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Mutant phosphodiesterase 3A protects from hypertension-induced cardiac damage

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Abstract

Background: Phosphodiesterase 3A (PDE3A) gain-of-function mutations cause hypertension with brachydactyly (HTNB) and lead to stroke. Increased peripheral vascular resistance, rather than salt retention is responsible. Surprisingly, the few HTNB patients examined so far did not develop cardiac hypertrophy or heart failure. We hypothesized that in the heart, PDE3A mutations could be protective.

Methods: We studied new patients. CRISPR-Cas9-engineered rat HTNB models were phenotyped by telemetric blood pressure measurements, echocardiography, μCT, RNA-seq and single nuclei RNA-seq. Human induced pluripotent stem cells (iPSCs) carrying PDE3A mutations were established, differentiated to cardiomyocytes, and analyzed by Ca²⁺ imaging. We employed Förster resonance energy transfer (FRET) and biochemical assays.

Results: We identified a new PDE3A mutation in a family with HTNB. It maps to exon 13 encoding the enzyme’s catalytic domain. All hitherto identified HTNB PDE3A mutations cluster in exon 4 encoding a region N-terminally from the catalytic domain of the enzyme. The mutations were recapitulated in rat models. Both exon 4 and 13 mutations led to aberrant phosphorylation, hyperactivity, and increased PDE3A enzyme self-assembly. The left ventricles of our HTNB patients and the rat models were normal despite preexisting hypertension. A catecholamine challenge elicited cardiac hypertrophy in HTNB rats only to the level of wild-type rats and improved the contractility of the mutant hearts, compared to wild-type rats. The β-adrenergic system, phosphodiesterase activity and cAMP levels in the mutant hearts resembled wild-type hearts, while phospholamban phosphorylation was decreased in the mutants. In our iPSC cardiomyocyte models, the PDE3A mutations caused adaptive changes of Ca²⁺ cycling. RNA-seq and single nuclei RNA-seq identified differences in mRNA expression between wild-type and mutants affecting amongst others metabolism and protein folding.
**Conclusions:** While in vascular smooth muscle, *PDE3A* mutations cause hypertension, in hearts they confer protection against hypertension-induced cardiac damage. Non-selective PDE3A inhibition is a final, short-term option in heart failure treatment to increase cardiac cAMP and improve contractility. Our data argue that mimicking the effect of *PDE3A* mutations in the heart rather than non-selective PDE3 inhibition is cardioprotective in the long-term. Our findings could facilitate the search for new treatments to prevent hypertension-induced cardiac damage.

**Non-standard Abbreviations and Acronyms**

- HTNB: Hypertension with brachydactyly type E
- PDE3A: Phosphodiesterase 3A
- hiPSC-CMs: human induced pluripotent stem cell-derived cardiomyocytes
- LV: left ventricular/ventricle
- HW/BW: weight/body weight
- IVSd: interventricular septal end diastole
- DEG: differentially expressed gene

**Key words:** Hypertension; Genetics; Phosphodiesterase; Cardiac hypertrophy; Heart failure
Clinical Perspective

What is new?

• This study provides evidence that phosphodiesterase 3A selective activation could protect the heart from hypertrophy and failure.

• The mechanism involves long-term adaptations of mRNA and protein expression as well as Ca\(^{2+}\) cycling.

What are the clinical implications?

• The findings underscore the heterogeneity of phosphodiesterase 3A signaling in different tissues. They provide a basis for new cell-type-based therapeutic strategies.
Introduction

Hypertension affects more than a billion people worldwide and is the primary risk factor for cardiovascular disease. Hypertension with brachydactyly type E (HTNB) is an autosomal dominant Mendelian disease resembling essential hypertension. Untreated HTNB patients die of stroke by age 50 years. We demonstrated gain-of-function phosphodiesterase (PDE)3A gene mutations cause HTNB by increasing peripheral vascular resistance. We studied a large HTNB family earlier and were puzzled that cardiac hypertrophy and heart failure did not occur, which would be expected after decades of hypertension. Studies of isolated patients with the amino acid T445N substitution and a patient with a G449S substitution showed no increased left ventricular (LV) mass despite a blood pressure of up to 190/100 mmHg requiring minoxidil treatment. How mutant PDE3A could protect against hypertension-induced cardiac damage is unclear.

PDEs comprise 11 enzyme families that degrade cyclic adenosine monophosphate (cAMP) and/or cyclic guanosine monophosphate (cGMP) and thereby terminate cyclic nucleotide signaling. PDE3A hydrolyzes cAMP and exists in three isoforms (PDE3A1-3) transcribed and translated from alternative start sites of a single gene. All isoforms are expressed in human myocardium. They are distinguished at their N termini, contain the same catalytic domain and possess similar hydrolytic activities. The HTNB-causing mutations identified hitherto reside in a 15-base pair (bp) mutational hotspot in PDE3A exon 4 and cause amino acid substitutions within the five amino acid residues 445-449, which are located N-terminally of the catalytic domain. They affect PDE3A1 and PDE3A2, but not PDE3A3 because the latter lacks the affected N terminus (Figure 1A).

We report clinical data from a patient with an exon 4 and new HTNB patients with an exon 13 PDE3A mutation, affecting the catalytic domain of PDE3A (Figure 1A). Analyses of rat models carrying mutations in exon 4 or 13 and human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) carrying the mutations provided evidence for a cardioprotective function of both mutations and insight into the underlying molecular mechanisms.
Methods

All supporting data are available within the article and the Data Supplement. The analytic methods will be made available to other researchers for purposes of reproducing the results in their laboratories on request.

Analysis of patient mutation

All patients confirmed participation by written informed consent, including consent for publication of results with images. The Ethical Committee of the Charité and local Internal Review Boards approved the studies. Patient DNA was Sanger-sequenced.

Rat models and phenotyping

State of Berlin authorities approved the rat studies according to American Physiological Society guidelines (license G 0435/17). The PDE3A-R862C Sprague-Dawley rat model was generated analogously to the PDE3A-Δ3aa and functional deletion (Del) rats. Male rats (5-8 months, \( \approx 450-650 \) g) were phenotyped as described. Individual experiments were carried out with age-matched animals. Saline (0.9 % NaCl, 0.02 % ascorbic acid) or isoproterenol (0.13 mg/kg/h) was administered through osmotic minipumps (Alzet 2ML2, Charles River Wiga, Sulzfeld, Germany).

Human induced pluripotent stem cell (hiPSC) models

hiPSCs from a healthy donor (Berlin Institute of Health Stem Cell Core facility; cell line BIH-049 A) were used to introduce the mutations encoding the T445N and R862C substitutions CRISPR/Cas9 and TALEN methods. The cells were differentiated to cardiomyocytes (hiPSC-CMs) as described.
Ca^{2+} imaging, Förster resonance energy transfer (FRET), biochemical and molecular biological approaches

The hiPSC-CMs were loaded with Fluo-8-AM (2 µM) and imaged on a Zeiss NLO. Files were converted from LSM to csv format in ImageJ Studio and processed using a MatLab-based algorithm (CalTrack, https://github.com/ToepferLab/CalTrack). Levels of cAMP were measured using radioimmunoassay (RIA). PDE3A2-R862C activity was determined using FRET, PDE activity in left ventricles (LV) biochemically. Immunoprecipitation, Western blotting, spot synthesis of PDE3A-derived peptides and overlays with purified Flag-tagged PDE3A1, and qRT-PCR were carried out as described.

RNA-seq and single nuclei RNA-seq of rat hearts

RNA-seq libraries with rat LV-derived RNA were prepared with TruSeq Stranded mRNA kit (Illumina, Cat. No 20020595), and sequenced on a HiSeq 4000 platform (Illumina). We used featureCounts to count reads aligning to genes in rat genome (mRatBN7.2), accompanied by assembly-matched RefSeq genome annotations (GCF_015227675.2) with STAR v2.7.0f. Quality control was performed and gene expression analyzed using DESeq2. Enrichment analysis was accomplished using Metascape with default ‘Express Analysis’ settings.

Single nuclei sequencing of rat LV was performed as described. Sample data were integrated using Harmony and analyzed using SCANPY. Different clusters were detected using the Leiden algorithm. Cardiomyocytes were identified based on the expression of cardiomyocyte-specific marker genes. Differentially expressed genes in cardiomyocytes were detected using the find maker function with a minimum log2 fold change of 0.3. Gene set enrichment was performed using the gseapy package.

Statistics

Telemetry data were analyzed by likelihood ratio comparisons of nested mixed effects models assessing whether genotype adds predictive power to a model already containing animal ID.
and time as random effects using the R lmtest and lme4 packages. For all other statistical analyses, GraphPad Prism 8.4.3 or 9.1.0 was used. All data sets were tested for normal distribution using Kolmogorov-Smirnov, Shapiro-Wilk or D'Agostino and Pearson normality test. Differences between groups with normally distributed data were analyzed using one-way ANOVA with Tukey’s multiple comparisons test. Non-normal distributions were examined by Mann-Whitney or Kruskal-Wallis with Dunn’s multiple comparisons test. Repeated echocardiographic measurements were analyzed using the mixed effects model with Tukey’s multiple comparisons test. P values <0.05 were regarded statistically significant. Outliers were removed using the ROUT method (Q=1%). Representative immunoblots were selected to represent the means of the quantified data. Representative images were selected by eye and based on good signal/noise ratios.
Results

HTNB-causing PDE3A exon 4 mutations are cardioprotective

We previously reported a HTNB patient with a PDE3A exon 4 mutation encoding the amino acid substitution G449S (Figure 1A). The now 58-year-old patient had severe hypertension despite the treatment with maximal doses of an ACE inhibitor, L-type channel blocker, β-blocker, α₁-adrenoceptor antagonist, α₂-adrenoceptor agonist, loop diuretic and the direct vasodilator minoxidil (Data Supplement). In a 16-hour blood pressure recording, the patient presented a mean blood pressure of 160/102 mmHg during daytime and dipping during the night. Echocardiography showed a virtually normal LV, underpinning the cardioprotective effect of the mutation: LV wall thickness was 8 mm septal and 10 mm in the posterior wall, no signs of hypertensive LV hypertrophy (Figure 1B; Data Supplement). Ejection fraction (4-chamber view) was normal: 56%. LV end-diastolic diameter was somewhat enlarged (61 mm) but ventricular wall motion showed no abnormalities. Aortic valve was tricuspid, its function regular. Most likely post-endocarditic, the anterior mitral valve leaflet was slightly myxomatous thickened with a mild to moderate mitral valve insufficiency (excentric jet, directed towards free wall) leading to volume load and the mild ventricular enlargement. The valve defect was not considered significant for further interventional treatment or replacement; as a consequence, the left atrium was parasternal slightly dilated (43 mm); planimetric in 4-chamber-view: 26 cm², in 2-chamber-view 25 cm² (female: "slightly abnormal" 20-30 cm²). The right ventricle was normal. The patient had a moderate tricuspid valve insufficiency with moderate pulmonary hypertension (P_max 38 mmHg + central venous pressure). Vena cava inferior was breath-modulated. Some small arteriosclerotic carotid and aortal lesions were probably the result of a known although treated hypercholesterinemia. Carotid intima thickness was 0.6 mm on average.

To validate a potential cardioprotective effect of HTNB-causing mutations in the absence of anti-hypertensive treatment, we utilized our CRISPR/Cas9-generated PDE3A-Δ3aa rat model of HTNB. The rats lack nine bp in their Pde3a exon 4 causing a deletion of the three
amino acid residues 441-443, analogous to the human T445del genotype. We also included our functional deletion (Del) rats where a 20 bp deletion in exon 4 causes a frameshift and truncation of PDE3A at amino acid residue 439. Telemetric blood pressure measurements confirmed the hypertension of the PDE3A-Δ3aa rats (144/108mmHg). The blood pressures of the wild-type and the functional Del rats were 125/92mmHg and 110/87mmHg, respectively (data is mean of days -4 to +1, Figure 2A). Despite the hypertension in the 5-8 months old PDE3A-Δ3aa animals that would be prone to induce cardiac hypertrophy if not heart failure, the hearts appeared morphologically similar when compared to wild-type rats (Figure 2B). However, the media to lumen ratio of cardiac arteries was increased in the PDE3A-Δ3aa rats compared to wild-types (Figure 2B), similar to their secondary mesenteric arteries. The cardiac vessels of the functional Del rats resembled those of wild-type animals (as their peripheral arteries) and their hearts appeared morphologically normal, most likely due to their low blood pressure. Echocardiography indicated similar cardiac functional parameters of PDE3A-Δ3aa and wild-type rats (Figure 2C and 4F). Therefore, HTNB-causing PDE3A mutations in humans and our rat model affect vascular morphology and blood pressure but not the heart.

Next, we tested whether mutant PDE3A conferred such cardioprotection under the combined effects of preexisting hypertension and chronic β-adrenergic stimulation. The positive inotropic β-adrenergic agonist, isoproterenol, increases heart rate, lowers blood pressure and induces cardiac damage including hypertrophy in rats. Isoproterenol lowered the blood pressure of all our rat models within hours to a similar extent (Figure 2A). Within the 14 days of treatment, the blood pressure of PDE3A-Δ3aa animals returned to the pre-treatment level (Figure 2A), indicating a more rapid decline of the effect of β-adrenergic stimulation on the blood pressure than in wild-type and functional Del animals. Isoproterenol increased the heart rate in all rat groups throughout the treatment (Figure 2A), confirming the responsiveness of all hearts to the agent. Physiological saline (NaCl) as a control did not alter blood pressure or heart rate (Figure 2A). Blood pressures of the NaCl and isoproterenol groups of each genotype were not significantly different before treatment (Data Supplement).
The hallmark of cardiac hypertrophy is an increased LV mass. Isoproterenol significantly increased LV mass and the heart weight/body weight (HW/BW) ratio in the wild-type animals (Figure 2C). Of note, the increase of the LV mass in the hypertensive PDE3A-Δ3aa rats did not reach statistical significance. Only the interventricular septal end diastole (IVSd) diameter of PDE3A-Δ3aa hearts increased significantly. Cardiac output and fractional shortening (FS) significantly increased only in the PDE3A-Δ3aa rats. Since their heart rate was similar to that of wild-type animals (Figure 2A), these changes could not be attributed to an isoproterenol-induced increase of heart rate. The functional Del rats responded to isoproterenol similar to the wild-type animals (Figure 2C). Since their pre-treatment blood pressure was lower than that of the wild-type animals, we hypothesize an enhanced sensitivity to chronic β-adrenoceptor stimulation. Expression of hypertrophy and fibrosis markers (fibronectin, WGA, Collagen 1, Anp, Bnp, Coll1, Coll4, αMhc, βMhc) were inconsistent. The Anp mRNA level significantly increased only in LVs of isoproterenol-treated PDE3A-Δ3aa mutants, while the number of ED1-positive macrophages significantly increased in the wild-type, functional Del and PDE3A-Δ3aa rats (Figure S1). Fibronectin only significantly increased in the wild-type and functional Del rats (Figure 2D). Taken together, the HTNB-causing PDE3A-Δ3aa mutant had a cardioprotective effect; it did not amplify the isoproterenol-induced cardiac damage which would be expected in a state of preexisting hypertension.

A gain-of-function PDE3A exon 13 mutation affects the catalytic domain, causes HTNB and confers cardioprotection

We identified a new family with autosomal-dominant HTNB (Table S1). Their heterozygous missense mutation (c.2584C>T) causing a R862C (arginine-to-cysteine) substitution mapped to PDE3A exon 13 encoding the catalytic domain of the enzyme (Figures 1A and 3). The 43 years-old patient III/3 had a blood pressure of 202/137mmHg. Echocardiography revealed an ejection fraction of 77 %. The mitral valve moved normally, there was no mitral or aortic regurgitation. The aortic valve was tricuspid and moved normally without evidence of stenosis or
regurgitation. Thus, despite the hypertension and similar to the G449S exon 4-patient, the echocardiogram of the R862C exon 13-patient was essentially normal, with some increase in the LV wall thickness (systolic posterior LV wall was 1.8 cm, diastolic 1.45 cm).

Using CRISPR/Cas 9, we generated a rat model expressing the R862C substitution (Figure 4A). Similar to the heterozygous PDE3A-Δ3aa rats, in heterozygous and homozygous PDE3A-R862C rats PDE3A1 and PDE3A2 protein expression was downregulated in aorta and heart (Figures 4B, 7A and G, Figure S2A). HTNB patients are shorter in stature. Accordingly, the PDE3A-R862C rats were shorter (Figure S2B) and about 20 % lighter than wild-type animals (Figure 4C). MicroCT of paws documented the brachydactyly (Figure 4D, Figure S2C).

Telemetric blood pressures in male homozygous rats aged 7 months confirmed the hypertension (139/106mmHg; Figure 4E). Blood pressure values of heterozygous rats appeared normal except for day 5, when it was elevated compared to wild-type rats. In contrast to all other rat groups, homozygous R862C rats showed no or very little day-night heart rate and blood pressure rhythm (Figures 2A and 4E). The underlying mechanism is unclear. However, the blood pressure values confirmed the effects of the R862C substitution on blood pressure, although the effects in heterozygotes were not as robust as in the homozygotes or exon 4 mutations.

BMI, serum (Na⁺, K⁺, Cl⁻, urea, creatinine, cystatin) and urine parameters (Na⁺, K⁺, Cl⁻, urea, creatinine, albumin) were similar in homozygous PDE3A-R862C and wild-type rats (Figures 2D-F). Cardiac histology (Figure 2B) and parameters estimated by echocardiography were not different between PDE3A-R862C, wild-type and PDE3A-Δ3aa rats (Figure 4F). Renin, the central blood pressure regulator of the renin-angiotensin-aldosterone system (RAAS) system, increases blood pressure. It is secreted from renal juxtaglomerular cells in response to cAMP elevation; PDE3A is involved by cAMP hydrolysis. Hyperactivity of mutant PDE3A could lower renin levels through lowering cAMP. Indeed, in PDE3A-Δ3aa rats the serum renin level was lower than in wild-type animals. The renal renin mRNA was significantly downregulated in PDE3A-Δ3aa and upregulated in functional Del rats, while unchanged in homozygous PDE3A-R862C compared to wild-type animals (Figure S2G). Therefore, the RAAS system...
does not provide hypertrophic signals to the hearts of HTNB rats, e.g. via angiotensin recep-
tors. Thus, similar to our PDE3A-Δ3aa HTNB rat model, the PDE3A-R862C mutant recapitu-
lated human HTNB, including the normal cardiac phenotype.

With regard to blood pressure, the homozygous PDE3A-R862C animals responded to
saline or isoproterenol similarly to the PDE3A-Δ3aa rats (Figure 2A). Isoproterenol did not
significantly affect cardiac parameters such as LV mass, IVSd or HW/BW, indicating less hy-
pertrophy in PDE3A-R862C than in the other genotypes (Figure 2C). However, the PDE3A-
R862C hearts responded with a significant increase of fractional shortening and ejection frac-
tion, and a slightly increased cardiac output. Thus, PDE3A mutations encoding amino acid
substitutions within- and N-terminally from the catalytic domain cause HTNB, protect from hy-
pertension-induced cardiac damage and improve contractility.

**Exon 4 and 13 PDE3A mutants display similar properties**

We had shown that the exon 4 mutants, G449S, PDE3A2-T445N and PDE3A2-Δ3aa, were
hyperactive.\(^5\) To determine whether PDE3A2-R862C is also hyperactive, we used the same
Förster resonance energy transfer (FRET) approach as before (Figure 5A). Fusions of
PDE3A2-wild-type or PDE3A2-R862C with mCherry were co-expressed with the FRET sen-
sor, ICUE3, in HEK293 cells. The sensor consists of cyan (CFP) and yellow fluorescent protein
(Venus) flanking an Epac-based cAMP-binding site. Its emission intensity was similar in the
presence of PDE3A2-wild-type and PDE3A2-R862C, indicating similar activities. A ΔFRET of
0 indicates baseline cAMP. Forskolin induces cAMP synthesis by adenylyl cyclase activation.
In forskolin-stimulated cells expressing PDE3A2-R862C, the ΔFRET was reduced compared
to cells expressing the wild-type, reflecting lower cAMP and hyperactivity of the mutant. The
difference between the mutant and the wild-type was abolished by the PDE3 inhibitor, ci-
lostamide, demonstrating similar sensitivities to this inhibitor.

Our previous analyses revealed aberrant phosphorylation of exon 4 PDE3A2 mutants.\(^3,5\) In
HEK293 cells expressing PDE3A2-wild-type and PDE3A2-R862C, the protein kinase C (PKC)
stimulator phorbol-12-myristate-13-acetate (PMA) increased the phosphorylation of S428 of PDE3A2-R862C more than of wild-type (Figure 5B). A forskolin-induced cAMP increase had no effect on S428 phosphorylation, ruling out an involvement of protein kinase A (PKA), the main effector of cAMP. PMA and forskolin did not change the S438 phosphorylation of PDE3A2-R862C compared to wild-type (Figure 5B). Proteins of the 14-3-3 family are ubiquitously expressed and bind phosphorylated serine residues of other proteins, and we previously found an increased interaction of 14-3-3 with exon 4 PDE3A2 mutants. The interaction of PDE3A2-R862C with 14-3-3 was slightly but not significantly increased compared to wild-type (Figure 5C). Thus, in HTNB aberrant phosphorylation of mutant PDE3A is common, while the interaction of the mutants with 14-3-3 may not play a general role.

Since all PDE3A mutations cause aberrant phosphorylation and hyperactivity of the enzyme, the involved phosphosite region S428/S438 most likely controls the catalytic domain (amino acid residues 810-1068) through a conformation-based mechanism. Our further biochemical studies (Figure S3) indicated increased dimerization/self-assembly of all mutant PDE3A. The introduction of the R862C substitution promoted disulfide bond formation. The catalytic domain crystallizes as a dimer and is active as a dimer. Thus, the mutations could affect PDE3A activity by modulating the dimerization.

**Gene expression changes and phospholamban phosphorylation is reduced in PDE3A mutant rat hearts**

To uncover molecular mechanisms downstream of mutant PDE3A that account for the cardioprotective effect, we analyzed LVs of wild-type, PDE3A-Δ3aa and functional Del rat hearts by RNA-seq. By determining the differential gene expression profiles, we found up- and down-regulated genes, especially in the LVs of the functional Del model under treatment conditions (Figure 6A and 6B; Table 1; Figure S4A and Excel files S1 and S2). Pde3a expression was higher in LVs of isoproterenol-treated PDE3A-Δ3aa rats when compared to wild-type. Reduced Pde3a mRNA in the functional Del was detected (Figure 6C). The number of statistically
significant differentially expressed genes (DEGs) in the LVs between the untreated or treated
wild-type and PDE3A-Δ3aa rats was low (Figure 6B). No significant DEG were common be-
tween isoproterenol- and NaCl-treated samples (Figure 6B). Functional enrichment analysis
of DEGs revealed PDE3A-related involvement in protein folding, metabolism and Ca\(^{2+}\) regula-
tion (Figures 6D-F). Since PDE3A plays a role in β-adrenergic signaling, Ca\(^{2+}\) reuptake into
the sarcoplasmic reticulum (SR) and thus relaxation of cardiomyocytes during diastole,\(^{21, 37, 38}\)
we focused on assessing components of the β-adrenergic system.

Western blotting revealed downregulation of PDE3A1 and PDE3A2 expression in LVs of
the untreated PDE3A-Δ3aa rats compared to wild-type (Figure 7A). Since Pde3a mRNA (Fig-
ure 6C), PDE3 and non-PDE3 activity (Figure 7B) and the cAMP levels (Figure S4B) in the
PDE3A-Δ3aa LVs and the wild-type were similar, the hyperactivity of mutant PDE3A is appar-
ently compensated by downregulation of the protein and does not affect global cAMP levels.
An autophagosomal-mediated degradation could explain the observation, as a partial PDE3A
degradation through an autophagosomal-lysosomal pathway during hypoxia occurred in a
mouse ischemia/reperfusion model.\(^{39}\) Our gene ontology (GO) analyses (Figure 6) identified
aberrations in the protein folding and protein stability machinery. Such proteins may be in-
volved in the control of the mutant PDE3A. The unfolded protein response (UPR) mediates a
cardioprotective effect, e.g. by limiting energy consumption.\(^{40}\)

Our anti-PDE3A antibody recognizing the C terminus of all three isoforms detected PDE3A
in the wild-type at the Z-lines and in between. The signals between Z-lines seemed reduced
in the PDE3A-Δ3aa LVs (Figure 7C). PDE3A1 is located in microsomal fractions such as the
SR, and PDE3A2 and A3 are both microsomal and cytosolic.\(^{10}\) Since PDE3A3 was not detect-
able by Western blotting (Figures 7A and 7G), the expression of PDE3A2 appeared downreg-
ulated in the cytosol. As expected, PDE3A was not found in functional Del LVs. RNA-seq data
and Western blotting did not detect differences in expression levels of components of the β-
adrenergic signaling pathway, the Ca\(^{2+}\) cycling and contraction machinery or hypertrophy
markers such as ANP and BNP between wild-type and PDE3A-Δ3aa animals (Figures 7D,
Differences in protein expression were restricted to the functional Del rats, e.g. Troponin I (Tnl), phosphorylated Troponin I (pTnl) and PDE4A were upregulated, while ANP and PDE1A were downregulated compared to wild-type (Figure 7D). Thus, in PDE3A-Δ3aa LVs global RNA and protein expression profiles were at physiological levels. However, PDE3A acts locally.

In cardiomyocytes, PDE3A1 and PDE3A2 interact with a protein complex comprising A-kinase anchoring protein (AKAP)18, protein kinase A (PKA), calmodulin kinase IIδ (CaMKIIδ), SR Ca\(^{2+}\)-ATPase (SERCA)2a and phospholamban (PLN), which controls Ca\(^{2+}\) reuptake into the SR and thereby relaxation.\(^{21, 37, 38}\) Within the complex, cAMP-activated PKA phosphorylates PLN causing dissociation of PLN from SERCA2a releasing its inhibitory effect on SERCA2a. As a result, SERCA2a pumps Ca\(^{2+}\) into the SR. In the PDE3A-Δ3aa LVs, the PLN phosphorylation of the serine-16 PKA phosphorylation site and of threonine-17 was decreased (Figure 7E), indicating that hyperactive PDE3A locally affects phosphorylation and suggesting an inhibitory effect of the mutant PDE3A on SERCA2a. In the light of the similar global cAMP levels in the PDE3A-Δ3aa and wild-type LVs, the hyperactive PDE3A mutant most likely locally lowers cAMP and thereby the activity of PKA in the vicinity of the protein complex.

To detect cardiomyocyte-specific DEGs, we carried out single nuclei (sn)RNA-seq of two LVs from each wild-type, PDE3A-Δ3aa and functional Del rats. The analysis revealed, for example, changes in insulin, cGMP and metabolic pathways (Figure S4D and Excel File S3). In the adrenergic signaling pathway, Camk2δ, Myl2, Kcnq1, Scn5a, Slc8a1 and Myh7 were upregulated in PDE3A-Δ3aa compared to wild-type, while Ppp1cb, Rps6ka5 and Adcy6 were downregulated (Figure 7F). The downregulation of Adcy6 (adenylyl cyclase 6) would cause a decrease of cAMP synthesis. The downregulation of Ppp1cb, encoding protein phosphatase 1 could increase protein phosphorylation. Therefore, proteins in addition to PLN may be differentially phosphorylated in HTNB.

Isoproterenol treatment of the wild-type rats significantly downregulated the PDE3A1 protein compared to the NaCl-treated animals (Figure 7G). The decreased expression level was
similar to that in NaCl- and isoproterenol-treated PDE3A-Δ3aa and PDE3A-R862C rats. Of note, compared to NaCl, isoproterenol increased PDE3A2 expression in both HTNB mutants, although the difference did not reach statistical significance. Thus, in contrast to PDE3A1, it appears that PDE3A2 in the HTNB mutants is differentially regulated in response to β-adrenergic stimulation. Despite the differences in PDE3A protein expression between the NaCl- and isoproterenol-treated PDE3A-Δ3aa and wild-type rats, the cAMP levels in their LVs were statistically not different (Figure S4B), underpinning that the hyperactivity of mutant PDE3A is compensated by downregulation of mutant PDE3A protein expression. NaCl or isoproterenol induced significant changes of the mRNA levels of Pde1C and Pde4B only in the functional Del rat LVs (Figure 6, Figure S4A), presumably to achieve similar cAMP levels as the wild-type (Figure S4B).

In line with the few changes in mRNA expression (Figure 6, Figure S4A), the expression levels of only a few proteins in the β-adrenergic signaling cascade changed in response to isoproterenol (Figure 7G). β1-adrenergic receptor expression was highest in LVs of NaCl-treated wild-type rats and decreased in the wild-type and HTNB mutants in response to isoproterenol. Isoproterenol increased the SERCA2a and decreased TnI levels, and S16- and T17-phosphorylated PLN in the HTNB mutants compared to wild-type. However, the differences did not reach statistical significance. Other proteins involved in maladaptive responses of LVs, including MAP kinases, ANP or BNP were not affected by isoproterenol (Figure 7G, Figure S4A and E).

Collectively, the RNA-seq, the snRNA-seq, protein expression and cAMP analyses of the LVs indicated that PDE3A mutations lead to a molecular state of the β-adrenergic system that resembled the wild-type despite the hypertension. The differences between wild-type and the HTNB hearts, involving for example metabolic processes, protein folding and the PI3K-AKT signaling pathway (Figures 6 and 7F), are most likely additional critical factors contributing to the cardioprotection conferred by HTNB-causing PDE3A mutations.
Ca²⁺ cycling in PDE3A mutant cardiomyocytes is different from wild-type

The increased fractional shortening of the hearts of the isoproterenol-treated PDE3A-Δ3aa and PDE3A-R862C rats, the increased ejection fraction of hearts of the isoproterenol-treated R862C rats (Figure 2C) and the ejection fractions (56 % and 73 %) of our HTNB patients (see above) pointed to adaptations in contractility. Moreover, the decreased PLN phosphorylation in the LVs of the untreated PDE3A-Δ3aa animals (Figure 7E) suggested effects of the mutations on Ca²⁺ cycling. We analyzed Ca²⁺ cycling in human induced pluripotent stem cells (iPSCs) expressing the PDE3A-T445N or PDE3A-R862C substitution that were differentiated to cardiomyocytes (Figure 8A, Figure S5). Mutant PDE3A was detected at the Z-lines (Figure 8B), and, as in our animal models, the protein expression of PDE3A1 and PDE3A2 was downregulated in the mutants (Figure 8C). The L-type Ca²⁺ channel (Ca,1.2) mediating Ca²⁺ entry, was downregulated in both mutants; components of the contractile apparatus, myosin-binding protein C3 (MyBPC3) and pTnI were also downregulated in the mutant cells; however, to a significant level only in the T445N cells.

The Ca²⁺ transient duration at 50 % amplitude (Figure 8D and E) in the presence of isoproterenol was significantly longer in the T445N and slightly longer in the R862C mutant compared to wild-type cells. The combination of isoproterenol and cilostamide caused a significantly extended duration at 50 % amplitude in both mutants compared to wild-type. The decay time to 50 % amplitude, i.e. the Ca²⁺ reuptake into the SR, was longer in the T445N cells than in the wild-type and similar between wild-type and R862C cells upon isoproterenol stimulation. The combination of isoproterenol and cilostamide had a similar effect. The rise time to 50 % amplitude, i.e. Ca²⁺ release from the SR, was similar in all cells. Together, these effects indicated a longer dwell time of Ca²⁺ in the cytosol of the mutants compared to wild-type, which, in turn, can promote contractility.
Discussion

Increased transmural wall tension occurs in HTNB patients and is an obvious stimulus for cardiac hypertrophy. However, the hearts of our patients were normal, except for post-endocarditic damage. Our study shows that PDE3A mutations affecting a region N-terminally of the catalytic domain or the catalytic domain cause HTNB, lead to hyperactive PDE3A enzymes and protect against hypertension-induced cardiac damage. Inhibition has opposite consequences. Non-selective PDE3 inhibition with milrinone is only a final, short-term option in heart failure treatment because milrinone increases mortality. Our data argue that activation of PDE3A rather than non-selective PDE3 inhibition is cardioprotective in the long-term. In line, the functional Del animals, mimicking treatment with a PDE3A-specific inhibitor, responded to the chronic β-adrenergic stimulation similar to the wild-type animals although their baseline blood pressure was lower, i.e. PDE3A loss/inhibition sensitized them to cardiac stress. The hearts of Pde3a knockout mice were not protected from ischemia/reperfusion injury.

The HTNB-causing PDE3A exon 4 mutations affect PDE3A1 and A2 while the exon 13 mutation affects all three isoforms. Therefore, at least PDE3A1 and A2 play a role in conferring cardioprotection. A role of PDE3A1 alone is unlikely because cardiac transgenic PDE3A1 overexpression, which increased cellular PDE3A activity, decreased cardiac performance by downregulation of the β-adrenergic system. However, similar to the situation in the PDE3A-Δ3aa hearts, the cardiac transgenic overexpression did not induce maladaptive fibrosis or apoptosis. In the transgenic mice compared to wild-type, angiotensin II-induced cardiac fibrosis was less, and the ischemia/reperfusion injury-induced myocardial infarct size and the number of apoptotic cells was reduced. PDE3A1 and A2 were detected in the LVs of our HTNB rat models, and as opposed to transgenic overexpression of only PDE3A1, our rat models maintained the cardiac β-adrenergic system in a state resembling the wild-type despite the hypertension, even upon additional isoproterenol-induced cardiac stress. Therefore, the cardioprotection conferred by HTNB-causing PDE3A mutations most likely involves PDE3A1 and PDE3A2.
We had shown that hypertension in HTNB is provoked by vascular smooth muscle and found an increased media to lumen ratio in secondary mesenteric arteries. Cardiac vessels of our HTNB rat models displayed a similarly increased ratio. Since heart function and morphology of the HTNB mutants resembled the wild-type, the cardioprotective effect appears to reside in the cardiac cells. Our RNA-seq and snRNA-seq data pointed to, amongst others, changes in metabolism in whole LVs and individual cardiomyocytes of the PDE3A-Δ3aa model, which could favorably modulate energy consumption. The observed adaptations of Ca²⁺ cycling in hiPSC-CM also argue for an involvement of cardiomyocytes.

PDE3A isoforms reside in different compartments of cardiomyocytes, PDE3A1 and PDE3A2 at the SR. The HTNB mutations caused local changes of signaling, as emphasized by the reduction of PLN phosphorylation in LVs of PDE3A-Δ3aa rats. The hyperactivity most likely lowers local cAMP levels and thereby local PKA activity and PLN phosphorylation. Thus, hyperactivity of the mutants in defined cellular compartments appears to play an important role in the cardioprotective effect although global mutant PDE3A protein expression is downregulated. Whether altered protein interactions of the HTNB PDE3A mutants modify tethering to compartments is unclear, as e.g. the interaction of 14-3-3 with different PDE3A mutants is not consistently increased. The aberrant phosphorylation of the exon 43 and R862C HTNB mutants is associated with their hyperactivity and underpins their better accessibility for kinases and/or phosphatases compared to wild-type enzymes.

C707R and A980V substitutions at the N and C termini of the PDE3A catalytic domain reduced enzyme activity, indicating that catalytic domain mutants do not generally increase PDE3A activity. Our data suggest increased dimerization/self-assembly of mutant PDE3A as a mechanism underlying the hyperactivity. Dimerization/self-assembly as a mechanism for activity control emerges as a common theme amongst PDEs. PDE3A and PDE4 isoforms are active as dimers. The PDE3B catalytic domain crystalized as dimer, or tetramer. Moreover, increasing activity of PDE2, a dimer, in mice by transgenic overexpression protected
against arrhythmias and improved contractility after ischemic insult, and PDE4B overexpression prevented systolic dysfunction, apoptosis, and fibrosis, and attenuated hypertrophy induced by chronic isoproterenol infusion. Recently, allosteric activators of PDE4 dimers were discovered. They could serve as a template towards development of novel PDEs activators for cardioprotection.

Our study shows that HTNB-causing PDE3A mutations protect against hypertension-induced cardiac damage and suggest mimicking their effects in the heart as a new strategy towards prevention of hypertension-induced cardiac damage and heart failure. Targeting PDE3A needs to consider that its three isoforms function cell type-specifically in different, highly regulated microdomains.
Acknowledgements

Sadly, Matthew A. Movsesian has passed away during the preparation of the manuscript. With “Matty”, we have lost a friend and colleague who was enthusiastic about PDE3. His contributions will be missed in the future. We thank our patients for their participation in these studies.

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Author Contributions

ME carried out FRET experiments, immunoprecipitation and Western blotting. MBM generated the HTNB iPSC model. MBM, PHD and BIMF differentiated iPSC to cardiomyocytes. MBM, TP and PHD characterized the iPSC-CM model. TP and MBM performed Ca²⁺ imaging. BIMF
and MBM carried out immunofluorescence microscopy with iPSC-CM model. MBM carried out snRNA-seq. LM, YE, TUPB, and SF carried out tissue preparation and staining and qPCRs, and evaluated blood pressure data. CS performed experiments with regard to self-assembly. AK discovered and phenotyped the PDE3A-R862C HTNB family. MG and AA carried out sequencing of DNA of the PDE3A-R862C HTNB family. AS carried out histological staining, Western blotting, and RNA-seq with validation of results. TL and FQ stained tissue and did immunofluorescence microscopy. BJM, DFL, DDL, DYS-F, TB and PGM evaluated RNA-seq data. SW examined the G449S patient. RWG was involved in Ca²⁺ imaging. CM performed Western blotting. KeZ carried out Western blotting and coordinated animal breeding. MR did RIA assays. StS, MT and AH carried out echocardiography. SP and FWH provided recombinant proteins. HN did µCT analyses. JE and ML were involved in FRET measurements. DM and SD supported the iPSC establishment. RL and DNM supported animal studies. MAM carried out PDE activity measurement. SB contributed to conceptualizing the study. NH was involved in snRNA-seq, genetic analysis and establishing the stem cell model. MB and EP designed and supported generation of animal models and animal experiments. EK designed and coordinated the study and wrote the manuscript.

Disclosures

No author has a competing private or commercial interest.

Supplemental Materials

Expanded Methods

Figures S1 – 5

Tables S1 – 2

Excel File S1 - 3
References


by partial phosphodiesterase-3A degradation enhances cardioprotection during hypoxia. 


Figure legends

Figure 1. Location of HTNB-causing mutations in the PDE3A gene.
A. Mutations cluster in a mutational hotspot in a regulatory region in exon 4 and one is located within exon 13 encoding the catalytic domain of the enzyme. B. Images and data of an echocardiographic examination of a 58-years old HTNB patient expressing the PDE3A-G449S substitution.

Figure 2. Hearts from wild-type and HTNB rats respond similarly to chronic β-adrenergic stimulation.
A. Radiotelemetry was employed to measure blood pressure (BP) and heart rates of two rat HTNB models, heterozygous PDE3A-Δ3aa (red) with a deletion of three amino acid residues within the mutational hotspot N-terminally of the catalytic domain, and homozygous PDE3A-R862C (purple) rats with a R862C substitution in the catalytic domain of PDE3A (see Figure 1A). In addition, the measurements were carried out using a rat model with a 20 bp deletion that gives rise to a frameshift and a truncated, functionally deleted protein (functional Del; green) and wild-type (black) rats. On day six after initiation of the measurements, osmotic minipumps for administration of isoproterenol (Iso, left) or physiological saline (NaCl, right) were implanted and measurements continued for 2 weeks. Shown are systolic and diastolic BP and heart rates (beats per minute; BPM) over time (horizontal axis, night phases marked in black). The curves represent loess fits. Gray intervals, 95 % CIs for loess parameters; horizontal dashed lines, model expectation values. Lower black bars depict night periods. PDE3A-Δ3aa: Iso, n = 9, NaCl, n = 6; R862C animals: Iso, n = 5, NaCl, n = 4; wild-type: Iso, n = 7, NaCl, n = 9; functional DEL: Iso, n = 8, NaCl, n = 6. The BPs of Iso-treated animals were significantly different between all genotypes; likewise, the BPs of the NaCl-treated animal groups were significantly different from each other. The heart rate of homozygous R862C rats differs significantly from the other animals. P values from likelihood ratio tests comparing
nested linear mixed models are listed in the Data Supplement. B. Hearts of wild-type, heterozygous PDE3A-Δ3aa, homozygous PDE3A-R862C and functional Del rats were stained with H&E and Picro Sirius Red. At least 25 non-overlapping image fields from 5 different samples in each experimental group were analyzed using ImageJ Studio. Cardiac hypertrophy was evaluated by measuring the cross-sectional area of cardiomyocytes. For the cardiac arteries, the media to lumen ratio was calculated by dividing the square of tunica media to the luminal area. The fibrosis index (%) was calculated as a percentage of collagen-positive areas to the total area of the image. Statistical analysis was carried out using a Kruskal-Wallis and Dunn’s multiple-comparison test; shown are mean ± SEM. *p < 0.05, **p < 0.01, ****p < 0.0001. C. During the BP measurements shown in A, the day before implantation of osmotic minipumps (day 0), and at the end of the experiment (day 14) the indicated cardiac parameters were determined by echocardiography. Statistical analysis was carried out using the mixed effects analysis with Tukey’s multiple comparisons test; shown are mean ± SD. *p < 0.05, **p < 0.01. Further parameters measured by echocardiography are indicated in Table S2. D. Fibronectin (FN; red) was stained and the percentage of Fn positive areas was determined. Statistical analysis was carried out using two-way ANOVA and Tukey’s multiple-comparison test; shown are mean ± SD. **p < 0.01.

Figure 3. Pedigree of HTNB family with missense mutation causing an R862C substitution in the catalytic domain of PDE3A.
Black, HTNB-affected; grey, not affected by hypertension at the age of 23 years. Roentgenograms of index patient III/3 are shown. Arrows indicate shortened metacarpal bones (white) and cone-shaped epiphysis (red), characteristic for brachydactyly type E. In addition, the terminal phalanx of both thumbs represents brachydactyly type D. Both phenotypes, brachydactyly type E and hypertension, vary in their severity in the affected subjects (Table S1). Sanger sequencing identified the heterozygous PDE3A mutation c.2584C>T causing a R862C amino
acid substitution in subjects III/3, IV/3, IV/4. Subject IV/2 did not give consent for sequencing.

The co-segregating haplotype indicated also carrying the mutation.

**Figure 4. A rat model with a mutation in PDE3A exon 13 recapitulates HTNB.**

**A.** A rat model encoding a R862C substitution within the catalytic domain of PDE3A was generated using CRISPR/Cas9. Amino acid sequences of human and rat PDE3A and the DNA sequences encoding wild-type and mutant rat Pde3a are aligned. **B.** Detection of PDE3A1 and PDE3A2 in aortas of wild-type (WT), homozygous PDE3A-R862C and functional Del rats by Western blotting. Signals were semi-quantitatively analyzed by densitometry. Wild-type (WT), n = 6; PDE3A-R862C, n = 3 and one functional Del. Statistical analysis was carried out using the Mann-Whitney test; shown are mean ± SEM; *p < 0.1. The analysis of aortas of heterozygous PDE3A-R862C rats, as well as of additional WT and functional Del animals is shown in Figure S2A.

**C.** Body weights of the indicated rat models are shown. Wild-type (WT), n = 13; heterozygous PDE3A-Δ3aa, n = 15; heterozygous (HET) R862C, n = 4; homozygous (HOM) R862C, n = 10; functional Del, n = 14. Statistical analysis was carried out one-way ANOVA and Tukey’s multiple comparison test; shown are mean ± SEM; **p < 0.01, ***p < 0.001, ****p < 0.0001. **D.** MicroCT images of the right front paws of wild-type (WT) and homozygous R862C rats and quantification of metacarpal bone III length. WT, n = 4; R862C, n = 3; Statistical comparison was carried out using the Mann-Whitney test, shown are mean ± SD, *p < 0.1. Analysis of paws of heterozygous PDE3A-R862C animals is presented in Figure S2B. **E.** Radio-telemetric blood pressure measurements of the indicated rat models over 6 days. The figure shows the measurements of the complete cohort of wild-type, functional Del and homozygous PDE3A-R862C animals that were used for the experiment in Figure 2A before separation into NaCl and isoproterenol treatment groups and before implantation of minipumps. In addition, heterozygous PDE3A-R862C rats were subjected to the telemetric measurements. The curves represent loess fits. Gray intervals, 95% CIs for loess parameters; horizontal dashed lines, model expectation values. Lower black bars depict night periods. Wild-type
All blood pressure curves were significantly different, only heterozygous R862C and WT were not. The heart rate of homozygous R862C rats differs significantly from the other animals. P values from likelihood ratio tests comparing nested linear mixed models are listed in the Data Supplement. F. Fractional shortening (FS), ejection fraction (ES) and cardiac output of WT, heterozygous Δ3aa, heterozygous (HET) and homozygous (HOM) R862C, and functional deleted (Del) PDE3A rats was estimated by echocardiography. The figure shows the measurements of the animals in Figure 2B before separation into NaCl and isoproterenol treatment groups and before implantation of minipumps. The statistical differences were calculated using one-way ANOVA and Tukey’s multi comparison test; shown are mean ± SEM; *p < 0.1, **p < 0.01, ***p < 0.001. Further parameters measured by echocardiography are indicated in Table S2.

Figure 5. The R862C substitution in the catalytic domain increases activity and causes aberrant phosphorylation of mutant PDE3A.

A. FRET to determine PDE3A2-R862C activity in a cell-based approach using HEK293 cells. Upper left, PDE3A2-R862C or PDE3A2-wild-type fused with mCherry (red) co-localize (orange) in HEK293 cells when transiently co-expressed with the FRET sensor, ICUE3 (cyan and yellow) Scale bar, 20 µm. The cytosolic ICUE3 sensor contains the cAMP binding domain of exchange protein directly activated by cAMP (Epac) flanked by the yellow fluorescent protein, Venus, and cyan fluorescent protein (CFP). The binding of cAMP induces a conformational change that increases the distance between the two fluorescent proteins and thereby decreases FRET. Upper Right, Detection of the PDE3A2-mCherry variants by Western blotting with anti-PDE3A antibody confirmed similar expression levels and confirmed lack of endogenous expression of PDE3A. The sensor was detected with an anti-GFP antibody. Hsp60 was used as the loading control. Box-Whisker plot illustrating the emission intensity of the WT and R862C PDE3A2 variants. Under resting conditions, the cAMP hydrolytic activity of wild-type
and the PDE3A2-R862C mutant was similar as indicated by the similar emission intensities.

Box-Whisker plot illustrating the $\Delta$FRET, with the black whiskers marking the 5th and 95th percentiles, and the symbols beyond these upper and lower bounds representing values that are considered outliers. 0 reflects the basal cAMP levels. An increased $\Delta$FRET indicates higher cAMP levels, consistent with decreased PDE3A activity. Forskolin stimulates adenylyl cyclases to synthesize cAMP, while cilostamide inhibits PDE3A. Upon forskolin stimulation, the PDE3A2-R862C mutant revealed significantly increased PDE3A activity compared to wild-type, as indicated by the lower $\Delta$FRET values. When the effect of forskolin alone (30 µM) was examined, the substance was added and the measurement immediately started. When the effect of cilostamide was investigated, the cells were incubated with the agent (10 µM) for 20 min prior to the addition of forskolin (30 µM). Mann-Whitney and Kolmogorov-Smirnov tests did not reveal statistically significant differences between emission intensities. For statistical analysis of the $\Delta$FRET values a two-way ANOVA and Tukey’s multiple comparisons test was carried out, n = 3 independent experiments and analysis of 34-49 individual cells per PDE3A2 variant and condition. Shown are mean ± SEM, ****p < 0.0001. B. Comparison of the phosphorylation of S428 and S438 in PDE3A2-R862C and PDE3A2-wild-type. The proteins were expressed in HEK293 cells and the cells were stimulated with forskolin (Fsk) for cAMP elevation and with the PKC stimulator, phorbol-12-myristate-13-acetate (PMA). The phosphorylation was detected by Western blotting with phosphosite-specific antibodies upon immunoprecipitation of the above-mentioned proteins. Statistical analysis was carried out using two-way ANOVA and Tukey’s multiple comparisons test. Shown are representative blots from n = 5-8 independent experiments. Shown are mean ± SEM, *p < 0.1, **p < 0.01, ****p < 0.0001. C. Effects of stimulation with forskolin or PMA on the interaction of the mutant PDE3A2-R862C with the adapter protein, 14-3-3-θ, compared to wild-type (WT). Relative interaction compared to control PDE3A2-wild-type-Flag is shown. Semiquantitative analysis was carried out by densitometry. Statistical analysis, carried out using two-way ANOVA and
Tukey's multiple comparisons test, did not reveal statistically significant differences. n = 5-9 per condition. Shown are mean ± SEM.

**Figure 6.** Differential gene expression in the left ventricles of hearts from wild-type and PDE3A-Δ3aa rats and from wild-type, PDE3A-Δ3aa and functional Del rats treated with isoprotenol or saline.

A. Volcano plots illustrating the distribution of differentially expressed genes (DEGs), FDR cutoff = 0.1, and log2 fold change (FC) cutoff = 1. FDR, false discovery rate. DEGs are listed in Excel file S2. B. Comparison of numbers of DEGs between the described experimental groups (see also Table 1). C. RNA-seq read counts for PDE3A in each treatment condition and for each genotype. The p-value was determined by Student's t-test by comparing treated animals to wildtype. WT, wildtype; Δ3aa, PDE3A-Δ3aa and PDE3A functional deletion. D.-F. Gene ontology (GO) analysis of the DEGs. n = 3 untreated WT, n = 3 NaCl WT, n = 2 Isoprotenol WT; n = 4 untreated PDE3A-Δ3aa, n = 2 NaCl PDE3A-Δ3aa, n = 3 Isoprotenol PDE3A-Δ3aa; n = 2 NaCl functional Del, n = 2 Isoprotenol functional Del.

**Figure 7.** The left ventricles of hearts from wild-type and HTNB mutant rats are similar.

A. Detection of PDE3A1 and PDE3A2 in the left ventricles of hearts from untreated wild-type (WT), PDE3A-Δ3aa and functional Del rat models by Western blotting with semi-quantitative analysis of the Western blot signals by densitometry. Statistical analysis was carried out using the Kruskal-Wallis and Dunn's multiple comparisons test, n = 5 per genotype, mean ± SEM. B. Measurement of cAMP-hydrolytic activity in the left ventricles of hearts from untreated wild-type (WT), PDE3A-Δ3aa and functional Del rat models. cAMP-hydrolytic activity was quantified at 30°C with [3H]cAMP (1 μM) as substrate; PDE3 activity was quantified by measuring activity in the absence and presence of milrinone. The amount of protein used per assay and the incubation times were adjusted to ensure that no more than 20 % of the total cyclic nucleotide was hydrolyzed during the assay. Statistical analysis was carried out using the Kruskal-
Wallis and Dunn's multiple comparisons test, n = 5 per genotype, mean ± SEM. C. Immunofluorescence microscopic detection of PDE3A (red) in sections from left ventricles of hearts from untreated wild-type, PDE3A-Δ3aa and functional Del rats. The antibody recognizes the C terminus of all PDE3A isoforms. As a negative control, detection was carried out in the absence of primary anti-PDE3A antibody. Shown are representative images of at least 5 different animals per rat model. Plotted is the PDE3A signal intensity along the indicated white line. The peaks of the signals correspond to the z-lines. D. The expression of the indicated proteins in the left ventricles of hearts from untreated wild-type, heterozygous PDE3A-Δ3aa and functional Del rats was compared. The signals were semi-quantitatively evaluated by densitometric analysis. Statistical analysis was carried out using the Kruskal-Wallis with Dunn's multiple comparisons test if value distribution was nonparametric and by one-way ANOVA with Tukey's multiple comparisons test for normally distributed values. n = 5-17 left ventricles for each genotype, mean ± SEM. The semiquantitative analysis of further proteins is shown in Figures S4C. E. Detection of phospholamban (PLN) and PLN phosphorylated at serine 16 (pSer16) and threonine 17 (pThr17) in the left ventricles of hearts from untreated wild-type (WT) and PDE3A-Δ3aa rats by Western blotting with semi-quantitative analysis of the Western blot signals by densitometry. Hsp60 was the loading control. Statistical analysis was carried out using the Mann-Whitney test, n = 5 per genotype, mean ± SEM. F. Single nuclei RNA-seq analysis of left ventricles of hearts from wild-type, PDE3A-Δ3aa and functional Del rats. n = 2 per genotype. Left, Uniform Manifold Approximation and Projection (UMAP) representation and cell types expressing Pde3a. Right, adrenergic/cAMP signaling in cardiomyocytes, and marker genes indicating identified cell types. The adrenergic/cAMP signaling scheme was generated using KEGG pathway analysis tools (Kanehisa Laboratories). Differential gene expression is color-coded: blue, upregulated PDE3A-Δ3aa and wild-type; red, upregulated in wild-type vs. PDE3A-Δ3aa; green, upregulated in functional Del vs. PDE3A-Δ3aa; orange, upregulated in PDE3A-Δ3aa vs. functional Del; yellow, upregulated in PDE3A-Δ3aa vs both.
G. The expression of the indicated proteins in the left ventricles of hearts from NaCl- or iso-
proterenol-treated wild-type, PDE3A-Δ3aa, homozygous PDE3A-R862C and functional Del
rats was compared. The signals were semi-quantitatively evaluated by densitometric analysis.
Statistical analysis was carried out using two-way ANOVA and Tukey's multiple comparison.

n = 2-6 per genotype, mean ± SEM. The semiquantitative analysis of further proteins is shown
in Figure S4E.

Figure 8. HTNB-causing PDE3A mutations in human iPSC that are differentiated to car-
diomyocytes lead to adaptations in Ca²⁺ cycling.
A. Sanger sequencing results confirming the introduction of T445N and R862C substitutions
in iPSCs. DNA and protein sequences are shown. Ref., reference sequence. B. PDE3A (red)
localizes to the Z-lines in hiPSC-CMs expressing the indicated version of PDE3A. Z-lines were
stained with specific anti-Actinin and Alexa488-coupled secondary antibody (green), and
PDE3A with specific primary and Alexa594-coupled secondary antibody. Nuclei were stained
with DAPI. C. Detection of key proteins involved in excitation contraction coupling (ECC) by
Western blotting. The signals were semi-quantitatively evaluated by densitometric analysis. n
= 8. Shown are mean ± SD. Statistical analyses were carried out using the Kruskal-Wallis with
Dunn's multiple comparisons test; *p < 0.1, **p < 0.01. D. Schematic representation of a car-
diomyocyte Ca²⁺ transient. The depicted parameters correspond to an increase of cytosolic
Ca²⁺(Ton(50)), Ca²⁺ removal from the cytosol (Toff(50)) and duration of the calcium transient
(CD50) at 50 % of the amplitude. E. The hiPSC cardiomyocytes were loaded with Fluo-8-AM
and treated with either DMSO (solvent, 0.2 %), 1 µM isoproterenol, 20 µM cilostamide or the
combination of both for 10 min. Imaging (40 x objective, laser = 488 nm, pinhole = open) was
carried out in line scan mode acquiring 20,000 line-scans with 1.92 ms per line. The graphs
show the effects of each treatment on the Ca²⁺ transients. Statistical testing was performed
using a Kruskal-Wallis test with Dunn's multiple comparisons test; *p < 0.1, **p < 0.01, ***p <
0.001, ****p < 0.0001.
Table 1. Differentially expressed genes in NaCl- or isoproterenol-treated rats. A total of 20,733 distinct genes were observed to be transcribed in rat hearts across all treatments and all examined genotypes: 19,284 in untreated, 20,031 in NaCl-treated and 20,167 in isoproterenol-treated. The identity of the differentially expressed genes is indicated in Excel files S1 and 2.

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Figure 1

A

PDE3 gene
12p12.2
PDE3A mRNA
Full protein
1141aa
PDE3A1
146 aa
PDE3A2
300 aa
PDE3A3
484 aa

Lebanon France Turkey USA Japan South Africa Canada II Germany Canada I The Netherlands New Zealand II New Zealand I

Catalytic domain

RRSLPPGLLRRVSTWTTTTT
Mutational hot spot

Parasternal long axis M mode

Parasternal long axis B mode

Parasternal short axis B mode

B

Four chamber view - EF

Parasternal short axis B mode
Figure 2

C

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<tr>
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<th>Δ3aa</th>
<th>functional Del</th>
<th>R862C Hom</th>
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<tr>
<td>Cardiac output (ml/min)</td>
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<td>FS (%)</td>
<td></td>
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<tr>
<td>EF (%)</td>
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Day

NaCl

Isoproterenol

Δ3aa

functional Del

R862C Hom

NaCl

Iso

0 20 40 60 80
0 50 100 150 200
0 0.1 0.2 0.3 0.4 0.5
0 0.5 1.0 1.5 2.0
0 500 1000 1500 2000
0 0.0 0.1 0.2 0.3 0.4 0.5
0 5 10 15 20 25
20 40 60 80 100
20 40 60 80 100
**Figure 4**

**A**

**Human mass spectrometry**

- **TSAPQAVLYNR862C1HAAAAWNLFSRPEYNFLINLDHVEFKHFRFLVIEAILATDLKHFDFVAKF**

**Rat mass spectrometry**

- **TSAPQAVLYNR862C1HAAAAWNLFSRPEYNFLVNLDDHVEFKHFRFLVIEAILATDLKHFDFVAKF**

**B**

- **WT**
- **R862C Hom**
- **functional Del**

**C**

- **WT**
- **Δ3aa**
- **R862CHet**
- **R862CHom**

**D**

**Metacarpal bone III**

- **WT**
- **R862C Hom**

**E**

- **R862C Hom**, **R862C Het**; **WT**: functional Del

**F**

- **EF (%)**
- **Cardiac output (ml/min)**

**Amino acids**

- **NDCSV**
- **R862C**

**Body weight (g)**

- **WT**
- **Δ3aa**
- **R862CHet**
- **R862CHom**

**PDE3A1**

- **WT**
- **R862C Hom**

**PDE3A2**

- **WT**
- **R862C Hom**

**Hsp60**

- **WT**
- **R862C Hom**

**Blood pressure**

- **Systolic BP (mmHg)**
- **Diastolic BP (mmHg)**
- **Heart rate (BPM)**

**Cardiac output (ml/min)**

- **WT**
- **R862C Het**, **R862C Hom**, **functional Del**
Figure 5

A

Emission intensity (λ=532nm)

B

PDE3A2

WT R862C

PDE3A2 pSer428

pS428

pS438

Flag-Hrp

Input

WT R862C

Flag-PDE3A2

Hsp60

WT R862C

Flag-PDE3A2

Hsp60

WT R862C

Flag-PDE3A2

Hsp60

C

IP: Flag-PDE3A2

WT R862C

14-3-3θ

Flag-PDE3A2

14-3-3θ

Flag-PDE3A2

14-3-3θ

Flag-PDE3A2

14-3-3θ
Figure 7

A  

![Image of Western Blot](image)

B  

![Image of Western Blot](image)

C  

![Image of Western Blot](image)

D  

![Image of Western Blot](image)

E  

![Image of Western Blot](image)
Figure 7

F

Cell types in the heart

Pde3a marker expression in the heart

Pde3a expression in rat heart

Adrenergic signaling in cardiomyocytes

Marker genes

Cardiomyocytes
Endothelial cells
Fibroblasts
Immune cells
Mural cells
Neuronal-like cells

G

WT Δ3aa R862C Hom functional Del

βAR/CAMP/PKA

PDE3A1
PDE3A2
β1-AR
β2-AR
SERCA2a
pS16-PLN
pT17-PLN
TnI
pTnI
ANP
BNP
p44/42 MAPK
pp44/42 MAPK
CREB
pCREB
14-3-3
PKC substrate
GAPDH
HSP60

β1 AR/CAPDH

β2 AR/CAPDH

SERCA2a/CAPDH

PDE3A1/Hsp60

PDE3A2/Hsp60

pT17-PLN/Hsp60

pS16-PLN/Hsp60

NaCl

WT Δ3aa R862C Hom functional Del

Cardiac damage

Contractility

Transcription

***

****

**

*
Figure 8

A

B

C

D

E

Calcium Transient Duration at 50% Amplitude (CD50)

Decay Time To 50% Amplitude (Toff50)

Rise Time To 50% Amplitude (Ton50)
Supplemental Material

Mutant phosphodiesterase 3A protects from hypertension-induced cardiac damage

Maria Ercu, PhD\textsuperscript{1,2,3}\# Michael B. Mücke, MD\textsuperscript{1,2,3}\# Tamara Pallien, MS\textsuperscript{1,2}\# Lajos Markó, MD, PhD\textsuperscript{2,3,4,5} Anastasii Sholokh, MS\textsuperscript{1,2,3} Carolin Schächterle, PhD\textsuperscript{1} Atakan Aydin, PhD\textsuperscript{1} Alexa Kidd MD\textsuperscript{5} Stephan Walter, MD\textsuperscript{6} Yasmin Esmati\textsuperscript{2,3,4} Brandon J. McMurray, BSc\textsuperscript{7} Daniella F. Lato, PhD\textsuperscript{7} Daniele Yumi Sunaga-Franze, PhD\textsuperscript{1} Philip H. Dierks,\textsuperscript{1} Barbara Isabel Montesinos Flores,\textsuperscript{1} Ryan Walker-Gray, PhD\textsuperscript{1} Maolian Gong, MD\textsuperscript{1,4} Claudia Merticariu, BS\textsuperscript{1} Kerstin Zühlke, PhD\textsuperscript{1} Michael Russwurm, PhD\textsuperscript{8} Tiannan Liu MD,\textsuperscript{1} Theda U.P. Bartolomeus, MS\textsuperscript{2,3,4} Sabine Pautz, MS\textsuperscript{9} Stefanie Schelenz\textsuperscript{1}, Martin Taube\textsuperscript{1}, Hanna Napieczynska, PhD\textsuperscript{1} Arnd Heuser, MD\textsuperscript{1} Jenny Eichhorst, Dipl.-Ing. (FH)\textsuperscript{10} Martin Lehmann, PhD\textsuperscript{10} Duncan C. Miller, PhD\textsuperscript{1,2} Sebastian Diecke, PhD\textsuperscript{1,2,11} Fatimunnisa Qadri, PhD\textsuperscript{1} Elena Popova, PhD\textsuperscript{1} Reika Langanki, BS\textsuperscript{1} Matthew A. Movsesian, MD\textsuperscript{1} Friedrich W. Herberg, PhD\textsuperscript{6} Sofia K. Forslund, PhD\textsuperscript{1,4,11,12} Dominik N. Müller, PhD\textsuperscript{1,2,4} Tatiana Borodina, PhD\textsuperscript{1} Philipp G. Maass, PhD\textsuperscript{1,3,4} Norbert Hübner, MD\textsuperscript{1,2,3,4,5} Michael Bader, PhD\textsuperscript{1,2,3,4} and Enno Klussmann, PhD\textsuperscript{1,2,4,5} These authors contributed equally.

Running head: Cardioprotective PDE3A mutations

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**Table S1.** Clinical parameters of subjects from the New Zealand (R862C) family depicted in the pedigree in Figure 3.

**Table S2** Parameters measured by echocardiography shown in Figures 2C and 4F.

**Excel files**

**Excel file S1.** RNA-seq_metadata data and quality control (QC).

**Excel file S2.** Differentially expressed genes (DEGs) identified by RNA-seq of left ventricles from PDE3A-wild-type, PDE3A-Δ3aa and functional Del HTNB rat models. The animals were untreated or treated with saline (NaCl) or isoproterenol.

**Excel file S3.** Differentially expressed genes identified by scRNA-seq of left ventricles from PDE3A-wild-type, PDE3A-Δ3aa and functional Del HTNB rat models.
Patient data

HTNB patient with G449S substitution

Current medication
Torasemid 5 mg od, Ramipril 10 mg od, Amlodipin 10 mg od, Metoprololsucc. 95 mg od, Moxonidin 0.2 mg od, Doxazosin 4 mg ret. od, Minoxidil 2.5 mg bid, Carbamazepin* 400 mg ret. Bid (1-0-1.5) (*due to seizures)

Physical examination
Body weight 63 kg; height 157 cm, BMI 25.24 kg/m²; Brachydactyly.
Heart regular, no murmurs, lungs clear to auscultation; blood pressure at presentation 180/100 mmHg, O₂ saturation 97 %.

Resting ECG
HF 69/min, left axis deviation, sinus rhythm, normal intervals. no ST segment changes. Tall T-waves in v3-v5.

Echocardiographic analysis of G449S HTNB patient
2 chamber view
mitral valve regurgitation
Clinical data from the New Zealand (R862C) HTNB family

Table S1. Clinical parameters of subjects from the New Zealand family (R862C) depicted in the pedigree in Figure 3. The affected family members whose DNA was sequenced all had a c.2584C>T mutation, leading to an R862C substitution. Age indicates age at assessment. SBP and DSP, systolic and diastolic blood pressure, respectively; HTN, hypertension; BDE, brachydactyly type E; sit., sitting; stand., standing; UKN, unknown.; ✝, death at the indicated age.

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Expanded Methods

Generation of the PDE3A-R862C HTNB model

State of Berlin authorities approved the rat studies according to American Physiological Society guidelines (license no. G 0435/17). The PDE3A-Δ3aa and functional deletion (Del) rat models were generated as in Ercu et al., Circulation 2020. The PDE3A-R862C rat model was generated by electroporation of Sprague-Dawley rat zygotes with a mixture of 1,280 ng/µl Cas9 protein (IDT, Skokie, IL, USA), 258 ng/µl of gRNA with the sequence 5’-GATGGTTCTCCAGAAACGGAA (IDT, Skokie, IL, USA), and 500 ng/µl of an oligonucleotide for the nucleotide substitution (5’-*A*AATTCCACATGTCAGGTTAACTAA-GAAGTTACTCCGCGGGACATGAAGAGATAAGTTACTCCGCGGGACATGAAGAGA TTCCAGGCTG-CAGCTGCGTATGGTTCTCCAGAAACGTACAGTCATTGTACACGAGG CCTAGGGTG-GAGAAGGCAGGAA*G* (*phosphothioate moieties). Post electroporation, the zygotes were cultured to the two-cell stage and transferred into foster mothers according to established methods. The offspring were genotyped by PCR with primers flanking the gRNA target region (PDE3a1, AAGCCTTCCAGTCCTTTGTG; PDE3a2, TGACTAGGAATCGGAAGTGC) and sequencing of the PCR fragment.

Animal phenotyping and interventions

Male rats (ca. 6-8 months) were used. Telemetric blood pressure measurements were carried out as in Ercu et al., Circulation 2020. In brief, the telemetry measurements started at least one week after recovery from implantation. Signals were recorded continuously at 5 min intervals for 10 s continuously day and night in freely moving animals. After at least six days of basal blood pressure measurements, an initial echocardiographic examination was carried out (Preclinical Research Center of the Max-Delbrück Center for Molecular Medicine, Berlin, Germany) as described. Three to five days later, osmotic minipumps (Alzet 2ml2, Charles River Wiga, Sulzfeld, Germany) were implanted for administration of saline (0.9 % NaCl + 0.02
% ascorbic acid) or isoproterenol (0.13 mg/kg/h). On day 14 after the implantation, a second echocardiographic examination was carried out.

The blood pressure data were analyzed using mixed-effects modeling as described. Animal identity and time point within a day were included as random effects. Genotype and day/night status at time of measurement were included as fixed effects, using the R lme4 package. The scopes of effects were obtained from the model parameters (slope and intercept parameters). The significance of each factor was assessed by comparing each linear model to a simpler model omitting the genotype as a predictor; here, likelihood ratio tests were performed as implemented in the lmtest R package. Data were visualized using loess regressions from the ggplot2 R package. The blood pressure traces are shown separately for each of the experimental groups for the period before minipump implantation. We have used the blood pressure data obtained before the implantation of the minipumps for comparing effects of the genotypes on blood pressure in untreated animals in Figure 4E.

µCT analysis was carried out similar as in Ercu et al., Circulation 2020. In brief, the front paws were scanned ex vivo using Skyscan 1276 (Bruker, Kontich, Belgium) and the following acquisition parameters: source voltage of 100 kV, source current of 200 µA, Cu 0.25 mm filter, exposure time of 645 ms, rotation step of 0.2°, and frame averaging of 3. The flat-field correction was applied. The images were reconstructed with NRecon (Bruker), with the ring artefact reduction = 11 and the beam hardening correction of 10 %. The images were analyzed quantitatively with Amira (ThermoFisher Scientific, Germany).

**Histological staining and immunofluorescence microscopy**

PDE3A in cryosections of hearts was detected by immunofluorescence microscopy using a custom-made anti-PDE3A antibody directed against the C-terminal amino acids 1095-1110 (Eurogentec; CLSGTENQAPDQAPLQ), secondary Alexa647R-coupled donkey anti-rabbit IgG and a Leica TCS SP5 confocal microscope.
Rat hearts were cut along the transverse axis, immediately fixed in 10 % formalin and stored at least 24 h. The samples were embedded in the paraffin and cooled overnight at 4 °C. Sections (2 or 5 μm thick) were prepared from paraffin blocks using a microtome and mounted on microscope slides. The samples were rehydrated by deparaffinizing twice in xylene (2 x 5 min) and running through a decreasing ethanol series (100 %, 96 %, 80 %, 70 % for 5 min each). For further processing, the slides were washed three times in 1x PBS.

**Picro Sirius Red**

The sections were incubated for 60 min with Picro Sirius Red solution (Morphisto, 13422) in the dark, washed 2 x 5 min with 0.005 % vinegar water, dehydrated in ethanol series (3 x 100 %), and immersed in xylene (2 x 5 min). Finally, the sections were covered with Eukitt.

**Hematoxylin & eosin**

After rehydration the sections were stained with hematoxylin (Sigma-Aldrich, GHS332) for 10 min, washed with tap water and differentiated with 0.3 % acidic alcohol. Then slides were washed in tap water, stained with eosin (Sigma-Aldrich, HT110116) for 2 min, dehydrated in ethanol with ascending concentrations (80, 90, 100 %), cleaned in isopropanol, followed by 30 min incubation in xylol and mounting with Eukitt.

High-quality bright-field images of the tissue sections were obtained using an All-in-One Light/Fluorescence Microscope BZ-9000 (Keyence). At least 25 non-overlapping fields from 5 different samples in each experimental group were imaged and analyzed using ImageJ studio. Cardiac hypertrophy was evaluated by measuring the cross-sectional areas by analyzing H&E slides after outlining round to cuboidal-shaped nucleated myocytes. Media to lumen ratios of cardiac arteries were calculated by dividing the square of tunica media with the luminal area. The fibrosis index (%) was calculated as a percentage of collagen-positive areas to the total area of the image.
**Wheat germ agglutinin (WGA)**

Visualization of cardiac myocytes was performed immediately after rehydration of the samples. After blocking nonspecific binding (60 min with 10 % NDS in 1x PBS at room temperature in a humidified chamber), the directly coupled WGA was applied (1:100 in 10 % NDS, 4 °C asl, humidified chamber). A secondary antibody was not required. After incubation, samples could be covered with Vectashield/DAPI.

**Fibronectin, collagen type 1, CD31, and ED1**

After rehydration, unmasking was performed by boiling in 1x citrate buffer for 20 min, followed by blocking of endogenous peroxidases by applying 3 % H₂O₂ for 15 min at room temperature and finally washing with distilled water for 3 x 5 min. Next, nonspecific binding was blocked with 10 % NDS (in 1x PBS) for 60 min at room temperature in a humidified chamber and finally the slides were incubated with the first antibody overnight at 4 °C. The next day, slides were washed 3 x 5 min with 1x PBS and incubated for 120 min at room temperature with the secondary antibody. The final step included washing the slides 3 x 5 min with 1x PBS and covering them with Vectashield/DAPI.

The concentration and incubation times of the primary and secondary antibodies vary depending on the staining:

**Primary antibodies:**
- Anti-fibronectin, 1:75 (in 10 % NDS), moisture chamber.
- Anti-type I collagen, 1:20 (in 10 % NDS), humidified chamber.
- Anti-CD31, 1:50 (in 10 % NDS), humidified chamber.
- Anti-ED1, 1:100 (in 10 % NDS), humidified chamber.

**Secondary antibodies:**
- Fibronectin: Cy3-conjugated donkey anti-rabbit IgG, 1:300 (in 1x PBS), humidified chamber.
- Col1: Cy3-conjugated donkey anti-goat IgG, 1:300 (in 1x PBS), humidified chamber
- CD31: Cy3-conjugated donkey anti-goat IgG, 1:100 (in 1x PBS), humidified chamber
- ED1: Cy3-conjugated donkey anti-mouse IgG, 1:300 (in 1x PBS), humidified chamber

Staining was evaluated as follows:

Using the 3D Histec Slide Scanner, all slices could be imaged and analyzed offline immediately after staining was completed. This prevented any bleaching effect or fading of fluorescence.

WGA: 50 round cardiac myocytes evenly distributed in the heart cross-section were selected per slice using the case viewer. WGA staining evaluated the potential hypertrophy of cardiac myocytes by measuring the mean circumference of each cell.

Fibronectin: 10 representative images (40x zoom) without vascular content were acquired per slice with Case Viewer and analyzed with ImageJ studio for the interstitial fibrotic content in each image.

Collagen I: Staining was used to determine perivascular fibrosis. All intact vessels in a heart were compared. The medial and fibrotic outlines of the vessels were recorded, and the ratio of fibrotic area to medial area was calculated.

Endothelial staining with anti-CD31 was used to determine capillary density, and 10 representative images (40x zoom) were acquired per section and counted with ImageJ.

Macrophages were labeled with anti-ED1 and counted in 10 representative sections (40x zoom) per slice per animal. The average numbers of ED1-positive cells per animal were calculated.
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<td>Anti-CD31</td>
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</table>

**Generation and characterization of mutation-carrying human induced pluripotent stem cells (hiPSCs) and differentiation to cardiac myocytes**

hiPSCs from a healthy donor were obtained from the Berlin Institute of Health Stem Cell Core facility (hPSCreg.org cell line BIHi-049-A). For the T445N mutation, an approach using transcription activator like effectors nucleases (TALENs) together with a piggyBac-based selection cassette was used to introduce the mutation into hiPSCs. 1.5 million cells were transfected with 1.1 µg of each TALEN and 8 µg of HDR-template using a Nucleofactor 4D (Amaxa, Protocol CM150). 72 h after transfection, cells were selected with 0.25 mg/ml puromycin for 7 days followed by 0.1 mg/ml. Single cell clones were picked, DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) and integration was verified using a three primer PCR (5’Arm: piggyBac: CGTCAATTAGTACTGATGATTATTTTAAC, FW: GGGGTATGACTGTGGTGCAA, RV: GAGGCTAATGACTGGGCTGG; 3’Arm: piggyBac: GCGACGGATTCGCTATTTAGAAAG, FW TTCATTGGCCAGAGATCTT, RV: ATCCAGGGCTAAGCAGTACAGGA). To excise the piggyBac cassette, cells were transfected with an excision-only transposase (hera BioLabs, Lexington KY, USA) and treated with 0.1 µM
Ganciclovir for 14 days. After selection, single cell clones were picked and excision was verified using the previous primers. Afterwards the genotype was verified using PCR and Sanger sequencing (Primers: FW: AGTCTTTTCCTAGCGCCTG, RV: TGGTCTTTGTGAGGGCTAAT).

For the R862C mutation, hiPSC were transfected with 122 pmol recombinant Cas9 protein (IDT, Skokie, IL, USA) together with 200 nmol trcrRNA, and crRNA dimer (sequence of the crRNA 5'-direction: ATATAACGATCGTTTCAGTTT) together with 100 nM of an asymmetric oligonucleotide (TTCTCATGATTTTTGTGATTATTCTTAAAAAGTTGAACTCTTAACTGTCTTATT TGCCATGCGGTGCTATAACGATTGTTTCAGTTTTGGAGAATCATACGCGAGCTGCTG CATGGAAATCTTTTCA; all IDT, Skokie, IL, USA). 48 h after transfection, cells were plated (45 cells/cm²), allowed to grow for 7 days and single cell colonies were picked and expanded. DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) and the genotype was confirmed using PCR and Sanger sequencing with the following Primers: (FW: GCATTGCATATTCTCATGATTTTTGT, RV: GCCAAAATTGCTTCAATGAAAG).

hiPSC were cultured in Essential (E8) (Thermo Fisher Scientific) medium on Matrigel (Corning)-coated (1:100) plates with daily medium changes under normoxic conditions (37°C, 5 % O₂). Cells were regularly tested for mycoplasma contamination using PCR Mycoplasma Test Kit I/C (PromoCell, Heidelberg, Germany) according to the manufacturer’s instructions. Cells were replated three days prior to differentiation in a ratio of 1:12 using PBS containing 0.5 mM EDTA. On day 0, the differentiation was initiated using cardiac priming medium (RPMI-1640, 1x B-27 minus insulin, 10 µM CHIR-99021; Thermo Fisher Scientific, Tocris). On day 1, 4 ml basal medium (RPMI-1640, 1x B-27 Minus Insulin) were added to each well. On day 3, the medium was replaced with 4 ml cardiac induction medium (RPMI-1640, 1 x B-27 minus insulin, 5 µM IWR-1 endo) per well. On day 5, 4 ml of basal medium were added, followed by a change to cardiac maintenance medium (RPMI-1640, 1x B-27) on day 7. Between day 9 and 13, car-
Cardiac myocytes were metabolically selected using cardiac selection medium (RPMI-1640 without glucose, L-ascorbic acid 2-phosphate 213 µg/mL, Human Recombinant Albumin 500 µg/ml, Sodium DL-lactate 5mM). After 2 days of recovery in cardiac maintenance medium, cells were replated on Matrigel-coated (1:60) 6-well plates at a density of 2 million cells per well.

The hiPSCs differentiation efficiency was monitored by flow cytometry. For this, hiPSC-CMs were dissociated on day 45, stained with VioBility 405/452 (Miltenyi Biotech) dye, followed by fixation using the FoxP3 Kit (Miltenyi Biotech) and stained with anti-TNNT2-FITC (Miltenyi Biotech) and anti-MLC2v-APC (Miltenyi Biotech) antibodies according to the manufacturer’s instructions. Cells were measured on a MACSQuant VYB (Miltenyi Biotech) device and data analyzed using FlowJo.

For protein extraction, cell pellets were lysed in 300 µl lysis buffer (10 mM K₂HPO₄, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.5 % Triton-X 100, 0.2 % sodium deoxycholate, pH 7.4) using syringes. Cell debris was removed by centrifugation at 14,000 rpm for 15 min at 4 °C. The protein concentration was determined using the Coomassie Plus Bradford protein assay (Thermo Fisher Scientific). SDS-PAGE and Western blotting were performed with 40 µg total protein being loaded per lane.

For staining of pluripotency markers, hiPSCs cells were seeded on a IBIDI u-slide (IBIDI, Munich, Germany #80806) at a density of 1,000 cells per chamber. They were cultured for 2 days with Rock inhibitor (1:1,000) followed by 3 days without. Cells were fixed using fresh 4 % PFA for 20 min at room temperature. Pluripotency markers were stained using the PSC 4-Marker Immunocytochemistry Kit (ThermoFisher, USA, #A24881) according to the manufacturer’s instructions. Images were acquired using a Keyence BZ-X810 microscope.

hiPSC-CMs were seeded on Matrigel-coated (1:60) IBIDI u-slides at a density of 80,000 cells per chamber. They were allowed to recover for 7 days. Cells were fixed using fresh 4 % PFA for 20 min at room temperature. Cells were washed 3 times using PBS, followed by blocking for 1 h at room temperature using 10 % normal goat serum, 3.5 % BSA and 0.01 % Triton-
X in PBS. Cells were stained using anti-PDE3A antibody (Bethyl A302-740A) and anti-alpha-actinin (Sigma A7811) in 5.5 % BSA and 0.01 % Triton X in PBS at 4°C overnight. After primary antibody incubation, cells were washed 3 times with PBS and incubated with Alexa488-coupled anti-mouse and Alexa 594-coupled anti-rabbit antibody for 2 h at 4°C. Cells were washed twice with PBS followed by incubation with NucBlue (ThermoFisher, #R37605) according to the manufacturer’s instructions. Cells were washed twice and finally covered in mounting medium (IBIDI, Germany, #50001). Images were acquired using an inverted confocal microscope (Leica TCS SP8).

Ca²⁺ imaging

The iPSC-CM were seeded on fibronectin-coated glass bottom dishes (35 mm, IBIDI) on day 45 and cultured with medium changes every 48 h for 7-10 days. Cells were loaded with 2 µM Fluo-8-AM in the presence of 0.01 % pluronic-F127 acid for 10 min at 37 °C, 5 % CO₂. The Fluo-8-AM is trapped inside the cells after de-esterification. Cells were washed two times with measurement buffer (135 mM NaCl, 4 mM KCl, 10 mM Hepes pH 7.3, 5 mM Glucose, 1.8 mM CaCl₂, 1 mM MgCl₂) and kept at 37 °C, 5 % CO₂ for 20 min. For the imaging, the cells were paced at 0.5 Hz with a 2 ms biphasic 10 V pulse at room temperature. Cells were imaged on a Zeiss NLO (40 x objective, laser = 488 nm, pinhole = open) in line scan mode acquiring 20,000 line-scans with 1.92 ms per line with a width of 512 pixels and a bit depth of 8 bit. For treatments, measurement buffer was replaced with 1 ml new buffer containing the compound (DMSO, 1 µM Isoproterenol, 20 µM cilostamide) and the cells incubated at room temperature for 10 min. Compounds were dissolved, the final DMSO concentration was 0.2 % in all treatments. Files were converted from .lsm format to csv format in Image J Studio, line intensities were summed up using a customized R script and data was further processed using a MatLab-based algorithm (CalTrack, [https://github.com/ToepferLab/CalTrack](https://github.com/ToepferLab/CalTrack)) with the following settings: frames per second: 520.833, frames discarded at beginning: 0, frames to be analyzed: 10,000. Traces were corrected for photo bleaching and the parameter function was adapted
to assign the baseline at the end of the average trace instead at 95% to account for decreased relaxation speed at room temperature.

**RNA-seq of rat hearts**

Rat left ventricles were homogenized with ceramic beads in Qiazol Lysis reagent (Cat. No. / ID: 79306), followed by phenol/chloroform extraction, and RNeasy Kit (Qiagen, Cat. No. / ID: 74004) to prepare RNA according to the manufacturer’s instruction. RNA quantification was measured using Qubit and RNA integrity was assessed by the High Sensitivity RNA Bioanalyzer (Agilent, 5067-5581).

RNA-seq libraries were prepared with TruSeq Stranded mRNA kit (Illumina, Cat. No 20020595), and sequenced on a HiSeq 4000 platform (Illumina) in a multiplexed dual indexed mode (1x151 + 8In + 8In).

Single-end reads were quality assessed with FastQC (v0.11.9), then mapped to the *R. norvegicus* genome (mRatBN7.2), accompanied by assembly-matched RefSeq genome annotations (GCF_015227675.2) with STAR v2.7.0f using the options: --sjdbGTFfile, --quant-Mode GeneCounts, --outSAMtype BAM SortedByCoordinate. Mapped samples were indexed with SAMtools (v1.9), and mapping quality was assessed with RNA-SeQC (v2.0.0). On average, 93.0% of reads mapped uniquely to the reference genome (range 83.4-95.5%, see Excel file S1). Sequence reads were assigned to annotated genomic features and counted with featureCounts (Subread v2.0.0). Differential expression analysis on the counts data was performed by R (v4.0.5) package DESeq2 (v1.28.1), using default tool specifications. 'Expressed genes' that were considered either significantly up- or down-regulated required to have 10 or more reads assigned, and a Benjamini-Hochberg adjusted false discovery rate (FDR, q value) of <10%. Principal component analysis (PCA) checked clustering of samples and conditions in DESeq2 after applying a variance stabilizing transformation using vsn package (v3.56.0) to the count data for each treatment type (untreated, isoproterenol-treated, NaCl-treated). Most
samples clustered according to their genotype and/or condition. *PDE3A* expression was comprehensively examined by constructing read count boxplots with ggplot2 package (v3.3.5) in all treatment groups after checking normality by group with Shapiro-Wilk test, and assessing equality of variance with Levene’s test using rstatix package (v0.7.0). Student’s t-test was applied to determine significance. Enrichment analysis was accomplished using Metascape with default ‘Express Analysis’ settings. A total of 20,733 distinct genes were observed to be transcribed in rat hearts across all treatments and all examined genotypes: 19,284 in untreated, 20,031 in NaCl-treated and 20,167 in isoproterenol-treated.

**Single nuclei sequencing (snRNA-seq) of rat hearts**

Snapfrozen rat LVs (20 - 50 mg) were homogenized using a Dounce homogenizer in homogenization buffer (250 mM sucrose, 25mM KCl, 5mM MgCl2, 10mM Tris HCl, 1µM DTT, 1x Protease-Inhibitors, 0.4U/µl RNaseIN plus, 0.2 U/µl SuperaseIN, 0.1 % Triton-X-1000). Homogenates were filtered through a 40 µm strainer and nuclei were sedimented by centrifugation and resuspended in storage buffer (PBS, 4 % BSA, 0.2 u/µl Protector RNaseIn). Nuclei were stained using NucBlue according to the manufacturer’s instructions and FACS-sorted using a BD FACSAria Fusion to remove cell debris. Sorted nuclei were counted utilizing Trypan Blue and adjusted to 600-1000 nuclei/µl. Nuclei suspensions were loaded onto the 10x Chromium Controller using the 10x Single-cell 3'-v3.1 kit, targeting a recovery of 5,000 nuclei. Generation of single nuclei RNA libraries was performed according to the manufacturer’s instruction. Libraries were sequenced according to the 10x Single-cell 3'-v3.1 kit recommendations to a targeted read depth of 30,000 reads per nucleus. Sequencing reads were mapped against a custom rat pre-mRNA reference genome using CellRanger (5.0.1). The reference genome was constructed from ensemble genome Rn6 using the 10X pipeline to create custom reference genomes. The .gtf file was modified to enable counting reads mapping to any sequence within the gene body.
Single nuclei sequencing data was analysed using SCANPY (1.5.1). Nuclei were removed if <400 or >3,500 genes, <300 or > 2,000 counts were detected. Genes were removed if they were detected in fewer than 5 nuclei. Doublets were predicted using the packages Scrublet (0.2.1), using log-transformation of raw counts and Solo (0.3). If nuclei were predicted to be doublets in at least one of the algorithms they were removed.

Data was normalized to $10^4$ counts. For predimensional reduction, highly variable genes were selected based on mean expression and dispersion, the number of counts and percentage of mitochondrial-mapped reads per nucleus were regressed out and data was scaled to a maximum value of 10 standard deviations. Principle components were harmonized per sample variability using Harmony (0.0.4) and dimensionality reduction performed using the uniform manifold approximation and projection algorithm (UMAP). Clusters were detected using the Leiden algorithm (0.8.1) at a resolution of 0.4. Cell types were assigned based on marker genes, as previously described. As we were only interested in the cardiac myocytes and to avoid over-clustering, clusters with similar transcriptional signatures were merged.

For differential expression analysis, nuclei with a chimeric marker gene signature were excluded. Differentially expressed genes in cardiac myocytes were detected using the function rank_genes_groups grouped by the genotype. Only the top 100 upregulated genes are reported, filtered for a minimum log2 foldchange of 0.3 and adjusted p value below 0.05. First, genotype-specific upregulated genes compared to all included genotypes (global) were calculated followed by pairwise comparisons between genotypes. Gene set enrichment analysis was performed on the filtered list of genes from rank_genes_groups using Enrichr within the python package gseapy (0.9.5). The significance level was set to 0.05 and p values were corrected using the Benjamini-Hochberg method. For differentially regulated enriched gene sets Enrichr was run on a concatenated list of up- and downregulated genes derived from the pair-wise genotype comparisons.

Radioimmunoassay (RIA) and Förster resonance energy transfer (FRET)
Left ventricles were removed immediately after sacrificing the animals under isoflurane anesthesia and cAMP was measured using radioimmunoassays (RIA) as described.

PDE3A2-R862C activity was measured in living cells using Förster resonance energy transfer (FRET) as described in Ercu et al., Circulation, 2020. The vector encoding Flag-tagged PDE3A1 was the source for generating mCherry-tagged PDE3A2 WT and R862C versions. pcDNA3-ICUE3 was a gift from Jin Zhang (Addgene plasmid # 61622; http://n2t.net/addgene:61622; RRID:Addgene_61622).

Detection of PDE3A self-assembly

For the detection of self-assembly of PDE3A2, HEK293 cells (7.5 x 10^5) were seeded in 6-well plates and cultured for 24 h. Cells were transfected with 5 µl PEI (1 mg/ml; Linear Polyethylenimine 25,000, Polysciences, Inc.) and 0.5 µg PDE3A2-Flag constructs (WT, T445N and R862C). The cells were cultured for another 24 h and lysed with RIPA buffer (50 mM Tris-HCl, pH 7.8, 10 % glycerol, 150 mM NaCl, 1 % Triton X, 0.025 % Na-deoxycholate and 1 mM EDTA), supplemented with protease and phosphatase inhibitors (Complete and PhosSTOP, Roche Diagnostics) on ice for 10 min. Lysates were cleared by centrifugation (21,250 x g, 10 min, 4°C) and protein concentration was determined. An equivalent of 20 µg of protein was supplemented with 4x non-reducing Laemmli (50 mM Tris-HCl, pH 6.8, 4 % glycerol, 1.6 % SDS without β-mercaptoethanol), denatured at 95°C for 5 min and subjected to Western blotting.

Antibodies

Antibodies against phosphoserine (pS) 428 and 438 of PDE3A were custom-made (Eurogentec). Also custom-made by Eurogentec were antibodies against a C-terminal epitope (residues 1095-1110 in A1) present in all PDE3A isoforms (CLSGTENQAPDQAPLQ).
The amino acid sequence of human PDE3A

1  mvapgdaarv rdkpvhsqys qaptgrdch hradpasprd sgcrqcgwdl vlqpirrssrk
61  issalcagsl sfallilvlvr qgvqrgcld qckeaaaeaee eeaapgaeqg vfpgprggap
121  gggarlpwl gqsalifsll caffwmglyl lragvrlpl a vallaacgcc ealvgiglyv
181  gedhllslpa agvvlslaa atwlvrlrl vlqvmialtsa vrtvslis lrfkvawrpyl
241  aylagvglil laryeqgil qaeaaprekh lgvsqiqgk edipvfkkrr rsssvasaem
301  sgcsskshrr tsldcipreq lmghsewdhk rgrqsgqssg tsiivdiamv geahglitdl
361  ladpslppnct cstravsnl lstgltfquia hkpvrnpvts isenytcsd eseeskdka
421  ipkrlrrslpgqlrrrsst wttttsatgl ptlepapvrr drstskile qgtpassldswn
481  npvmmtlhtks rsftssyais aahvkkakkq srpjalakcis plspcsspl qgtpasslvs
541  kisavqfpses adttaksgl srhartytqg apdlspsglt ppvicscgrg pysqgpnadp
601  plersgvtartpsrddtaq vtsdyetnnn sdsdsvqne detecrepl rkasacstya
661  petmmflkpl ilapeplmvnl nidsimeqln twnfpiqdlv enigrcqgr lqvsyrlf
721  dmglfseakf pierefmyfh elidyrdip yhnrhathdv lhawyltgt pipgltinstv
781  dhgstsdads dsgfgthqmg yvfqtykntv ddkygcslqun dpaalmlay vaaamhdydh
841  pqgtnaflvta tsapsqavlmsdvslnheaaa wnlsmfr pnyenflmld hvfkhfrfl
901  vieailatlkk hkhdfvdakfq kvnqddvgl dwtndnrtl vcmqcmcklad inprakckel
961  hlqtsgyqyn veygggdeea siglpispfms drsagqlanl gesfishvqv plcnspydsag
1021 lmpgkwveds desgtdddpe eeeepapnn eeeetennes pkkkfrkrrk iyqcigtqhil
1081 qnhkmwkkvi eeqerlagie ngssldqtpgs hsseiqgaik eeeseekgpr geeiptqkd
1141 q

SPOT-synthesized peptides representing amino acid residues 145-1141 of human PDE3A. The numbers correspond to the numbers in Figure S2A.

**Spot**  **Sequence**
**Results**

**Statistical comparison of blood pressure of the wild-type, functional Del and HTNB rat models depicted in Figure 2A of the main manuscript.**

Listed are p values for the indicated comparisons.

**Systolic BP (mmHg)**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Genotype</th>
<th>Days</th>
<th>Mean ± SEM</th>
</tr>
</thead>
</table>
| all days, full set, P (condition) | 3.1792840051051e-05
| all days, full set, P (genotype) | 1.80065987419032e-19
| all days, full set, P (condition X genotype) | 0.00047745036140452
| ISO, all days, P (genotype) | 2.24575142507014e-12
| NaCl, all days, P (genotype) | 3.48993903560027e-11
| WT, NaCl, all days, mean = 123.134787087665 ±-13.2019087612956
| WT, NaCl, days -4 to 1, mean = 123.915267110777 ±-14.0784455986177
| WT, NaCl, days 2+, mean = 122.88978143551 ±-12.9050037082604
| Δ3aa, NaCl, all days, mean = 150.747109061598 ±-14.7953119732042
| Δ3aa, NaCl, days -4 to 1, mean = 147.542581753404 ±-12.4612757687857
| Δ3aa, NaCl, days 2+, mean = 152.085706920378 ±-15.4726755770512
| Functional Del, NaCl, all days, mean = 110.390156452959 ±-10.0078656556358
| Functional Del, NaCl, days -4 to 1, mean = 109.19245315204 ±-10.4698487586212
| Functional Del, NaCl, days 2+, mean = 110.887789841302 ±-9.76669966555573
| R862C, NaCl, all days, mean = 132.936215611164 ±-13.6844686986109
| R862C, NaCl, days -4 to 1, mean = 135.234314381271 ±-13.9033060208311
| R862C, NaCl, days 2+, mean = 132.095916162783 ±-13.5067679290779
| WT, ISO, all days, mean = 115.010817927401 ±-13.7173383703413
| WT, ISO, days -4 to 1, mean = 123.932051978663 ±-11.3589274282282
| WT, ISO, days 2+, mean = 110.581793619511 ±-12.585845372889
| Δ3aa, ISO, all days, mean = 134.191603401426 ±-16.4890094626684
| Δ3aa, ISO, days -4 to 1, mean = 140.6173705907 ±-14.9386710071833
| Δ3aa, ISO, days 2+, mean = 130.22739729143 ±-16.1483463802637
| Functional Del, ISO, all days, mean = 105.145191099476 ±-13.5567361610883
| Functional Del, ISO, days -4 to 1, mean = 111.01488610729 ±-9.10319963649266
| Functional Del, ISO, days 2+, mean = 102.123474437049 ±-14.4525022136603
| R862C, ISO, all days, mean = 136.360338929111 ±-20.3462600065227
| R862C, ISO, days -4 to 1, mean = 144.068164688427 ±-15.5358711212741
| R862C, ISO, days 2+, mean = 133.536621915426 ±-21.1537398508708
P (all days, WT vs Δ3aa) = 2.14878564469269e-12  
P (days -4 to 1, WT vs Δ3aa) = 6.2391422612534e-09  
P (days 2+, WT vs Δ3aa) = 3.6409888384234e-10  
P (all days, WT vs functional Del) = 2.70462955488118e-06  
P (days -4 to 1, WT vs functional Del) = 8.91562730665803e-07  
P (days 2+, WT vs functional Del) = 0.000208997110860927  
P (all days, WT vs R862C) = 3.26450460631035e-06  
P (days -4 to 1, WT vs R862C) = 2.62065000017533e-05  
P (days 2+, WT vs R862C) = 2.21221191046212e-05  
P (all days, Δ3aa vs functional Del) = 1.55571302500694e-15  
P (days -4 to 1, Δ3aa vs functional Del) = 6.05028499099353e-15  
P (days 2+, Δ3aa vs functional Del) = 1.1144147270246e-12  
P (all days, Δ3aa vs R862C) = 0.0601630335549635  
P (days -4 to 1, Δ3aa vs R862C) = 0.29841722098672  
P (days 2+, Δ3aa vs R862C) = 0.0694572056223863  
P (all days, functional Del vs R862C) = 1.30167905808461e-10  
P (days -4 to 1, functional Del vs R862C) = 8.93311031992043e-11  
P (days 2+, functional Del vs R862C) = 3.58329882233102e-09

**Diastolic BP (mmHg)**

all days, full set, P (condition) = 0.300947242826468  
all days, full set, P (genotype) = 1.11188235255332e-09  
all days, full set, P (condition X genotype) = 0.0101022648961665  
ISO, all days, P (genotype) = 5.05719910328334e-05  
NaCl, all days, P (genotype) = 1.29626946304023e-07  
WT, NaCl, all days, mean = 90.6449353613143 +12.2067667283097  
WT, NaCl, days -4 to 1, mean = 91.142628774041 +13.1926284105371  
WT, NaCl, days 2+, mean = 90.4887011265515 +11.8763642040232  
Δ3aa, NaCl, all days, mean = 115.0087431110111 +18.807410561212  
Δ3aa, NaCl, days -4 to 1, mean = 109.825387199389 +15.4827866042806  
Δ3aa, NaCl, days 2+, mean = 117.173938928458 +19.6327044152157  
Functional Del, NaCl, all days, mean = 85.5046625303871 +7.27251448630198  
Functional Del, NaCl, days -4 to 1, mean = 85.0724260815822 +7.41160984963933  
Functional Del, NaCl, days 2+, mean = 85.684252323969 +7.20650580417908
R862C, NaCl, all days, mean = 96.3520058703547 +\text{-}13.1399328033208
R862C, NaCl, days -4 to 1, mean = 98.9485878855444 +\text{-}12.348188762722
R862C, NaCl, days 2+, mean = 95.4025660710646 +\text{-}13.292143955113
WT, ISO, all days, mean = 86.7437253683431 +\text{-}10.7808476179825
WT, ISO, days -4 to 1, mean = 92.2217304304677 +\text{-}11.2125422071248
WT, ISO, days 2+, mean = 84.0241220668843 +\text{-}9.445894418489
Δ3aa, ISO, all days, mean = 102.829994725233 +\text{-}16.352915758643
Δ3aa, ISO, days -4 to 1, mean = 105.760940008379 +\text{-}16.6193441601137
Δ3aa, ISO, days 2+, mean = 101.021826010545 +\text{-}15.9198861527942
Functional Del, ISO, all days, mean = 85.9904077848417 +\text{-}14.1007002469123
Functional Del, ISO, days -4 to 1, mean = 88.912220082531 +\text{-}10.0696559752645
Functional Del, ISO, days 2+, mean = 84.4862599726195 +\text{-}15.5671811682717
R862C, ISO, all days, mean = 106.072331529955 +\text{-}21.8789518031958
R862C, ISO, days -4 to 1, mean = 110.705829376855 +\text{-}16.7206633799212
R862C, ISO, days 2+, mean = 104.374876617024 +\text{-}23.2573136568225

P (all days, WT vs Δ3aa) = 6.95570833933003e\text{-}08
P (days -4 to 1, WT vs Δ3aa) = 1.51171425167247e\text{-}05
P (days 2+, WT vs Δ3aa) = 9.7098516843578e\text{-}07
P (all days, WT vs functional Del) = 0.128101225371132
P (days -4 to 1, WT vs functional Del) = 0.0960362605140835
P (days 2+, WT vs functional Del) = 0.193386287625263
P (all days, WT vs R862C) = 0.00101556773611119
P (days -4 to 1, WT vs R862C) = 0.00076546639314539
P (days 2+, WT vs R862C) = 0.0039815745535844
P (all days, Δ3aa vs functional Del) = 1.72646698567518e\text{-}08
P (days -4 to 1, Δ3aa vs functional Del) = 9.60332061018201e\text{-}08
P (days 2+, Δ3aa vs functional Del) = 3.85536544937237e\text{-}07
P (all days, Δ3aa vs R862C) = 0.148788707112506
P (days -4 to 1, Δ3aa vs R862C) = 0.62310181315921
P (days 2+, Δ3aa vs R862C) = 0.118865763702442
P (all days, functional Del vs R862C) = 4.18403548972194e\text{-}05
P (days -4 to 1, functional Del vs R862C) = 3.41330079107209e\text{-}06
P (days 2+, functional Del vs R862C) = 0.000196595758079309
Heart rate (BPM)

all days, full set, P (condition) = 7.30439497678725e-13
all days, full set, P (genotype) = 0.027208680075181
all days, full set, P (condition X genotype) = 0.279134766519194
ISO, all days, P (genotype) = 0.0781136170528911
NaCl, all days, P (genotype) = 0.0396369501303714
WT, NaCl, all days, mean = 329.639700012835 +71.5683586655788
WT, NaCl, days -4 to 1, mean = 308.894190071989 +48.5773674785426
WT, NaCl, days 2+, mean = 336.152060408797 +76.2347820836142
Δ3aa, NaCl, all days, mean = 307.403830015371 +50.148540830131
Δ3aa, NaCl, days -4 to 1, mean = 308.099248632141 +49.8736496356713
Δ3aa, NaCl, days 2+, mean = 307.11339162326 +50.2613955971408
Functional Del, NaCl, all days, mean = 335.784218606001 +47.5877719942612
Functional Del, NaCl, days -4 to 1, mean = 340.25851631644 +49.2054581641867
Functional Del, NaCl, days 2+, mean = 333.925193980792 +46.7748311292342
R862C, NaCl, all days, mean = 290.675914133625 +41.5323912822064
R862C, NaCl, days -4 to 1, mean = 296.199533630621 +40.258804898904
R862C, NaCl, days 2+, mean = 288.656203546437 +41.807928947744
WT, ISO, all days, mean = 391.782020166269 +75.7236584058948
WT, ISO, days -4 to 1, mean = 316.993982508374 +52.3522892407814
WT, ISO, days 2+, mean = 428.911192532349 +55.3600794306367
Δ3aa, ISO, all days, mean = 371.094649531665 +81.4345746815601
Δ3aa, ISO, days -4 to 1, mean = 301.596753833264 +51.1905489931011
Δ3aa, ISO, days 2+, mean = 413.969527929464 +65.4842551038261
Functional Del, ISO, all days, mean = 386.353685334081 +67.6342374210703
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Functional Del, ISO, days 2+, mean = 416.306805079545 +55.1312204706244
R862C, ISO, all days, mean = 379.170203277906 +77.6871371644984
R862C, ISO, days -4 to 1, mean = 291.171243323442 +49.0194070458711
R862C, ISO, days 2+, mean = 411.408109033591 +59.0745572656604

P (all days, WT vs Δ3aa) = 0.111660671292058
P (days -4 to 1, WT vs Δ3aa) = 0.0535488916820319
P (days 2+, WT vs Δ3aa) = 0.0618584600730447
P (all days, WT vs functional Del) = 0.626969532154837
Increased dimerization/self-assembly of mutant PDE3A enzyme

Since all PDE3A mutations cause aberrant phosphorylation and hyperactivity of the enzyme, the involved phosphosite region S428/S438 most likely controls the catalytic domain (amino acid residues 810-1068) through an allosteric conformation-based mechanism. However, 3D structures of full-length PDE3A showing the position of the N terminus within the protein are not available and Alphafold 2 did not allow for a 3D structure prediction for larger parts of the protein than the catalytic domain. The N terminus is involved in self-association. The catalytic domain crystallizes as a dimer and is active as a dimer. Thus, the mutations could affect PDE3A activity by modulating the dimerization. Initially, we sought to map regions of PDE3A mediating dimerization/self-assembly and to elucidate whether the phosphosites S428 and S438 and the mutational hotspot region between amino acid residues 445-449 are involved.

We spot-synthesized full-length human PDE3A1 (amino acid residues 145-1141) as 25mer overlapping peptides and overlaid them with wild-type PDE3A1 (Figure S3A). Amino acid residues 1-144 contain hydrophobic membrane-associating regions that are not expressed in the
myocardium and were therefore omitted. PDE3A1 bound several peptides throughout the protein, including the region comprising residues S428-S438 and 445-449. In the catalytic domain, the interaction was mediated by C-terminal residues and the region between K956 and P995 (Figure S3A), which represents the core dimerization region in the catalytic domain.

In the center of the catalytic domain, interactions were not apparent. Arginine (R) is positively charged and may prevent dimerization in the central region through electrostatic repulsion. We substituted R862 with the cysteine (C) as in the new HTNB patients (Figure 3). This approach removes the charge and could induce disulfide bond formation with C in close proximity and thereby promote or stabilize dimerization. Initial co-immunoprecipitation studies using Flag- and HA-tagged versions of PDE3A2 showed self-assembly of PDE3A2-wild-type, PDE3A2-R862C and PDE3A2-T445N (Figure S2B). Expression in HEK293 cells, which do not endogenously express PDE3A (Ercu et al., Circulation 2020), and detection by Western blotting revealed increased complex formation of the R862C version compared to PDE3A2-wild-type and PDE3A2-T445N (Figure S3C). PDE3A2-R862C complex formation was very sensitive to the reducing agent β-mercaptoethanol, confirming disulfide bond formation between PDE3A2-R862C molecules (Figure S3D). These studies indicated increased self-assembly of mutant PDE3A and point to a role of enzyme conformation in inducing the hyperactivity of the mutants.
Figure S1

Histological analyses

- WGA staining (AU)
- Coll I media
- Coll I fibrose
- CD31_area_fraction

qPCR analyses

- ANP expression (AU)
- BNP expression (AU)
- Coll I expression (AU)
- Coll IV expression (AU)
- aMHC expression (AU)
- bMHC expression (AU)
Figure S1. Hearts from wild-type and HTNB rats respond similarly to chronic β-adrenergic stimulation.

This figure extends Figure 2D of the main manuscript. It shows the detection of the indicated hypertrophy and fibrosis markers by histological analysis or PCR. Macrophages were labeled with anti-ED1 (red dots) and counted in 10 representative sections per slice per animal. Each symbol represents the average numbers of ED1-positive cells per animal. Statistical analysis
was carried out using two-way ANOVA and Tukey's multiple-comparison; shown are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure S2

A kDa

150
100
60

PDE3A1
PDE3A2

Hsp60

WT
R862C
Het
functional Del

B

C

Metacarpal bone III

WT
R862C
Het

D

E

Serum parameters

F

Urine parameters

G

Relative renin expression (GAPDH)

**

***

****

WT
R862C
Het
functional Del

**

**

BMI

WT
R862C
Het
functional Del
Figure S2. Characterization of the PDE3A-R862C HTNB rat model.

A. Detection of PDE3A1 and PDE3A2 in aortas of wild-type (WT), heterozygous PDE3A-R862C and functional Del rats by Western blotting. Signals were semi-quantitatively analyzed by densitometry. Wild-type (WT), n = 4; PDE3A-R862C and functional Del, n = 3 each. Statistical analyses using one-way ANOVA and Dunnett's multiple comparisons test, shown are mean ± SD, **p < 0.01, ***p < 0.001, ****p < 0.0001.

B. Body lengths of 8-11 weeks old wild-type and homozygous PDE3A-R862C rats. Statistical comparison was carried out using the Mann-Whitney test, shown are mean ± SD, *p < 0.1.

C. MicroCT images of the right front paws of heterozygous R862C and wild-type (WT) rats and quantification of metacarpal bone III length and volume. WT: n = 4; R862C: n = 7; Statistical comparison was carried out using Mann-Whitney test, shown are mean ± SD, **p < 0.01.

D. The body mass index (BMI) of 8-11 weeks old wild-type and homozygous PDE3A-R862C was calculated using the formula: weight (g)/length (cm²). Statistical comparison using the Mann-Whitney test did not reveal statistically significant differences; n = 10 WT and n = 4 homozygous PDE3A-R862C rats, shown are mean ± SD.

E. Serum and F. urine parameters of 8-11 weeks old wild-type and homozygous PDE3A-R862C were determined. Statistical comparison using the Mann-Whitney test did not reveal differences between the two genotypes. n = 4 WT and n = 4 PDE3A-R862C; values are means ± SD.

G. Renin expression was determined by qPCR. WT: n = 7; D3aa: n = 11; R862C Hom: n = 3; Functional Del, n = 7. Statistical analysis was carried out using the Kruskal-Wallis and Dunn's multiple comparison test, shown are mean ± SEM, *p < 0.1, **p < 0.01.
Figure S3A

spotted peptide amino acids

L516 80 90 100 110 120 T810

T810

K956

Y1016
176 L-W-P-G-K-W-V-E-D-S-D-P-S-G-D-T-D-D-P-R

M145

S292 S312 S428 S438 S465 S492

transmembrane domains

phospho sites ml hot spot

PDE3A1 WT #1


S1033 T1036

S1060

S1110

Q1081

Q1141
**Figure S3**

**B**

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**C**

![Graph showing relative oligomerization](image)

**D**

![Graph showing upper signal/lower signal relative to GAPDH](image)
**Figure S3. The R862C substitution in the catalytic domain of PDE3A enhances dimerization/oligomerization and increases catalytic activity.**

**A. Upper,** Amino acid residues 145-1141 of PDE3A were spot-synthesized as 25mer overlapping peptides with an offset of five amino acids. Peptide numbers are indicated. The numbers of amino acid residues refer to the full length human PDE3A1. The peptides were overlaid with full-length PDE3A1-Flag affinity-purified via its Flag tag from HEK293 cells transiently expressing the construct. Binding of the protein to the peptides was detected using anti-PDE3A antibodies in a procedure analogous to Western blotting. **Lower,** Numbers and sequences of interacting peptides are indicated. Mt. hot spot, mutational hot spot. **B.** Flag-tagged PDE3A2-R862C, PDE3A2-T445N or wild-type were co-expressed with HA-tagged PDE3A2-R862C, PDE3A2-T445N or wild-type as indicated. The proteins were affinity-precipitated (IP) via the Flag tag and detected by Western blotting with anti-HA and anti-Flag tag antibodies. GAPDH was detected as loading control and to confirm specificity of the precipitation (absent the IP samples). Shown are representative results from n = 3 independent experiments. Shown are mean ± SEM. **C. and D.** PDE3A2-R862C, PDE3A2-T445N or wild-type were transiently expressed in HEK293 cells. **C.** The cells were lysed and proteins separated by SDS-PAGE under non-reducing conditions, i.e. with Laemmli sample buffer devoid of β-mercaptoethanol or **D.** under reducing conditions, i.e. in the presence of the indicated % of β-mercaptoethanol in the Laemmlı sample buffer. PDE3A was detected by Western blotting. Shown are representative results from n = 8 and 3 independent experiments (C. and D., respectively). Shown are mean ± SEM. Statistical analysis was carried out using one-way ANOVA and Bonferroni multi-comparison, **p < 0.01.
Figure S4

A

Δ3aa Het vs. WT

β-AR/cAMP/PKA signaling

Ca²⁺ cycling

Contractility

Cardiac damage

PDEs

Transcription factors

Δ3aa Het vs. WT

log2 FC

β-AR/cAMP/PKA signaling

Ca²⁺ cycling

Contractility

Cardiac damage

PDEs

Transcription factors

NaCl

Iso

B

cAMP (pmol/mg)

NaCl

Iso

WT

Δ3aa

functional Del

R862C

- 39 -
Figure S4

- 40 -
Figure S4

D  

cGMP-PKG signaling pathway

E

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MyBPC

pMyBPC

ICER

PKA substrate

Hsp60

GAPDH

PDE3A1-Hsp60

PDE3A2-Hsp60

Myosin

CaM

MLC

Ca

2+

Lower calcium

Increased calcium

Decrease in intracellular PDE2

 Decrease in intracellular pCREB

Delta 3aa

Δ3aa

R862C

Δ3aa

Δ3aa

Δ3aa

Δ3aa

Δ3aa

Δ3aa

Δ3aa

Δ3aa

Δ3aa

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Figure S4. The left ventricles of hearts from wild-type and HTNB mutant rats are similar.

A. RNAseq analysis derived from data depicted in Figure 6 of the main manuscript and from Excel files S2 and S3. Left, heat map showing differentially expressed mRNAs in the left ventricles of hearts from untreated heterozygous (HET) PDE3A-Δ3aa and wild-type rats. WT, n = 3; PDE3A-Δ3aa, n = 4. Log2FC indicates fold changes. The differences in expression of the indicated mRNAs did not reach statistical significance. Right, heat maps showing differentially expressed mRNAs in the left ventricles of hearts from NaCl- or isoproterenol-treated wild-type, PDE3A-Δ3aa and functional Del rats compared to wild-type. n = 3 for each genotype. Log2FC indicates fold changes. Shown are mean ± SEM, *p < 0.05.

B. The concentration of cAMP in left ventricles from hearts of untreated, NaCl- or isoproterenol-treated wild-type, PDE3A-Δ3aa, PDE3A-R862C and functional Del rats was determined by radioimmunoassay. n = 3 untreated WT, n = 2 NaCl WT, n = 2 isoproterenol WT; n = 3 untreated PDE3A-Δ3aa, n = 3 NaCl PDE3A-Δ3aa, n = 3 isoproterenol PDE3A-Δ3aa; n = 3 untreated functional Del, n = 4 NaCl functional Del, n = 4 isoproterenol functional Del; n = 3 untreated PDE3A-R862C, n = 5 NaCl PDE3A-R862C, n = 5 isoproterenol PDE3A-R862C. As expected, the two tested wild-type animals responded to isoproterenol with a clear increase in cAMP. Shown are mean ± SEM for experimental groups. A two-way ANOVA did not detect statistically significant differences.

C. and E. represent extensions of Figure 7 of the main manuscript. C. Semiquantitative analyses of the Western blots of untreated animals. D. cGMP signaling pathway in cardiac myocytes derived from single nuclei RNA seq analysis depicted in Figure 7F of the main manuscript. The scheme was generated using KEGG pathway analysis tools (Kanehisa Laboratories). Differential gene expression is color-coded: blue, upregulated PDE3A-Δ3aa and wild-type; red, upregulated in wild-type vs. PDE3A-Δ3aa; green, upregulated in functional Del vs. PDE3A-Δ3aa; orange, upregulated in PDE3A-Δ3aa vs. functional Del; yellow, upregulated in PDE3A-Δ3aa vs. both. E. Semiquantitative analyses of the Western blots of NaCl- or isoproterenol-treated rats. It also shows all statistically significant differences between PDE3A1 and PDE3A2 expression in the left ventricles of hearts from NaCl- and isoproterenol-treated rat models. Statistical analysis in C. was carried out using one-way ANOVA and Tukey’s multiple comparison test if values were normally distributed or using the non-parametric Kruskal-Wallis and Dunn’s multiple comparison test. In E., statistical analysis was carried out using two-way ANOVA and Tukey’s multiple comparison; shown are mean ± SEM is plotted. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure S5

A. TALENs and piggyBac targeting of T445N

- Homologous recombination
- Integration of selection cassette and selection
- Transposase
- Genome edited locus after excision of selection cassette

B. CRISPR/Cas9 based targeting of R862C

- Homologous recombination
- Genome edited locus

C. Human iPSC culture
Cardiomyocyte differentiation
Maintenance culture
Experiments

D. WT
- Cardiac gene expression
- Tnt2 and Tnnt2a expression

T445N HET
- Cardiac gene expression
- Tnt2 and Tnnt2a expression

R862C HOM
- Cardiac gene expression
- Tnt2 and Tnnt2a expression
Figure S5. Introduction of HTNB substitutions and characterization of the hiPSC and hiPSC-CMs.

A. Schematic representation of the TALENs and CRISPR/Cas9 approach to introduce T445N and R862C substitutions, respectively. B. hiPSC were stained for pluripotency markers. Wild-type (WT) and mutant cells show expression of the pluripotency markers TRA-1-60 and SOX2. C. Scheme for the differentiation of hiPSCs to cardiac myocytes. CHIR-99021 and IWR-1 small molecules induces shift into cardiac phenotype. Cardiomyocytes were enriched by metabolic selection and seeded for experimental evaluation at Day 45. D. hiPSC-CMs express cardiac marker proteins TNNT2 and MLC2v. Lower right panel: Quantitative analysis of the flow cytometry data shows no significant difference in expression of cardiac marker proteins between WT and mutant cells. Graphs show mean ± SEM (WT n = 4, T445N n = 3, R862C n = 3).
Table S2. Parameters measured by echocardiography shown in Figures 2C and 4F.

Table extending Figure 2C*.

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Table extending Fig 4F*.

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*shown are means ± SD  
hom, homozygous  
Het, heterozygous