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# Various effects of AAV9-mediated $\beta$ ARKct gene therapy on the heart in dystrophin-deficient (*mdx*) mice and $\delta$ -sarcoglycan-deficient (*Sgcd*<sup>-/-</sup>) mice

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## 1. Introduction

Mutations in genes coding for major protein components of the cytoskeleton in heart and skeletal muscle cells clinically lead to a heterogeneous group of muscular dystrophies frequently associated with severe cardiomyopathy [1]. In Duchenne muscular dystrophy (DMD) and limb girdle muscular dystrophy type 2 F (LGMD2F), absence of dystrophin or  $\delta$ -sarcoglycan (*Sgcd*) disrupts the dystrophin-glycoprotein-complex (DGC) progressively damaging cardiac and skeletal

muscle cells [2]. In almost every patient with DMD or LGMD2F subclinical or clinical cardiomyopathy can be found with strong impact on mortality.

However, it remains unclear whether universally evidence based treatment strategies of heart failure are applicable to every causally distinct form of cardiomyopathy. The contribution of alterations in  $\beta$ -adrenergic receptor ( $\beta$ AR) signalling such as upregulation of G protein-coupled receptor kinase-2 (GRK2) in maintaining cardiac dysfunction in heart failure is well documented [3,4]. However, it is not well investigated in cardiomyopathies associated with muscular dystrophies. Likewise, although large studies have shown protective effects of  $\beta$ -blocker treatment in patients with heart failure [5], the benefit of these drugs in treating the specific subgroup

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of patients with muscular dystrophy and cardiomyopathy is not yet established [6]. In a previous study we demonstrated a genotype-dependent response to pharmacological treatment with  $\beta$ -blockers, with beneficial effects in dystrophin-deficient *mdx* mice and rather harmful effects in *Sgcd*-deficient mice (*Sgcd*<sup>-/-</sup>) despite a functional interaction between dystrophin and the membrane-bound sarcoglycan complex [7]. In this earlier study the underlying pathophysiological mechanisms remained elusive, although the finding is clinically relevant with regard to the need of “individualized” treatment of patients with cardiomyopathy with or without muscle involvement. The distinct response pattern to pharmacological  $\beta$ -blockade indicates the need to develop more specific treatment strategies for patients with muscular dystrophy associated cardiomyopathy.

A promising approach to target maladaptive increase of GRK2 in heart failure represents a competitive inhibition of the GRK2- $\beta$ AR-interaction through  $\beta$ ARKct.  $\beta$ ARKct is a polypeptide which consists of the last 194 C-terminal amino acid residues of GRK2 [8–10]. Although  $\beta$ ARKct is effective also in other non-cardiac tissues such as vessels and nervous system so far no harmful side effects of  $\beta$ ARKct-treatment were documented in previous studies [11,12]. Previously we found that in *mdx* mice GRK2 is upregulated whereas in *Sgcd*<sup>-/-</sup> mice GRK2 was not changed compared to wildtype mice. Therefore, we hypothesized that differences of GRK2 may lead to different  $\beta$ AR signaling in these two mouse models.

Therapeutic gene transfer of  $\beta$ ARKct using adeno-associated virus (AAV)-vectors has been used for long-term, cardiac gene transfer in small and large animal models of heart failure and can significantly improve hemodynamic parameters and adverse ventricular remodelling [13,14]. The use of AAV vectors of serotype 9 enabled a highly efficient cardiac gene transfer after systemic application in mice making this vector system an ideal candidate for the study of novel gene therapeutic approaches in mouse models [15,16].

The aim of the present study was therefore to investigate the efficiency of an inhibition of GRK2 through AAV9-mediated cardiac overexpression of  $\beta$ ARKct (*AAV9/ $\beta$ ARKct*) to prevent the development of cardiomyopathy in *mdx* and *Sgcd*<sup>-/-</sup> mice, established mouse models for DMD and LGMD2, respectively. We found that *AAV9/ $\beta$ ARKct* treatment particularly improved left ventricular function and ameliorated hypertrophy in dystrophin-deficient *mdx* mice. By contrast in *Sgcd*<sup>-/-</sup> mice beneficial effects on development of heart failure were mild without any protection from hypertrophy. Neither GRK2 nor nuclear factor-kappaB (NF $\kappa$ B) were upregulated in *Sgcd*<sup>-/-</sup> mice, potentially explaining the different therapeutic response in these mouse models.

## 2. Material and methods

### 2.1. Generation of the $\beta$ ARKct construct and AAV9 vectors

For human codon usage optimized  $\beta$ ARKct-cDNA was generated (Geneart, Regensburg, Germany) and inserted into

pdsCMV-MLC0.26-EGFP resulting in pdsCMV-MLC0.26- $\beta$ ARKct [17,18]. For production of AAV9/ $\beta$ ARKct and AAV9/EGFP vectors, this plasmid was used for co-transfection of 293T cells together with p5E18-VD2-9, encoding the AAV-9 cap sequence, and pDGdelVP, containing the AAV-2 rep gene as well as adenoviral helper sequences [19,20]. AAV vectors were purified and titrated using SYBR green PCR with CMVenh329fwd (5'-TGCCCAGTACATGACCTTATGG-3') as forward and CMVenh462rev (5'-GAAATCCCCGTGAGTCAAACC-3') as reverse primer [21].

### 2.2. Animals and systemic in vivo vector delivery

Male *mdx* mice ( $n=32$ ) and C57Bl/10 wildtype control mice ( $n=11$ ) were obtained as a gift from Prof. Dr. R. Fink (Institute of Physiology, University of Heidelberg). *Sgcd*<sup>-/-</sup> mice ( $n=28$ ) (B6.129-Sgcdtm1Mcn/J) were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and bred in our animal facility. Wildtype littermates ( $n=10$ ) were used as controls for *Sgcd*<sup>-/-</sup> mice. Mice were housed under controlled temperature (17–28 °C) and light conditions (12:12 h light:dark cycle) and had free access to food and water. All procedures involving the use and care of animals were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and the German animal protection code. Approval was also granted by the local ethics review board. 10<sup>12</sup> viral genomes of AAV9 were injected into the tail vein of 8 weeks-old *mdx* ( $n=11$ ) and *Sgcd*<sup>-/-</sup> ( $n=10$ ) mice as a 150  $\mu$ l bolus using a sterile 29-gauge needle. At the end of the study, animals were euthanized by cervical dislocation and hearts were rapidly frozen in liquid nitrogen after dissection. To control for potential side effects of high dose AAV9 vectors, further *mdx* ( $n=10$ ) and *Sgcd*<sup>-/-</sup> mice ( $n=8$ ) were injected with an AAV9/EGFP-vector. There were no differences between untreated and AAV9/EGFP-treated *mdx* mice in morphology and cardiac hemodynamics at the age of 12 months (Supplementary table).

### 2.3. Immunoblotting

Protein samples from hearts were homogenized in RIPA-buffer (with 0,1% SDS) (Roche) using a tissue lyser (MM301, Quiagen, Hilden, Germany). Lysates were cleared from cellular debris by centrifugation for 5 minutes at 13,000 rpm and a total amount of 50  $\mu$ g of protein was loaded onto a 3–8% gradient gel (TRIS-glycine gel, Invitrogen, Karlsruhe, Germany). Protein was blotted on a PVDF membrane (Millipore, Billerica, USA) using  $\beta$ ARKct and GRK2 (sc-562, C-15, rabbit, polyclonal, Santa Cruz Biotechnology, 1:5,000), phospho-NF- $\kappa$ B p65 (Ser536) (rabbit, monoclonal, Cell signaling technology) and Glycerinaldehyd-3-phosphat-Dehydrogenase (GAPDH) (clone 6C5, mouse, monoclonal, Millipore, Billerica, Massachusetts, USA, 1:20,000) as primary antibodies (directed against mouse, rat and chicken). A fluorophore

labeled goat anti rabbit (IRDye 800) and a goat anti mouse (IRDye 680, LI-COR, Lincoln, USA), diluted 1:10.000 in 1x Roti-Block, were used to visualize protein bands by Odyssey infrared imaging (LI-COR, Lincoln, USA). To perform relative quantification of the bands obtained by immunoblotting, we applied ImageJ software based analysis (<http://rsb.info.nih.gov/ij/>). The area under curve (AUC) of the specific signal was corrected for the AUC of the loading control GAPDH.  $N = 5$  for all animal groups.

#### 2.4. Real-time polymerase chain reaction

For analysis of mRNA expression levels of brain natriuretic peptide (BNP), the  $\alpha$ -subunit of the stimulatory G-protein ( $G_s\alpha$ ), phospholamban (pln) and sarcoplasmic reticulum  $Ca^{2+}$ -ATPase (SERCA2A) total RNA was extracted from cardiac tissue lysates of untreated and *AAV9/βARKct*-treated *mdx*, *Sgcd*<sup>-/-</sup> and wild type control mice with the Rneasy Fibrous Tissue Mini Kit (Qiagen®, Hilden, Germany) ( $n = 8$  each animal group). SuperScript III reverse transcriptase (Invitrogen, Karlsruhe, Germany) was used to randomly reverse transcribe 1 μg of RNA into cDNA according to the manufacturer's specifications. Quantitative real time PCR was performed on an ABI PRISM 7000 (Applied Biosystems, Foster City, USA) using QuantiTect primer assays (Qiagen®, Hilden, Germany; assay name/catalog numbers: BNP [Mm\_Nppb\_1\_SG/QT00107541];  $G_s\alpha$  [Mm\_Gnas\_1\_SG/QT00134127]; pln [Mm\_Pln\_1\_SG/QT00254366]; SERCA2A [Mm\_Atp2a2\_1\_SG/QT00149121]; GAPDH [Mm\_Gapdh\_3\_SG/QT01658692]) and SYBR GreenER qPCR SuperMix (Invitrogen®, Karlsruhe, Germany). For detection of  $\beta$ ARKct the primers *-forward: gga agt gcc cga gac cgt c and reverse: gct cat gta gcc gtg cat g-* were generated.

#### 2.5. Histological analysis and determination of cardiomyocyte cross-sectional areas

Frozen 10 μm transverse sections were cut from the mid-part of hearts. To analyse cardiomyocyte cross-sectional areas color images were made of H&E-stained heart sections of untreated, *AAV9/βARKct*-treated *mdx*, *Sgcd*<sup>-/-</sup> and wildtype mice ( $n = 5$  per group) at 20x total magnification using a Nikon Eclipse 90i upright automated microscope with a DS-Ri1 color camera (Nikon, Düsseldorf, Germany). For appropriate measurement of cardiomyocyte cross-sectional areas, sections were chosen which include the papillary muscles and microphotographs were taken from the area adjacent to the papillary muscles [[22] and supplemental Figure 1]. Images were opened in ImageJ and after setting the threshold 25 cardiomyocytes per animal were analysed.

Masson-Trichrome staining was performed to analyse fibrosis of heart sections of untreated, *AAV9/βARKct*-treated *mdx*, *Sgcd*<sup>-/-</sup> and wild type mice ( $n = 5$  per group). Areas of fibrosis, appearing blue with Masson-Trichrome staining were highlighted, and the fibrotic area was analysed at 4x

total magnification with Image J software and calculated in percentage area of fibrosis.

#### 2.6. Transthoracic echocardiography

The ventral chest area of mice was depilated one day before echocardiography. For echocardiography, a Sonos 5500 (Philips, Eindhoven, NL) with a S12 transducer (12 MHz) was used and the performing person was blinded during procedure. To evaluate cardiac function, three consecutive beats were analysed to obtain the left ventricular end-diastolic diameter (LVEDD) and the left ventricular end-systolic diameter (LVESD). Cardiac function is represented as fractional shortening (FS) which was calculated as  $FS \% = [(LVEDD - LVESD) / LVEDD] \times 100$ . The thickness of left ventricular posterior wall was measured in diastole (PWTd) (number of animals examined s. Table 1).

#### 2.7. Pressure volume (PV) loops

Measurements were made in closed-chest, spontaneously breathing mice (number of animals examined s. Table 1). Mice were anaesthetized by intraperitoneal injection of Medetomidin (0,5 μg/g body weight), Fentanyl (0,05 μg/g body weight) and Midazolam (5 μg/g body weight). Body temperature was maintained at 37 °C using a homeothermic blanket (Harvard apparatus Ltdw, Edenbridge, UK). A 1.2 Fr catheter (Model FT111B Scisense Inc., London, ON, Canada) was inserted into the left ventricle of the mouse through the carotid artery to simultaneously measure pressure and volumes. Left ventricular volumes were extrapolated from admittance magnitude and admittance phase in real time using the ADVantage PV system (Scisense Inc., London, ON, Canada).

Pressure and volume data were recorded using a Scisense 404–16 Bit Four Channel Recorder with LabScribe2 Software (Scisense Inc., London, ON, Canada). Indices of systolic function included end-systolic pressures (ESP), stroke volume (SV) and stroke work (SW). Load dependent myocardial contractility was assessed by maximal rate of pressure development ( $dp/dt_{max}$ ). Transient inferior vena caval compressions were applied to reduce preload and determine end-systolic elastance (Ees) as a parameter of load-independent myocardial contractility as previously described [23]. Diastolic function was assessed by end-diastolic pressure (EDP), tau (Weiss method, regression of log of pressure vs. time) and the maximal rate of pressure decay ( $dp/dt_{min}$ ). Left ventricular size was determined by end-diastolic volumes (EDV). Two *mdx* mice died during the procedure without obtaining any loops (number of animals examined s. Table 1). C57Bl/10 present higher left ventricular volumes compared to C57Bl/6 mice in our PV-loops experiments (Table 1).

#### 2.8. Statistical analysis

For statistical analysis we used the GraphPad prism® software version 5.01. All data are presented as the mean  $\pm$

Table 1

Comparison of hemodynamic variables for untreated and AAV9/ $\beta$ ARKct-treated *mdx* and *Sgcd*<sup>-/-</sup> mice and the corresponding wildtype control mice.

PV loops	C57Bl/10 (n=5)	<i>mdx</i> (n=6)	<i>Mdx</i> + $\beta$ ARKct (n=8)	C57Bl/6 (n=8)	<i>Sgcd</i> <sup>-/-</sup> (n=8)	<i>Sgcd</i> <sup>-/-</sup> + $\beta$ ARKct (n=8)
HR (bpm)	458 ( $\pm$ 12)	356 ( $\pm$ 40)	410 ( $\pm$ 17)	466 ( $\pm$ 32)	403 ( $\pm$ 17)	447 ( $\pm$ 14)
ESP (mmHg)	125 ( $\pm$ 3)	83 ( $\pm$ 6)*	96 ( $\pm$ 5)	109 ( $\pm$ 6)	87 ( $\pm$ 3)**	94 ( $\pm$ 3)
Dp/dt <sub>max</sub> (mmHg/s)	12,355 ( $\pm$ 932)	5624 ( $\pm$ 229)*	7607 ( $\pm$ 210)**	11,385 ( $\pm$ 661)	6436 ( $\pm$ 361)***	7914 ( $\pm$ 629)
SV ( $\mu$ l)	23 ( $\pm$ 2)	9 ( $\pm$ 1)*	18 ( $\pm$ 1)**	18 ( $\pm$ 1)	12 ( $\pm$ 1)**	11 ( $\pm$ 1)
SW ( $\mu$ l*mmHg)	3054 ( $\pm$ 339)	768 ( $\pm$ 234)*	1910 ( $\pm$ 132)**	2061 ( $\pm$ 150)	1141 ( $\pm$ 120)***	1120 ( $\pm$ 53)
Ees (mmHg/ $\mu$ l)	10,1 ( $\pm$ 0,4)	4,8 ( $\pm$ 0,7)*	9,9 ( $\pm$ 2,1)**	8,9 ( $\pm$ 1,9)	3,9 ( $\pm$ 0,4)***	6,3 ( $\pm$ 0,9)
EDV ( $\mu$ l)	40 ( $\pm$ 4)	38 ( $\pm$ 2)	35 ( $\pm$ 4)	26 ( $\pm$ 1)	37 ( $\pm$ 1)**	33 ( $\pm$ 1)
EDP (mmHg)	9 ( $\pm$ 5)	8 ( $\pm$ 3)	8 ( $\pm$ 1)	7 ( $\pm$ 0,7)	12 ( $\pm$ 0,4)***	10 ( $\pm$ 0,9)
Tau weiss (msec)	7,8 ( $\pm$ 1,9)	11,4 ( $\pm$ 1,4)*	8,1 ( $\pm$ 0,6)	6,5 ( $\pm$ 0,4)	9,1 ( $\pm$ 0,4)***	7,2 ( $\pm$ 0,3)
Dp/dt <sub>min</sub> (mmHg/s)	-8257 ( $\pm$ 1117)	-5469 ( $\pm$ 551)*	-6510 ( $\pm$ 288)	-9389 ( $\pm$ 540)	-5394 ( $\pm$ 330)***	-6859 ( $\pm$ 414)
E <sub>a</sub> (mmHg/ $\mu$ l)	9,8 ( $\pm$ 2,3)	7,8 ( $\pm$ 1,1)	7,6 ( $\pm$ 1,2)	7,8 ( $\pm$ 1,3)	8,1 ( $\pm$ 2,3)	8,7 ( $\pm$ 1,3)
Echocardiography	(n=5)	(n=11)	(n=11)	(n=10)	(n=10)	(n=10)
FS (%)	74 ( $\pm$ 3)	49 ( $\pm$ 4)*	65 ( $\pm$ 2)**	60 ( $\pm$ 2)	32 ( $\pm$ 4)***	44 ( $\pm$ 1)****
PWTd (mm)	1,1 ( $\pm$ 0,07)	1,8 ( $\pm$ 0,06)*	1,2 ( $\pm$ 0,07)**	1,1 ( $\pm$ 0,07)	1,0 ( $\pm$ 0,07)	0,9 ( $\pm$ 0,07)

Values presented as mean+[standard error].

HR, heart rates; ESP, end-systolic pressures; dp/dt<sub>max</sub>, maximal rate of pressure development; SV, stroke volume; SW, stroke work; Ees, end-systolic elastance; dp/dt<sub>min</sub>, maximal rate of pressure decay; E<sub>a</sub>, arterial elastance; FS, fractional shortening.

\*  $p < 0.05$  *mdx* untreated vs. C57Bl/10.

\*\*  $p < 0.05$ , *mdx* untreated vs. *mdx* +  $\beta$ ARKct.

\*\*\*  $p < 0.05$  *Sgcd*<sup>-/-</sup> untreated vs. C57Bl/6.

\*\*\*\*  $p < 0.05$ , *Sgcd*<sup>-/-</sup> untreated vs. *Sgcd*<sup>-/-</sup> +  $\beta$ ARKct.

standard error. To test for statistical significance between groups, a one-way ANOVA with Bonferroni subgroup analysis was applied. If appropriate, Student's *t*-test was employed (two-sided). Tests for normal distribution of values were performed using the Shapiro–Wilk method. Error bars represent standard error (SEM). Differences in data were considered significant at  $p < 0.05$ .

### 3. Results

#### 3.1. Long-term cardiac $\beta$ ARKct transgene expression in *mdx* and *Sgcd*<sup>-/-</sup> mice

We generated an AAV9 vector containing codon-optimised  $\beta$ ARKct cDNA (AAV9/ $\beta$ ARKct) under transcriptional control of the cardiac-specific CMV-MLC promoter (Fig. 1A). We injected this construct into mice at 8 weeks of age and followed them to 12 months of age. At the age of 12 months AAV9/ $\beta$ ARKct-injected *mdx* and *Sgcd*<sup>-/-</sup> mice showed a similar expression of  $\beta$ ARKct mRNA (Fig. 1B) following 10 months of treatment ( $n = 8$  each group).

#### 3.2. AAV9/ $\beta$ ARKct-treatment improves cardiac haemodynamic function in *mdx* and *Sgcd*<sup>-/-</sup> mice

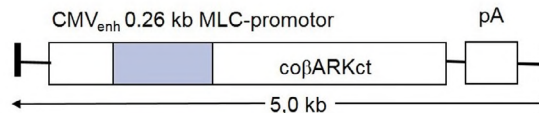
Cardiac function echocardiography was normal at the age of 8 weeks when treatment was started in *mdx* and *Sgcd*<sup>-/-</sup> mice (supplemental Figure 2 and Table 1). At the age of 6 months fractional shortening was significantly decreased only in *Sgcd*<sup>-/-</sup> mice compared to wildtype (supplemental Figure 2). At the age of 12 months, both *mdx* and *Sgcd*<sup>-/-</sup> mice had developed ventricular dysfunction (number of animals examined s. Table 1). Untreated *mdx* and *Sgcd*<sup>-/-</sup> mice showed compromised fractional shortening (FS) in echocardiography.

Pressure volume loops revealed reduced myocardial contractility (dp/dt<sub>max</sub> and end-systolic elastance (Ees)), decreased stroke volume (SV) and stroke work (SW) (Table 1 and Fig. 2A-C). PV-loops demonstrated improved myocardial contractility with increased dp/dt<sub>max</sub> (preload-dependent) and Ees (preload-independent) as well as improvements in the systolic parameters SV and SW in AAV9/ $\beta$ ARKct-treated *mdx* mice (Table 1, Fig. 2A and B). Furthermore, fractional shortening (FS) in echocardiography was increased in AAV9/ $\beta$ ARKct-treated *mdx* mice compared to untreated *mdx* mice (Table 1, Fig. 2C). Echocardiographic analyses of *Sgcd*<sup>-/-</sup> mice showed a significant increase in FS after AAV9/ $\beta$ ARKct treatment compared with untreated animals and a trend towards an increased dp/dt<sub>max</sub> and Ees (Fig. 2, Table 1). In *mdx* and *Sgcd*<sup>-/-</sup> mice, PV loops showed diastolic dysfunction with impaired active (low Tau and dp/dt<sub>min</sub>) and passive relaxation (EDP only in *Sgcd*<sup>-/-</sup> mice), which remained unchanged after treatment with AAV9/ $\beta$ ARKct (Table 1). In the examined mouse models  $\beta$ ARKct has obviously no effects on loading conditions as afterload measured by arterial elastance (E<sub>a</sub>) does not differ between the groups. In parallel to the improved left ventricular function, cardiac expression of  $\beta$ ARKct resulted in a significantly lower cardiac expression of brain-natriuretic peptide (BNP) mRNA expression in *mdx* mice (Fig. 2D). In contrast to *mdx* mice, cardiac BNP mRNA expression in *Sgcd*<sup>-/-</sup> mice was not different from wild type mice and there were no changes after treatment with  $\beta$ ARKct (Fig. 2D).

#### 3.3. Attenuation of myocardial hypertrophy in *mdx* mice

In echocardiography significant hypertrophy compared to wildtype mice can already be detected in 6-month-old *mdx* mice (supplemental Figure 1, Table 1). At the age of 12 months *mdx* mice revealed hypertrophic cardiomyopathy

## A dsAAV\_CMV<sub>enh</sub>/MLC0.26-βARKct



## B βARKct mRNA expression

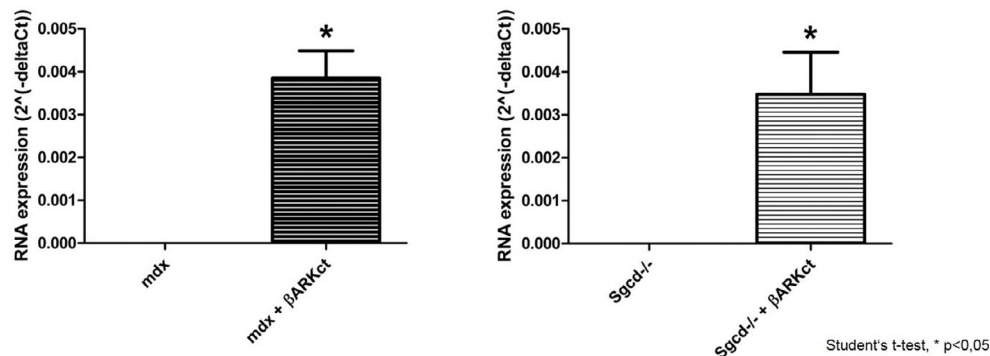


Fig. 1. Long-term cardiac βARKct transgene expression in mdx and Sgcd<sup>-/-</sup> mice.

(A) Schematic presentation of the double-stranded AAV9 genome plasmid (AAV9/βARKct) carrying a codon-optimized βARKct-cDNA (coβARKct) under transcriptional control of a CMV-enhanced (CMV<sub>enh</sub>), 0,26kb myosin light chain (MLC0.26) promoter. (B) RT-PCR showed a similar expression of βARKct mRNA in 12 months-old AAV9/βARKct-treated mdx and 12 months-old AAV9/βARKct-treated Sgcd<sup>-/-</sup> mice compared to untreated control mice ( $n = 8$  each animal group) (student's *t*-test, \* $p < 0,05$ ).

with increased enddiastolic posterior-wall-thickness (PWTd) in echocardiography, significant increase of cardiac cell size as assessed by cell surface area measurements and a higher heart-to-body weight ratio (mg/g) compared to age-matched wild type mice (Table 1 and Fig. 3). Chronic gene transfer of AAV9/βARKct into mdx mice resulted in an attenuation of PWTd, smaller cardiomyocytes and lower heart-to-body-weight ratio compared to untreated mdx mice (Fig. 3A–C).

In contrast to mdx mice, Sgcd<sup>-/-</sup> mice revealed subtle left ventricular dilatation with slightly increased EDV and discrete myocardial hypertrophy as indicated by a higher heart-to body weight ratio and slightly increased cardiomyocyte size compared to wild type controls (Table 1 and Fig. 3).

Treatment with AAV9/βARKct had no positive effects on myocardial hypertrophy in Sgcd<sup>-/-</sup> mice. Interestingly, we found subtle hints for aggravation of cardiac hypertrophy in AAV9/βARKct-treated Sgcd<sup>-/-</sup> mice demonstrated by an increased size of cardiomyocytes, whereas heart-to body ratio and PWTd remained unchanged (Fig. 3). However, βARKct-gene transfer has no influence on fibrosis in both mdx and Sgcd<sup>-/-</sup> mice (supplemental Figure 3).

### 3.4. Inhibition of GRK2 by βARKct gene transfer is effective in mdx but not in Sgcd<sup>-/-</sup> mice

Upregulation of GRK2 plays a key role in the pathogenesis of heart failure. Therapeutic inhibition through βARKct

had positive inotropic effects and reversed adverse ventricular remodelling in heart failure. Immunoblot analysis shows upregulated cardiac GRK2 protein levels in hearts of mdx mice compared to wild type controls whereas GRK2-protein levels in Sgcd<sup>-/-</sup> mice are not different from wild type littermates (Fig. 4). In mdx mice, treatment with βARKct leads to a reduction of cardiac GRK2-protein levels comparable to those of C57Bl/10 control mice. AAV9/βARKct-treated Sgcd<sup>-/-</sup> mice show cardiac GRK2- protein levels similar to untreated Sgcd<sup>-/-</sup> mice and C57Bl/6 controls. This finding further underlines the hypothesis of substantial differences in the pathogenesis of cardiomyopathies in the two mouse models and may explain stronger beneficial effects of AAV9/βARKct-treatment in mdx mice compared to Sgcd<sup>-/-</sup> mice. These data suggests that βAR signalling is not a driving force in this cardiomyopathy, which is consistent also with the lack of an effect of β-blockers in this model.

To further analyse effects of AAV9/βARKct-treatment on βAR-signalling we measured cardiac mRNA expression of the stimulatory G protein alpha subunit G<sub>s</sub>α. As expected for heart failure models, stimulatory G<sub>s</sub>α mRNA is down-regulated in hearts of mdx and Sgcd<sup>-/-</sup> mice compared to wild type controls (supplemental Figure 4A). AAV9/βARKct-treatment had no effect on G<sub>s</sub>α mRNA expression in both mouse models suggesting that there is no significant influence of AAV9/βARKct on adenylyl cyclase (AC)-activity. Furthermore, downstream major components of AC-mediated β-

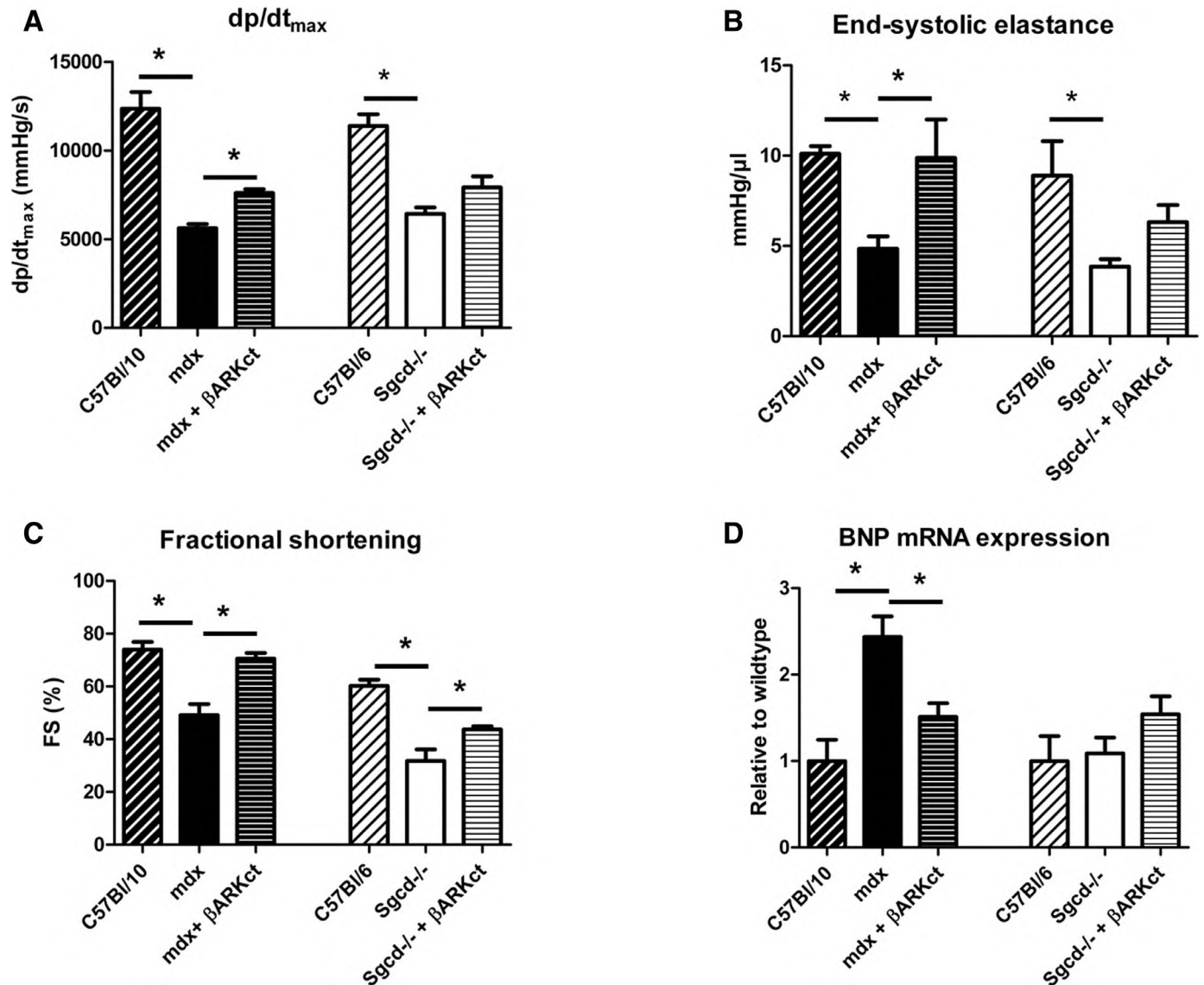


Fig. 2. Cardiac haemodynamics after treatment with AAV9/βARKct.

Animal numbers see Table 1. In 12-month-old *mdx* and *Sgcd*<sup>-/-</sup> mice impaired myocardial contractility was assessed by measurement of (A) dp/dt<sub>max</sub>, (B) end-systolic elastance (Ees) and (C) fractional shortening (FS) by PV-loops and echocardiography. (A)–(C) Treatment with AAV9/βARKct lead to improved myocardial contractility with higher dp/dt<sub>max</sub>, Ees and increased FS in *mdx* mice. In *Sgcd*<sup>-/-</sup> mice the invasively measured parameters for myocardial contractility dp/dt<sub>max</sub> and Ees remain unchanged. However, FS in echocardiography is increased in both *mdx* and *Sgcd*<sup>-/-</sup> mice treated with AAV9/βARKct. (D) Cardiac BNP expression was significantly increased in 12 months-old untreated *mdx* mice compared to C57Bl/10 control mice (2,4(±0,2)-fold, <sup>+</sup>*p* < 0,05). Treatment with AAV9/βARKct significantly reduced cardiac BNP expression in *mdx* mice (2,4(±0,2)- vs. 1,5(±0,2)-fold). There were no differences in BNP expression between AAV9/βARKct-treated *Sgcd*<sup>-/-</sup> mice, untreated *Sgcd*<sup>-/-</sup> mice and C57Bl/6 control mice. \**p* < 0,05 one-way ANOVA (*n* = 8 each animal group for BNP analysis).

adrenergic signalling calcium handling were analysed. Levels of cardiac sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA2A) mRNA were downregulated in *mdx* and *Sgcd*<sup>-/-</sup> mice compared to wildtype mice, however did not change after AAV9/βARKct-treatment (supplemental Figure 4B).

### 3.5. AAV9/ βARKct reduces activation of NF-kappaB in hearts of *mdx* mice

Several studies have indicated that activation of the transcription factor nuclear factor kappa-light-chain-enhancer of

activated B cells (NFκB), besides modulating inflammation, may play an important role in the development of cardiac hypertrophy and remodelling [24,25]. Similar to cardiac GRK2 protein expression, immunoblot analysis reveals upregulated cardiac protein levels of phosphorylated p65 (pp65) in hearts of *mdx* mice compared to wildtype controls (Fig. 5). In contrast, pp65-protein levels in *Sgcd*<sup>-/-</sup> mice are not different from wildtype littermates. In accordance to reduced myocardial hypertrophy, AAV9/ βARKct-treated *mdx* mice showed significantly lower cardiac pp65 protein levels compared to untreated mice. In conjunction with increased cardiomyocyte

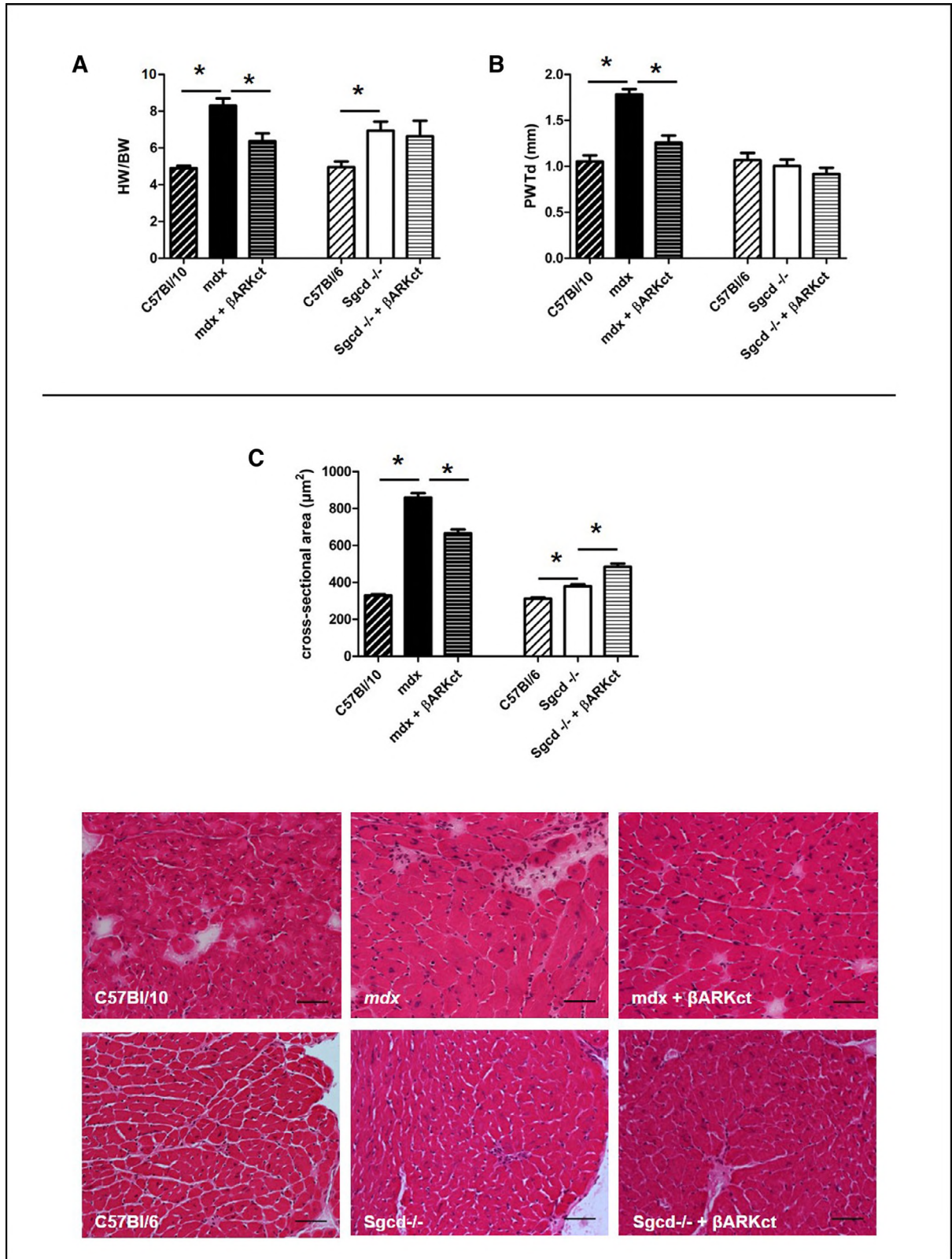


Fig. 3. Attenuation of myocardial hypertrophy in *mdx* but not *Sgcd*<sup>-/-</sup> mice after treatment with AAV9/ $\beta$ ARKct.

(A) Heart-to bodyweight ratios (HW/BW in mg/g) were increased in both 12-month-old untreated *mdx* and *Sgcd*<sup>-/-</sup> mice compared to C57Bl/10 and C57Bl/6 control mice. Treatment with AAV9/ $\beta$ ARKct attenuated the increased HW/BW in *mdx* but not *Sgcd*<sup>-/-</sup> mice. (B) Diastolic posterior wall thickness (PWTd) was significantly increased in untreated *mdx* mice and could significantly be reduced by AAV9/ $\beta$ ARKct treatment. No changes were observed in *Sgcd*<sup>-/-</sup> mice (A+B  $n = 10$  to 11). (C) Cardiomyocyte cross-sectional areas were increased in cardiomyocytes from untreated *mdx* and *Sgcd*<sup>-/-</sup> mice compared to control mice and attenuated in *mdx* but not *Sgcd*<sup>-/-</sup> mice (each group  $n = 5$ ). (D) Representative cardiac cross-sections from individual groups (20x total magnification). \* $p < 0.05$  one-way ANOVA.

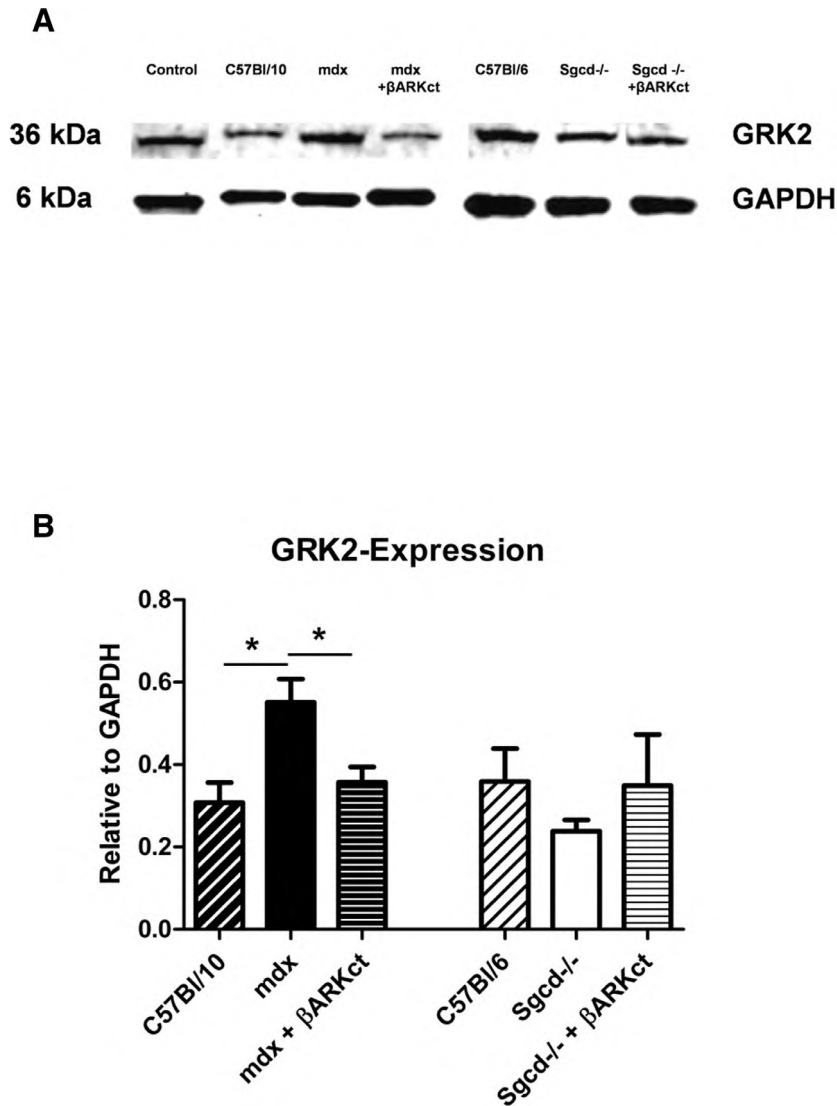


Fig. 4. Different GRK2 expression in *mdx* and *Sgcd*<sup>-/-</sup> mice.

(A) Representative immunoblots for GRK2 and GAPDH. (B) Quantification of immunoblots for GRK2 relative to GAPDH: Untreated *mdx* mice showed upregulated cardiac GRK2 protein levels compared to C57Bl/10 control mice, which were normalised by treatment with AAV9/ $\beta$ ARKct. GRK2-protein levels in untreated *Sgcd*<sup>-/-</sup> mice were not different from C57Bl/6 control mice and there were no changes after AAV9/ $\beta$ ARKct-treatment in *Sgcd*<sup>-/-</sup> mice.  $N = 5$  for all groups. \* $p < 0.05$  one-way ANOVA.

cross-sectional areas representing subtle signs of hypertrophy on the cellular level, the markedly increased pp65 protein levels in hearts of AAV9/ $\beta$ ARKct-treated *Sgcd*<sup>-/-</sup> mice compared to untreated mice, may support its potential role in modulating hypertrophy in these mice.

#### 4. Discussion

Cardiac involvement increasingly determines mortality of patients with muscular dystrophies such as DMD or sarcoglycanopathies like LGMD2F. These muscular dystrophies are characterised by an impaired mechanical interaction of the cytoskeleton and the extra-cellular matrix.  $\beta$ -blocker treatment in mouse models for DMD (*mdx*) and LGMD2F (*Sgcd*<sup>-/-</sup>) revealed conflicting results. This indicates that despite the close biochemical association between dystrophin

and the membrane-bound sarcoglycan complex, therapeutic responses to therapies may differ even between similar cardiomyopathies [7]. In this study we investigate the effectiveness of AAV-mediated cardiac gene transfer of  $\beta$ ARKct, a peptide inhibitor of GRK2, on the progression of cardiomyopathy in *mdx* and *Sgcd*<sup>-/-</sup> mice. Our data show an improvement of cardiac haemodynamics and a reduction of hypertrophy by AAV9/ $\beta$ ARKct treatment in *mdx* mice, whereas *Sgcd*<sup>-/-</sup> mice revealed a moderate improvement of left ventricular function (only fractional shortening, but not +dp/dt) and no change in compensatory hypertrophy. These findings are in line with the results observed after  $\beta$ -blocker treatment in these two models [7] and indicate that the response to  $\beta$ ARKct-treatment depends on the underlying molecular cause, activated pathways and potentially to the level of adrenergic dysregulation.

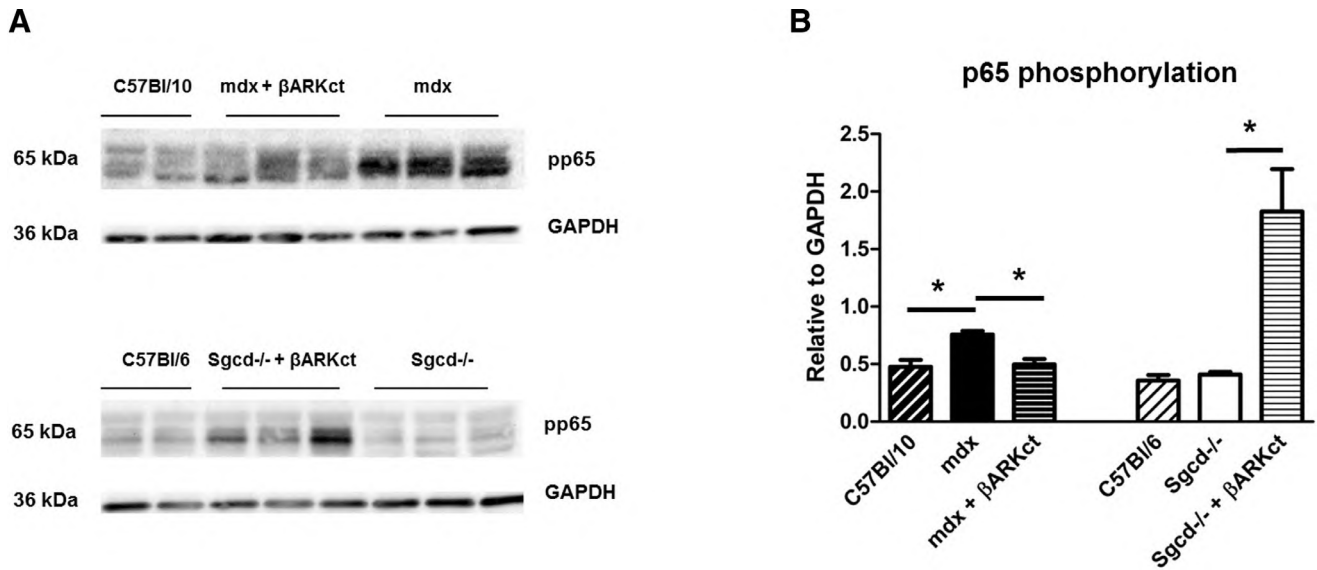


Fig. 5. AAV9/βARKct reduces activation of NF-kappaB in hearts of *mdx* mice.

(A) Representative Immunoblots for pp65 and GAPDH in heart tissue from experimental groups as indicated on the blot. (B) Quantification of immunoblots for pp65 relative to GAPDH: Untreated *mdx* mice showed upregulated cardiac pp65 protein levels compared to C57Bl/10 control mice. AAV9/βARKct-treated *mdx* mice show reduced cardiac protein levels of pp65. Pp65-protein levels in untreated *Sgcd*<sup>-/-</sup> mice were not different from C57Bl/6 control mice. AAV9/βARKct-treatment leads to an overexpression of cardiac pp65-protein levels in *Sgcd*<sup>-/-</sup> mice. *N* = 5 for all groups. \**p* < 0.05 one-way ANOVA.

In order to further explain the different response to AAV9/βARKct a closer look on its mode of action might be helpful. Overexpression of GRK2 is considered to play a key role in the pathogenesis of many forms of heart failure and its therapeutic inhibition through βARKct is a promising gene therapy strategy showing beneficial effects in several different animal models [9,13,26]. Restoration of impaired βAR-mediated contractile responsiveness in heart failure has been proposed as a major underlying molecular mechanism for the inotropic effect of βARKct. However, comprehensive cAMP-dependent downstream effects have not yet been determined [27]. Moreover, a previous in vitro study showed that the inotropic effects of βARKct in normal and diseased cardiomyocytes are independent of the classical cAMP-related pathway downstream of activated βARs supporting our finding of missing effects of βARKct on major components of cAMP-mediated calcium handling in both mouse models [28].

It has been shown that stimulation of Gβγ protein-dependent signalling inhibits L-type Ca<sup>2+</sup> channels (LLC) currents and the Gβγ sequestering peptide βARKct exerts its effects by interruption of this inhibitory pathway [28]. While LLC currents are reduced in *mdx* mice, calcium influx through LLC is even excessive in *Sgcd*<sup>-/-</sup> mice and disinhibition of LLC currents in cardiomyocytes by βARKct might even exacerbate cardiomyopathy in *Sgcd*<sup>-/-</sup> mice [29].

Besides Ca<sup>2+</sup> handling, there are further distinct molecular differences between *mdx* and *Sgcd*<sup>-/-</sup> mice. Upregulation of GRK2 was only found in *mdx* mice, a fact which may at least partially explain stronger treatment effects of AAV9/βARKct in *mdx* mice compared to *Sgcd*<sup>-/-</sup> mice. This is in line with the data from a previous study showing that beta-adrenergic response of cardiac muscle in *Sgcd*<sup>-/-</sup> mice is not altered compared to wildtype mice [30].

Another explanation for the different susceptibility to AAV9/βARKct-treatment might be well characterized differences in pathomechanisms causing cardiomyopathy in these two models. In *mdx* mice, cardiomyopathy likely develops from membrane instability during contraction with consecutive excessive influx of calcium (Ca<sup>2+</sup>) in affected cardiomyocytes [31,32]. In *Sgcd*<sup>-/-</sup> mice the integrity of the sarcoglycan complex is not critical and passive extension-induced membrane damage may not be causal [33,34]. Instead, cardiomyopathy in *Sgcd*<sup>-/-</sup> mice was explained by vascular constrictions and intermittent ischemic-like events due to vascular dysfunction associated with perturbation of the sarcoglycan-sarcospan complex (SSC) in vascular smooth muscles as well as paracrine effects from diseased cardiomyocytes [35,36].

A further difference between the two models might be the role of NFκB -signalling which plays an important role in the development of cardiac hypertrophy [25,26]. Furthermore, in earlier studies a linkage of enhanced GRK2 expression to myocardial hypertrophy in a pressure overload mouse model and transgenic mice that overexpress an amino-terminal peptide of GRK2 (βARKnt) develop cardiac hypertrophy has been shown [37,38]. A new aspect in the present study may be an interaction of GRK2 with subunits of the transcription factor NFκB-subunits in *mdx* mice, thereby promoting cardiac hypertrophy. A study by Patial et al. demonstrates that overexpression of GRK2 or GRK5 enhances tumor necrosis alpha (TNFα)-mediated NFκB-activity suggesting them as important mediators of a non-traditional NFκB-signalling pathway [39].

*Mdx* mice revealed an increased activity of NFκB (measured as phosphorylation of the subunit p65) while NFκB - activity in hearts of untreated *Sgcd*<sup>-/-</sup> mice was not altered compared to wildtype. βARKct-treated *mdx* mice were

characterised by reduced phosphorylation of the NF $\kappa$ B subunit p65 associated with improved contractility and reduced myocardial hypertrophy compared to untreated mice. These findings are consistent with a previous study reporting amelioration of pressure-overload induced cardiac hypertrophy due to cardiac specific ablation of the NF $\kappa$ B p65 subunit in mice [26]. Furthermore, it has been shown that cardiac contractile dysfunction in utrophin/dystrophin-deficient mice can be significantly improved by a peptide-based inhibition of NF $\kappa$ B [40].

AAV9/ $\beta$ ARKct treatment of *Sgcd*<sup>-/-</sup> mice was associated with increased phosphorylation of p65 and subtle signs of hypertrophy on the cellular level, which points to the complexity of pathological determinants of cardiomyopathy in different mouse models.

The increased NF $\kappa$ B -activity in *mdx* mice might be triggered by increased intracellular resting Ca<sup>2+</sup>-levels. Similar to our findings in hearts of *mdx* mice, Altamirano et al. found an elevated transcriptional activity of NF $\kappa$ B in *mdx* skeletal muscle cells and showed that a reduction of intracellular Ca<sup>2+</sup> reverses activated NF $\kappa$ B – signalling [41]. Furthermore, a reduction or inhibition of p65 was able to ameliorate pathology in skeletal muscles of *mdx* mice [42,43].

A limitation of the study could be the different timing of disease progression in *mdx* and *Sgcd*<sup>-/-</sup> mice. *Mdx* mice show a cardiac phenotype not before the age of 4 months, whereas in *Sgcd*<sup>-/-</sup> mice cardiac pathology can be found from the age of 3 months. However, *Sgcd*<sup>-/-</sup> mice show early histopathology in skeletal muscle partially already at the age of 2 weeks. It is therefore possible that  $\beta$ ARKct-treatment has been started at different stages of disease progression between the two models possibly contributing to the different effects of the therapy in these models. *Mdx* and *Sgcd*<sup>-/-</sup>-mice were bred in different genetic backgrounds and may not be directly comparable. To address these limitation *mdx* and *Sgcd*<sup>-/-</sup>-mice were compared to wildtype controls of the same background (C57Bl/10 and C57Bl/6, respectively).

We cannot exclude that systemic effects of anaesthetics may be different in the two background strains and may influence cardiac hemodynamics in pressure-volume loops experiments. However, doses used were similar between the groups and heart rates, representing a similar level of cardiac activation, were not different between the two strains. The trend to lower heart rates in untreated cardiomyopathic *mdx* and *Sgcd*<sup>-/-</sup>-mice rather reflects impaired cardiac function and increased vulnerability to external stress factors in these mice.

Taken together, chronic inhibition of GRK2 through AAV9-mediated cardiac overexpression of  $\beta$ ARKct is effective in the *mdx* mouse model of DMD and represents a promising tool to treat cardiomyopathies. However, effectiveness of this therapy may vary between different genetic causes of heart failure, which may involve different pathways and levels of adrenergic dysregulation including GRK2 upregulation. As shown here, even despite the tight functional association of dystrophin and  $\delta$ -sarcoglycan, the pathomechanisms of cardiomyopathy in *Sgcd*<sup>-/-</sup> and *mdx* mice are distinctly

different, which may explain the less beneficial effectiveness following chronic  $\beta$ ARKct expression in the heart of *Sgcd*<sup>-/-</sup> mice.

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## Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nmd.2018.12.006.

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