743–1752. doi: 10.1093/cvr/cvx151
- Heijman J, Volders PG, Westra RL, Rudy Y. Local control of beta-adrenergic stimulation: effects on ventricular myocyte electrophysiology and Ca(2+)-transient. *J Mol Cell Cardiol*. 2011;50:863–871. doi: 10.1016/j.jmcc.2011.02.007
- Stenphenson DT, Coskran TM, Wilhelms MB, Adamowicz WO, O'Donnell MM, Muravnick KB, Menniti FS, Kleiman RJ, Morton D. Immunohistochemical localization of phosphodiesterase 2A in multiple mammalian species. *J Histochem Cytochem*. 2009;57:933–949. doi: 10.1369/jhc.2009.953471
- Zaccolo M, Movsesian MA. cAMP and cGMP signaling cross-talk: role of phosphodiesterases and implications for cardiac pathophysiology. *Circ Res*. 2007;100:1569–1578. doi: 10.1161/circresaha.106.144501
- Rivet-Bastide M, Vandecasteele G, Hatem S, Verde I, Benardeau A, Mercadier JJ, Fischmeister R. cGMP-stimulated cyclic nucleotide phosphodiesterase regulates the basal calcium current in human atrial myocytes. *J Clin Invest*. 1997;99:2710–2718. doi: 10.1172/jci.19460
- Vandecasteele G, Verde I, Rucker-Martin C, Donzeau-Gouge P, Fischmeister R. Cyclic GMP regulation of the L-type Ca(2+) channel current in human atrial myocytes. *J Physiol*. 2001;533:329–340. doi: 10.1111/j.1469-7793.2001.0329a.x
- He Y, Liu Y, Zhou M, Xie K, Tang Y, Huang H, Huang C. C-type natriuretic peptide suppresses ventricular arrhythmias in rats with acute myocardial ischemia. *Peptides*. 2020;126:170238. doi: 10.1016/j.peptides.2019.170238
- Swaminathan PD, Purohit A, Hund TJ, Anderson ME. Calmodulin-dependent protein kinase II: linking heart failure and arrhythmias. *Circ Res*. 2012;110:1661–1677. doi: 10.1161/circresaha.111.243956

33. Zhang Q, Scholz PM, Pilzak A, Su J, Weiss HR. Role of phospholamban in cyclic GMP mediated signaling in cardiac myocytes. *Cell Physiol Biochem*. 2007;20:157–166. doi: 10.1159/000104163
34. Su J, Scholz PM, Weiss HR. Differential effects of cGMP produced by soluble and particulate guanylyl cyclase on mouse ventricular myocytes. *Exp Biol Med*. 2005;230:242–250. doi: 10.1177/153537020523000403
35. Su J, Zhang Q, Moalem J, Tse J, Scholz PM, Weiss HR. Functional effects of C-type natriuretic peptide and nitric oxide are attenuated in hypertrophic myocytes from pressure-overloaded mouse hearts. *Am J Physiol Heart Circ Physiol*. 2005;288:H1367–H1373. doi: 10.1152/ajpheart.00880.2004
36. Moltzau LR, Aronsen JM, Meier S, Skogestad J, Hougen K, Orstavik O, Sjaastad I, Christensen G, Skomedal T, Osnes JB, et al. SERCA2 activity is involved in the CNP-mediated functional responses in failing rat myocardium. *Br J Pharmacol*. 2013;170:366–379. doi: 10.1111/bph.12282
37. Brusq JM, Mayoux E, Guigui L, Kirilovsky J. Effects of C-type natriuretic peptide on rat cardiac contractility. *Br J Pharmacol*. 1999;128:206–212. doi: 10.1038/sj.bjp.0702766
38. Manfra O, Calamera G, Froese A, Arunthavarajah D, Surdo NC, Meier S, Melleby AO, Aasrum M, Aronsen JM, Nikolaev VO, et al. CNP regulates cardiac contractility and increases cGMP near both SERCA and TnI: difference from BNP visualized by targeted cGMP biosensors. *Cardiovasc Res*. 2022;118:1506–1519. doi: 10.1093/cvr/cvab167
39. Wollert KC, Yurukova S, Kilic A, Begrow F, Fiedler B, Gambaryan S, Walter U, Lohmann SM, Kuhn M. Increased effects of C-type natriuretic peptide on contractility and calcium regulation in murine hearts overexpressing cyclic GMP-dependent protein kinase I. *Br J Pharmacol*. 2003;140:1227–1236. doi: 10.1038/sj.bjp.0705567
40. Beaulieu P, Cardinal R, Page P, Francoeur F, Tremblay J, Lambert C. Positive chronotropic and inotropic effects of C-type natriuretic peptide in dogs. *Am J Physiol*. 1997;273:H1933–H1940. doi: 10.1152/ajpheart.1997.273.4.H1933
41. Moltzau LR, Aronsen JM, Meier S, Skogestad J, Orstavik O, Lothe GB, Sjaastad I, Skomedal T, Osnes JB, Levy FO, et al. Different compartmentation of responses to brain natriuretic peptide and C-type natriuretic peptide in failing rat ventricle. *J Pharmacol Exp Ther*. 2014;350:681–690. doi: 10.1124/jpet.114.214882
42. Qvigstad E, Moltzau LR, Aronsen JM, Nguyen CH, Hougen K, Sjaastad I, Levy FO, Skomedal T, Osnes JB. Natriuretic peptides increase beta1-adrenoceptor signalling in failing hearts through phosphodiesterase 3 inhibition. *Cardiovasc Res*. 2010;85:763–772. doi: 10.1093/cvr/cvp364
43. Szarouzyk M, Kattih B, Martin-Garrido A, Trogisch FA, Dittrich GM, Grund A, Abouissa A, Derlin K, Meier M, Holler T, et al. Skeletal muscle derived myoclin protects the heart during pathological overload. *Nat Commun*. 2022;13:149. doi: 10.1038/s41467-021-27634-5
44. Moltzau LR, Aronsen JM, Meier S, Nguyen CHT, Hougen K, Orstavik O, Sjaastad I, Christensen G, Skomedal T, Osnes J-B, et al. SERCA2 activity is involved in the CNP-mediated functional responses in failing rat myocardium. *Br J Pharmacol*. 2013;170:366–379. doi: 10.1111/bph.12282
45. Meier S, Andressen KW, Aronsen JM, Sjaastad I, Hougen K, Skomedal T, Osnes JB, Qvigstad E, Levy FO, Moltzau LR. PDE3 inhibition by C-type natriuretic peptide-induced cGMP enhances cAMP-mediated signaling in both non-failing and failing hearts. *Eur J Pharmacol*. 2017;812:174–183. doi: 10.1016/j.ejphar.2017.07.014
46. Dunkerly-Eyring B, Kass DA. Myocardial phosphodiesterases and their role in cGMP regulation. *J Cardiovasc Pharmacol*. 2020;75:483–493. doi: 10.1097/fjc.0000000000000773
47. Calamera G, Moltzau LR, Levy FO, Andressen KW. Phosphodiesterases and compartmentation of cAMP and cGMP signaling in regulation of cardiac contractility in normal and failing hearts. *Int J Mol Sci*. 2022;23:2145. doi: 10.3390/ijms23042145
48. Richter W, Xie M, Scheitrum C, Krall J, Movsesian MA, Conti M. Conserved expression and functions of PDE4 in rodent and human heart. *Basic Res Cardiol*. 2011;106:249–262. doi: 10.1007/s00395-010-0138-8
49. Baillie GS, Tejada GS, Kelly MP. Therapeutic targeting of 3',5'-cyclic nucleotide phosphodiesterases: inhibition and beyond. *Nat Rev Drug Discov*. 2019;18:770–796. doi: 10.1038/s41573-019-0033-4
50. Chen Y, Zheng Y, Iyer SR, Harders GE, Pan S, Chen HH, Ichiki T, Burnett JC Jr, Sangaralingham SJ. C53: A novel particulate guanylyl cyclase B receptor activator that has sustained activity in vivo with anti-fibrotic actions in human cardiac and renal fibroblasts. *J Mol Cell Cardiol*. 2019;130:140–150. doi: 10.1016/j.yjmcc.2019.03.024
51. Duggan S. Vosoritide: first approval. *Drugs*. 2021;81:2057–2062. doi: 10.1007/s40265-021-01623-w
52. Boess FG, Hendrix M, van der Staay FJ, Erb C, Schreiber R, van Staveren W, de Vente J, Prickaerts J, Blokland A, Koenig G. Inhibition of phosphodiesterase 2 increases neuronal cGMP, synaptic plasticity and memory performance. *Neuropharmacology*. 2004;47:1081–1092. doi: 10.1016/j.neuropharm.2004.07.040
53. Maurice DH, Ke H, Ahmad F, Wang Y, Chung J, Manganiello VC. Advances in targeting cyclic nucleotide phosphodiesterases. *Nat Rev Drug Discov*. 2014;13:290–314. doi: 10.1038/nrd4228
54. Skarnes WC, Rosen B, West AP, Koutourakis M, Bushell W, Iyer V, Mujica AO, Thomas M, Harrow J, Cox T, et al. A conditional knockout resource for the genome-wide study of mouse gene function. *Nature*. 2011;474:337–342. doi: 10.1038/nature10163
55. Stables CL, Curtis MJ. Development and characterization of a mouse in vitro model of ischaemia-induced ventricular fibrillation. *Cardiovasc Res*. 2009;83:397–404. doi: 10.1093/cvr/cvp068
56. Grouzmann E, Cavadas C, Grand D, Moratel M, Aubert JF, Brunner HR, Mazzolai L. Blood sampling methodology is crucial for precise measurement of plasma catecholamines concentrations in mice. *Pflugers Arch*. 2003;447:254–258. doi: 10.1007/s00424-003-1140-x
57. Cyganek L, Tiburcy M, Sekeres K, Gerstenberg K, Bohnenberger H, Lenz C, Henze S, Stauske M, Salinas G, Zimmermann WH, et al. Deep phenotyping of human induced pluripotent stem cell-derived atrial and ventricular cardiomyocytes. *JCI Insight*. 2018;3:e99941. doi: 10.1172/jci.insight.99941
58. Luo X, Li W, Kunzel K, Henze S, Cyganek L, Strano A, Poetsch MS, Schubert M, Guan K. IP3R-mediated compensatory mechanism for calcium handling in human induced pluripotent stem cell-derived cardiomyocytes with cardiac ryanodine receptor deficiency. *Front Cell Dev Biol*. 2020;8:772. doi: 10.3389/fcell.2020.00772
59. Wacker C, Dams N, Schauer A, Ritzer A, Volk T, Wagner M. Region-specific mechanisms of corticosteroid-mediated inotropy in rat cardiomyocytes. *Sci Rep*. 2020;10:11604. doi: 10.1038/s41598-020-68308-4
60. Mehendale AC, Doyle JM, Kolin CM, Kroehle JP Jr. Unlock the information in your data: Software to find, classify, and report on data patterns and arrhythmias. *J Pharmacol Toxicol Methods*. 2016;81:99–106. doi: 10.1016/j.vascn.2016.05.007
61. Curtis MJ, Hancox JC, Farkas A, Wainwright CL, Stables CL, Saint DA, Clements-Jewery H, Lambiase PD, Billman GE, Janse MJ, et al. The Lambeth conventions (II): guidelines for the study of animal and human ventricular and supraventricular arrhythmias. *Pharmacol Ther*. 2013;139:213–248. doi: 10.1016/j.pharmthera.2013.04.008
62. Streckfuss-Bomeke K, Wolf F, Azizian A, Stauske M, Tiburcy M, Wagner S, Hubscher D, Dressler R, Chen S, Jende J, et al. Comparative study of human-induced pluripotent stem cells derived from bone marrow cells, hair keratinocytes, and skin fibroblasts. *Eur Heart J*. 2013;34:2618–2629. doi: 10.1093/eurheartj/ehs203