Transgenic simulation of human heart failure-like L-type Ca\(^{2+}\)-channels: implications for fibrosis and heart rate in mice

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**Aims** Cardiac L-type Ca\(^{2+}\)-currents show distinct alterations in chronic heart failure, including increased single-channel activity and blunted adrenergic stimulation, but minor changes of whole-cell currents. Expression of L-type Ca\(^{2+}\)-channel \(\beta\)-subunits is enhanced in human failing hearts. In order to determine whether prolonged alteration of Ca\(^{2+}\)-channel gating by \(\beta\)-subunits contributes to heart failure pathogenesis, we generated and characterized transgenic mice with cardiac overexpression of a \(\beta_{2a}\)-subunit or the pore Ca\(_{1,2}\) or both, respectively.

**Methods and results** Four weeks induction of cardiac-specific overexpression of rat \(\beta_{2a}\)-subunits shifted steady-state activation and inactivation of whole-cell currents towards more negative potentials, leading to increased Ca\(^{2+}\)-current density at more negative test potentials. Activity of single Ca\(^{2+}\)-channels was increased in myocytes isolated from \(\beta_{2a}\)-transgenic mice. Ca\(^{2+}\)-current stimulation by 8-Br-cAMP and okadaic acid was blunted in \(\beta_{2a}\)-transgenic myocytes. In vivo investigation revealed hypotension and bradycardia upon Ca\(_{1,2}\)-transgene expression but not in mice only overexpressing \(\beta_{2a}\). Double-transgenic showed cardiac arrhythmia. Intersitial fibrosis was aggravated by the \(\beta_{2a}\)-transgene compared with Ca\(_{1,2}\)-transgene expression alone. Overt cardiac hypertrophy was not observed in any model.

**Conclusion** Cardiac overexpression of a Ca\(^{2+}\)-channel \(\beta_{2a}\)-subunit alone is sufficient to induce Ca\(^{2+}\)-channel properties characteristic of chronic human heart failure. \(\beta_{2a}\)-overexpression by itself did not induce cardiac hypertrophy or contractile dysfunction, but aggravated the development of arrhythmia and fibrosis in Ca\(_{1,2}\)-transgenic mice.

1. Introduction
Calcium entry through L-type channels, multimers of Ca\(_{1,2}\)-, \(\beta\)-, and \(\alpha_{2\delta}\)-subunits, triggers cardiac contraction. There is consensus that whole-cell current as readout of Ca\(^{2+}\)-channel activity is largely unchanged in heart failure.\(^1,2\) However, our observation of a huge, counterintuitive increase of single-channel activity in ventricular myocytes from human failing heart led to a more cautious interpretation and a more complex view of Ca\(^{2+}\)-channel remodelling in heart failure,\(^3-5\) involving perhaps regulatory or structural alterations which escape detection by standard whole-cell analysis.

\(\beta_2\)-subunits confer the high-activity gating pattern typical for failing human heart,\(^6\) in rodent cardiac cells\(^2\) and recombinant systems,\(^8,9\) respectively. Recently, we provided proof-of-concept that the single-channel phenotype of human heart failure can be reproduced in mouse heart by short-term overexpression of a \(\beta_{2a}\)-subunit (tg \(\beta_{2a}\)).\(^9\) Acute \(\beta_{2a}\)-subunit overexpression has been shown to cause cell death\(^7\) and apoptosis\(^10\) in isolated myocytes and lifetime overexpression in a transgenic mouse model resulted...
in hypertrophy, failure, and premature death, albeit under conditions where whole-cell current density—in contrast to human heart failure—was vastly elevated.\(^{11}\) This finding may just confirm that chronic increases in calcium load through elevated L-type channel expression and function in the sarcoclemma can lead to hypertrophy and failure.\(^{12}\) Conversely, and of more pathophysiological relevance, in vivo knockdown of β2-subunits in a rat model of aortic banding reduced Ca\(^{2+}\)-current density and attenuated cardiac hypertrophy.\(^{13}\) Although considered to serve as a functional protein kinase A substrate, \(^{14}\) β2-subunit overexpression was recently reported to blunt adrenergic regulation of Ca\(^{2+}\)-currents in cardiomyocytes,\(^{15}\) mimicking another feature of human heart failure.\(^{6,16,17}\)

In order to further elucidate the putative role of β2-subunits in heart failure,\(^{13}\) we simulated human pathophysiology with respect to Ca\(^{2+}\)-channel function, using a genetic mouse approach. Cardiac overexpression of β2a-subunits was induced \textit{in vivo} for a period of 4 weeks in mice through continuous application of the selective inducing drug tefubenozide via osmotic pumps. Our aim was to elucidate whether the functional and structural Ca\(^{2+}\)-channel remodelling—as it occurs in human heart failure—will by itself provoke pathophysiological consequences on cardiac morphology, contraction, or rhythm. To compare, contrast, and combine this strategy with a more conventional approach—an overt and long-term increase in Ca\(^{2+}\)-current—we also examined the effects of stable cardiac overexpression of the Ca\(_{1.2}\) pore-subunit (tg Ca\(_{1.2}\)), a model of calcium-dependent cardiac hypertrophy and failure.\(^{18,19}\) We investigated animals at an age of 4–5 months, i.e. at an age where tg Ca\(_{1.2}\) not yet develop significant hypertrophy or increased activity (‘heart-failure phenotype’) of ventricular L-type Ca\(^{2+}\)-channels, respectively.\(^{9,19}\)

2. Methods

2.1 Mouse models

All procedures complied with respective laws and local regulations (no IRB approval required) and conform with 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). The generation of transgenic mice with inducible, cardiac-specific expression of rat β\(_{2a}\) Ca\(^{2+}\)-channel subunits (tg β\(_{2a}\). Figure 1A) has been described previously.\(^6\) Transgenic mice with cardiac expression of the human pore-forming Ca\(^{2+}\) subunit (tg Ca\(_{1.2}\)) are described elsewhere.\(^{18,19}\) All strains were maintained on an FVB/N background with wild-type littermates serving as control. Experiments were performed with mice of both sexes at an age of 4–5 months. Animals (including wild-type mice) were used for experiments 26–28 days after implantation of osmotic minipumps, which contained the inducing drug tefubenzide (kind gift of Dow AgroSciences, Munich, Germany).

2.2 Implantation of osmotic pumps

Mice were anaesthetized by intraperitoneal injection of S-ketamin (Ketanest \(^5\)\(^g\), Pfizer; 125 mg/kg) and medetomidine (Domitor\(^\circ\)\(^m\), Pfizer; 250 μg/kg). Osmotic pumps (Alzet\(^\circ\)\(^m\), Model 2004) were prepared as described by the producer’s instructions. During operation, pumps were placed subcutaneously at the animals’ back with a connected catheter placed intraperitoneally.

2.3 Isolation of ventricular myocytes

Single-ventricular myocytes were isolated from murine hearts by enzymatic dissociation using the method described earlier.\(^5\) In brief, hearts were perfused with a collagenase solution (Worthington type I and II, 75 U/L; CellSystems, St Katharinen, Germany) in a Langendorff setup at 37°C and subsequently cut into small chunks. Myocytes were harvested by pouring the suspension through cheesecloth. Viability of yielded cells did not differ between the several genotypes.

2.4 Electrophysiological investigation of single-ventricular myocytes

All experiments were performed at room temperature. Single-channel recordings were performed by using the cell-attached configuration of the patch-clamp technique.\(^9\) Cells were placed in disposable Petri-dishes containing 3 mL of a high-potassium solution (mM: 25 KCl, 120 K-glutamate, 2 MgCl\(_2\), 10 HEPES, 2 EGTA, 10 dextrose, 1 CaCl\(_2\), 1 Na\(_2\)-ATP; pH 7.3 with KOH). Patch pipettes (borosilicate glass, 6–8 MΩ) were filled with pipette solution (mM: 70 BaCl\(_2\), 110 sucrose, and 10 HEPES; pH 7.4 with TEA-OH). Ba\(^{2+}\)-currents were elicited by voltage steps (150 ms, 1.66 Hz) from −100 to +10 mV or +20 mV, respectively (≥180 sweeps per experiment). Data were sampled at 10 kHz and filtered at 2 kHz (3 dB, four-pole Bessel) by using an Axopatch 1D amplifier (Axon Instruments, Foster City, USA). PCLAMP software (CLAMPEX, FETCHAN, and PSTAT 6) was used for data acquisition and analysis (Axon Instruments). When necessary, analysis of single-channel recordings was performed with correction for multiple channels in a patch.\(^6\)

Whole-cell recordings were obtained as described.\(^20\) We used an external solution containing (mM): 137 NaCl, 5.4 CsCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 dextrose, 10 HEPES (pH 7.4 with NaOH). Pipettes (2–4 MΩ borosilicate glass) were filled with (mM): 120 CsCl, 1 MgCl\(_2\), 5 HEPES, 10 EGTA, 4 Mg-ATP (pH 7.2 with CsOH). Giga-Ohm seals (2–5 GΩ) were formed by gentle suction. Before series resistance compensation, membrane capacitance was measured by means of fast depolarizing ramp pulses (from −40 to −35 mV; 5 ms duration) at the beginning of each experiment. Membrane currents were low-pass filtered at 2 kHz. Starting from a holding potential of −80 mV cell membranes were depolarized to −40 mV for 50 ms to inactivate Na\(^+\)- and T-type Ca\(^{2+}\)-channels. To obtain current–voltage relationship Ca\(^{2+}\)-currents were then elicited by 150 ms depolarizing pulses at 10 mV steps (range −30 to +50 mV). Analysis of whole-cell Ca\(^{2+}\)-current kinetics was performed as described before.\(^20\) In brief, steady-state activation and inactivation curves were fitted using a Boltzmann equation (Microcal Origin\(^\circ\)\(^m\) 6.0 software).

2.5. Haemodynamic measurements

Electrocardiograms in conscious, unrestrained mice were recorded by telemetry (DSI, Transoma Medical, USA, TA10EA-F20 transmitters) more than 7 days after implantation of transducers as previously described.\(^21\) Thirty seconds recordings were obtained every 10 min at a sampling frequency of 2 kHz. Periods when mice were at rest (activity = 0) were analysed using the ECG module of ADI Chart v5.5.5 (AD Instruments, Castle Hill, Australia). Aortic and left-ventricular catheterization was performed using a 1.4F pressure-volume catheter. Data were recorded and analysed with Chart v5.4 as described before.\(^22\) Mice were anaesthetized with isoflurane (2 vol% in O\(_2\)) and their body temperature was kept at 37°C.

2.6 Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qPCR) was performed as described previously.\(^23\) Briefly, ventricles were rapidly removed from decapitated mice, separated from atria, and cut transversally. The ventricle basis was immediately frozen in liquid
nitrogen. RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). A total of 1 \( \text{mg} \) RNA was DNase treated and reverse transcribed according to the manufacturer's protocol by means of the QuantiTect Reverse Transcription Kit (Qiagen). qPCR reactions were run in triplicate on an MX3000P detector (Stratagene, Amsterdam, The Netherlands) using Quantitect SYBR Green mastermix (Qiagen). Cycling conditions: 15 min polymerase activation at 95\(^\circ\)C and 40 cycles at 95\(^\circ\)C for 15 s, at 58\(^\circ\)C for 30 s, and at 72\(^\circ\)C for 30 s. Gene expression was normalized to \( \beta\)-actin values.

2.7 Cardiac histology

After haemodynamic evaluation or ECG recording, hearts were fixed with 4\% paraformaldehyde in phosphate-buffered saline, embedded in paraffin, cut in 3 \( \mu \text{m} \) slices, and stained either with haematoxylin–eosin or Sirius-red as described before.\(^{22}\) To allow for determination of myocyte cross-sectional area, heart sections were additionally stained with a green fluorescent dye coupled to wheat germ agglutinin (WGA, Alexa Fluor\textsuperscript{®} 488 conjugate, Invitrogen) and nuclei were counterstained with propidium iodide. Left-ventricular myocyte cross-sectional areas were analysed by computer-assisted morphometry\(^{22}\) using a Zeiss AxioCam MRc camera on an Axiovert 200 inverted microscope with \( \times 40 \) magnification and the AxioVision Rel. 4.5 software. Quantification of fibrosis revealed by Sirius-red staining was performed by measuring the extent of fibrotic area in heart cross-sections.

2.8 Data analyses and statistics

Data are presented as means \( \pm \) SEM. To evaluate effects of \( \beta_{2a} \) overexpression, the following groups were compared using Student's \( t \)-tests unless otherwise indicated: \( \text{tg}_{\text{ind}} \beta_{2a} \) vs. wild-type, \( \text{tg CaV1.2} \text{tg}_{\text{ind}} \beta_{2a} \) vs. \( \text{tg CaV1.2} \), and \( \text{tg CaV1.2} \) vs. wild-type. \( P \)-values < 0.05 were considered significant.
3. Results

3.1 Transgenic mouse models

We studied three transgenic mouse-lines expressing distinct \( \text{Ca}^{2+} \)-channel subunits specifically in cardiac myocytes in vivo (Figure 1A). Cardiac myocyte-specific expression of a rat \( \text{Ca}^{2+} \)-channel \( \beta_2a \) subunit (tgind \( \beta_2a \)) was induced by specific activation of a hybrid drosophila/bombay ec dysone receptor by tebufenozide.\(^9,24\) The second mouse-line, tg CaV1.2, exhibits a constitutive cardiac overexpression of the human L-type \( \text{Ca}^{2+} \)-channel pore (CaV1.2) and represents an established model of cardiac hypertrophy.\(^18,19\)

Mice combining inducible cardiac expression of the rat \( \beta_2a \)-subunit with constitutive cardiac overexpression of the human CaV1.2 subunit were obtained by cross-breeding (tg CaV1.2 x tgind \( \beta_2a \)). Wild-type littermates from either breeding of FVB/N and tg CaV1.2, ortgind \( \beta_2a \) or FvBN mice served as controls.

For the present study, mice of all four genotypes received the ec dysone receptor agonist tebufenozide. Treatment for 4 weeks resulted in cardiac-specific overexpression of \( \beta_2a \) subunits in tgind \( \beta_2a \) at the mRNA level (see Supplementary material online, Figure S1A). In our previous study we had shown that \( \beta_2a \) overexpression also occurred at the protein level after short-term induction.\(^9\) Human CaV1.2 mRNA was exclusively detected in tg CaV1.2 hearts (see Supplementary material online, Figure S1B). In order to test whether the transgenes affected expression of the endogenous CaV1.2- channel subunits, mRNA from wild-type and transgenic hearts was analysed by quantitative RT-PCR using murine-specific primers (see Supplementary material online, Table S1). Importantly, expression of the endogenous CaV1.2- or \( \beta \)-subunits did not differ between genotypes (see Supplementary material online, Table S2).

3.2 Whole-cell \( \text{Ca}^{2+} \) currents

Cardiac overexpression of the \( \beta_2a \)-subunit for 4 weeks led to an increase of the \( \text{Ca}^{2+} \)-current density at its maximum (wild-type: \(-10.3 \pm 0.9 \) pA/pF, \( n = 9 \) vs. \( \text{tgind} \beta_2a \): \(-12.7 \pm 0.7 \) pA/pF, \( n = 13 \); \( P < 0.05 \)) and at negative test voltages (Figure 1B), while current density at potentials positive to 0 mV suggested rather unchanged maximum conductance \( G_{\text{max}} \). A more pronounced elevation across the whole voltage range (Figure 1C) was observed on top of a constitutive cardiac overexpression of CaV1.2 (tg CaV1.2: \(-12.5 \pm 1.0 \) pA/pF, \( n = 12 \) vs. \( \text{tgind} \beta_2a \): \(-16.6 \pm 1.1 \) pA/pF, \( n = 6 \); \( P < 0.05 \)). \( \beta_2a \)-overexpression shifted \( \text{I–V} \) curves, derived steady-state activation curves \( (V_{0.5} \) values of activation for wild-type: \(-10.6 \pm 1.0 \) mV, \( n = 12 \) vs. \( \text{tgind} \beta_2a \): \(-16.9 \pm 1.0 \) mV, \( n = 14 \); \( P < 0.01 \)); \( \text{tg CaV1.2} \): \(-13.6 \pm 1.1 \) mV, \( n = 13 \) vs. \( \text{tg CaV1.2} \times \text{tgind} \beta_2a \): \(-15.2 \pm 1.0 \) mV, \( n = 6 \); \( P = 0.36 \), Figure 1D, E) and inactivation curves \( (V_{0.5} \) values of inactivation for wild-type: \(-16.8 \pm 1.0 \) mV, \( n = 10 \) vs. \( \text{tgind} \beta_2a \): \(-23.2 \pm 1.1 \) mV, \( n = 14 \); \( P < 0.01 \)); \( \text{tg CaV1.2} \): \(-20.4 \pm 0.9 \) mV, \( n = 16 \) vs. \( \text{tg CaV1.2} \times \text{tgind} \beta_2a \): \(-23.4 \pm 1.3 \) mV, \( n = 7 \); \( P = 0.06 \), Figure 1D, E) towards more negative membrane potentials. Cell capacitance was not different between genotypes (data not shown). Taken together, long-term overexpression of cardiac \( \beta_2 \)-subunits alone changed whole-cell \( \text{Ca}^{2+} \)-currents in a manner resembling findings from human failing myocardium.\(^1,16\)

3.3 Activity of single ventricular L-type \( \text{Ca}^{2+} \)-channels

Single-channel recording revealed increased activity after maintained cardiac \( \beta_2 \)-overexpression for 4 weeks (Figure 2A). This was the case for \( \beta_2 \)-overexpression alone (wild-type: \( \lambda_{\text{peak}} \) = 30 ± 7 fA, \( n = 5 \) vs. \( \text{tgind} \beta_2a \): \( \lambda_{\text{peak}} \) = 72 ± 21 fA, \( n = 6 \); \( P = 0.08 \)), and against the CaV1.2-transgenic background (tg CaV1.2: \( \lambda_{\text{peak}} \) = 20 ± 4 fA, \( n = 8 \) vs. \( \text{tg CaV1.2} \times \text{tgind} \beta_2a \): \( \lambda_{\text{peak}} \) = 40 ± 4 fA, \( n = 14 \); \( P < 0.01 \)). This increase was mainly due to significantly enhanced open probability and fraction of active sweeps (Figure 2B, D). In accordance with the leftward shift of whole-cell current activation, effects of \( \beta_2 \)-overexpression on single-channel gating were more pronounced at +10 mV compared with +20 mV (Figure 2B–E vs. Supplementary material online, Figure S2A–D). In summary, cardiac overexpression of \( \beta_2 \)-subunits caused L-type \( \text{Ca}^{2+} \)-channel properties similar to those obtained with myocytes from human failing ventricle.\(^6\)

3.4 Effect of \( \beta_2 \)-subunit overexpression on cAMP-dependent modulation of whole-cell \( \text{Ca}^{2+} \) currents

To examine phosphorylation-dependent channel modulation, we tested the effects of 8-Br-cAMP (1 mM) and the phosphatase inhibitor Okadaic acid (1 \( \mu \)M) on whole-cell currents in ventricular myocytes from wild-type and \( \text{tgind} \beta_2a \) mice (see Supplementary material online, Figure S3). Here, basal levels were only slightly higher in \( \text{tgind} \beta_2a \) myocytes (\(-9.5 \pm 2.9 \) pA/pF vs. wild-type: \(-7.5 \pm 1.6 \) pA/pF; \( P > 0.05 \), \( n = 5–6 \)). Significant cAMP-dependent stimulation was evident in wild-type myocytes only (wild-type: \(158 \pm 13 \)%; \( n = 6 \), \( P < 0.05 \); \( \text{tgind} \beta_2a \): \(116 \pm 9 \% \), \( n = 5 \), \( P = 0.1 \)). Hence, \( \beta_2 \)-subunit expression resembled another important hallmark of human heart failure\(^6,16\) that also has been shown for CaV1.2-transgenic mice.\(^12\)

3.5 Haemodynamic function of transgenic mice during anaesthesia

The effects of transgenic expression of \( \beta_2 \) and CaV1.2 subunits on haemodynamic function were assessed by left-ventricular microtip catheterization during isoflurane anaesthesia (Figure 3). Mice with a single transgene expressing only the hybrid drosophila/bombay ec dysone receptor (\( \alpha \)-MHV VgBnECR) did not differ in their haemodynamic function or cardiac structure from non-transgenic littermates (data not shown). Expression of the inducible \( \beta_2a \)-subunit did not affect aortic blood pressure when compared with either wild-type or \( \text{tg CaV1.2} \) animals (Figure 3A). Systolic and diastolic aortic pressures were significantly lower in \( \text{CaV1.2} \) transgenic mice when compared with wild-type mice (Figure 3A). No significant differences in left-ventricular contractility (dp/dt\(_{\text{max}} \)) were detected between wild-type and transgenic mice (Figure 3B). However, LV relaxation (dp/dt\(_{\text{min}} \)) was significantly reduced in CaV1.2 transgenic mice (Figure 3C). Somewhat unexpected, resting heart rate was only 408 ± 20 min\(^{-1} \) in tg CaV1.2 mice as opposed to 551 ± 20 min\(^{-1} \) in wild-type mice (\( P < 0.01 \), Figure 3D). Moreover, during aortic and left-ventricular catheterization, irregularities in cardiac rhythm were observed in double-transgenic but not in control or
single transgenic mice (Figure 4B, C). Aortic pressure recording revealed $35 \pm 14$ arrhythmic b.p.m. in double-transgenic mice as opposed to 0.2 arrhythmic b.p.m. in tg CaV1.2 mice ($P < 0.01$, Figure 4C).

### 3.6 Electrocardiography in awake animals

In order to test whether bradycardia and arrhythmia were also present in awake, freely moving animals, telemetric ECG transducers were implanted and electrocardiograms were recorded for successive days (Figure 4A, D, E). All four mouse strains showed expected diurnal heart-rate variations (data not shown) and tg CaV1.2 mice exhibited significant bradycardia during night and daytime (Figure 4E). Complementing our catheter data, episodes of arrhythmic beats were observed in 6/10 double-transgenic mice but only in 1/11 tg CaV1.2 animals (Figure 4D). No such rhythm disturbances were seen in wild-type or tgind β2a mice. Further ECG analysis revealed ventricular and supraventricular extrasystoles in tg CaV1.2 x tgind β2a mice (Figure 4A). Note, that none of these mice had atrial fibrillation and PR interval was unaffected (tg CaV1.2 x tgind β2a $32.1 \pm 2.0$ ms vs. wild-type $30.0 \pm 0.9$ ms, $P = 0.35$).
3.7 Cardiac histopathology and cardiac morphological parameters

Transgenic mice overexpressing CaV1.2, β2a or both Ca2+-channel subunits did not display any signs of left-ventricular hypertrophy at 4–5 months of age (Figure 5A–C). Ventricle-to-body-weight (Figure 5A) or ventricle-weight-to-tibia-length ratios (data not shown) did not differ between transgenic strains and wild-type mice. Similarly, wheat germ agglutinin (WGA) staining of mid-ventricular transverse sections (Figure 5B) to determine single cardiomyocytes’ cross-sectional area did not reveal significant differences between groups (Figure 5C). Nppa (atrial natriuretic peptide) expression, however, which is a sensitive and early marker for cardiac load and myocyte hypertrophy, was significantly upregulated 5.0-fold and 4.4-fold in ventricles of tg CaV1.2 and tg CaV1.2/C2tgindβ2a mice compared with wild-type, respectively (Figure 5D).

Furthermore, transgenic tg CaV1.2 and tg CaV1.2 × tββ2a mice developed pronounced cardiac fibrosis (Figure 6). Interstitial fibrotic area in mid-ventricular transverse sections was increased by 120% in tg CaV1.2 and by 231% in tg CaV1.2 × tββ2a mice compared with wild-type (Figure 6B). Left-ventricular fibrosis was significantly aggravated in double-transgenic mice when compared with wild-type CaV1.2 mice (tg CaV1.2 × tββ2a 8.2 ± 1.2% vs. tg CaV1.2 5.4 ± 0.6%). However, no significant changes in fibrotic area were observed by overexpression of the β2a subunit alone (Figure 6B). mRNA expression of connective tissue growth factor (Ctgf), a marker of fibrosis, was significantly enhanced in both tg CaV1.2 and tg CaV1.2 × tββ2a mice compared with wild-type or tββ2a animals (Figure 6C), respectively.

4. Discussion

Activity of single-ventricular L-type Ca2+-channels is significantly increased in human heart failure.6 Subtle alterations of Ca2+-current biophysics and pharmacology were also reported at the whole-cell level.16,17 These data are consistent with—though not proof of—the idea that Ca2+-current in human heart failure is carried by a reduced number of channels, each individually contributing more current at the expense of a reduced regulatory bandwidth. In order to further elucidate the pathophysiological relevance of the Ca2+-channel β2-subunit overexpression that we recently found in human heart failure,9 we now used an induction strategy with continuous administration of the inducing agent tebufenozide for 4 weeks, allowing for examination of long-term effects in vivo. It is important to emphasize that the continued induction of only the β2-subunit
transgene was totally sufficient to resemble the $\text{Ca}^{2+}$-channel phenotype of human heart failure:

- little change (even unchanged at positive test potentials) of whole-cell current density,\textsuperscript{1,2} 
- leftward shift of voltage-dependent activation,\textsuperscript{16} 
- marked enhancement of single-channel activity,\textsuperscript{6} and 
- blunting of the $\text{Ca}^{2+}$-current response towards phosphor-yating conditions.\textsuperscript{6,16}

These findings do not resolve the algebraic conundrum why in failing heart single channels—at least those located at the surface sarcolemma—are twice as active as would be predicted from largely unchanged whole-cell currents, with no direct evidence of a reduced expression of

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**Figure 4** Arrhythmia in transgenic mice expressing $\beta_2a$ and $\text{Ca}_{1.2} \text{Ca}^{2+}$-channel subunits. (A and B) Original traces of recordings obtained by ECG telemetry in awake, freely moving mice (A) from wild-type (upper panel) and double-transgenic mice (lower panels) or by microtip catheterization in anaesthetized animals (B), respectively. (A) In case of wild-type mice (top row) we observed sinus rhythm as displayed. Tg $\text{Ca}_{1.2} \times \text{tgind } \beta_2a$ mice showed several forms of rhythm disturbances including supraventricular extrasystoles (second row, SV), ventricular extrasystoles (third row, V), atrio-ventricular block (fourth row), and sinus pause/sinus bradycardia (bottom row). (B) Catheter data revealed the haemodynamic relevance of rhythm disturbances in double-transgenic mice. (C) The incidence of arrhythmic beats during microtip catheterization was significantly increased in $\text{tg Ca}_{1.2} \times \text{tgind } \beta_2a$ mice ($**P < 0.01$). No arrhythmic beats were detected in wild-type or $\text{tgind } \beta_2a$ mice. (D) Overall, arrhythmic episodes during ECG or catheterization were only seen in 1/11 (9.1%) tgs $\text{Ca}_{1.2}$, but in 6/10 (60%) double-transgenic mice ($**P < 0.01$; $\chi^2$ test). (E) Heart rate as determined by ECG telemetry in awake animals was significantly lowered in tgs $\text{Ca}_{1.2}$ mice ($n = 5–9$ per genotype, 3/5 examined tgs $\text{Ca}_{1.2}$ mice did not receive tebufenozide, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).
channel pore protein. Nor do they completely elucidate the mechanistic basis of hyperactivity of single channels (hyperphosphorylation, change in subunit isoform composition, or a combination of both). Our data do, however, prove that the entire spectrum of functional Ca\(^{2+}\)-channel remodelling as it occurs in human heart failure can be reproduced by continuing overexpression of a \(b_2\)-subunit alone. This serves as the basis to address the important question whether \(b_2\)-subunit overexpression is an adaptive or maladaptive phenomenon, and—consequently—whether interference with structural remodelling would constitute not only a technically feasible but also a therapeutically promising approach.

In vivo overexpression of \(b_2\)-subunits did not affect systolic contractile function, nor did we observe significant changes in tg Ca\(_{\,1.2}\) or double-transgenic hearts. This argues against a major adaptive role of \(b_2\)-subunit overexpression at least in young animals (4–5 months) as studied here, i.e., during a stage when heart failure is not yet developed. Lower blood pressure in tg Ca\(_{\,1.2}\) and tg Ca\(_{\,1.2}\) x \(b_\text{ind} \) mice may be explained by the significant bradycardia, as left ventricular \(dp/dt\text{max}\) was not altered in these mice. In this context, it is intriguing to speculate that reduced ventricular relaxation in Ca\(_{\,1.2}\) transgenic mice may be due to diastolic dysfunction associated with ventricular interstitial fibrosis. Based on these haemodynamic results it should be stressed that all the changes reported below are not indirectly mediated by a grossly distorted haemodynamic state.

Cardiac hypertrophy is not induced by \(b_2\)-subunit overexpression as such. This is highlighted by gross and normalized heart weight, myocyte capacitance and morphometric size, and the lack of ANP increase. Hypertrophy is a known consequence of long-term and extensive increase of Ca\(^{2+}\)-current density, regardless of whether induced by overexpression of Ca\(_{\,1.2}\) or \(b_2\)-subunits. Our results indicate that cardiac hypertrophy is not an immediate, direct consequence of Ca\(^{2+}\)-channel remodelling. It requires a timeframe of more than 4 weeks and/or a substantial increase of whole-cell Ca\(^{2+}\)-current density, making indirect mechanisms much more likely.

\(b_2\)-Subunit overexpression alone did not induce fibrosis, but Ca\(^{2+}\)-channel remodelling led to morphological changes even within a 4 week-period in apparently healthy young animals: cardiac fibrosis developing with Ca\(_{\,1.2}\) overexpression aggravates by additional \(b_2\)-subunit overexpression, along with a potentiated increase of the whole-cell Ca\(^{2+}\)-current density, making indirect mechanisms much more likely.

Figure 5 Heart weight, morphology, and atrial natriuretic peptide (Nppa) expression. (A) Ventricle-to-body-weight ratios did not differ between genotypes (n = 8–15 per genotype). (B and C) Cardiac myocyte cross-sectional area assessed by wheat germ agglutinin (WGA) staining of mid-ventricular transverse heart sections (B, representative pictures, bars: 50 \(\mu\)m) showed no differences between genotypes (n = 5–11 per genotype). (D) Nppa mRNA levels reached significant higher levels in tg Ca\(_{\,1.2}\) and tg Ca\(_{\,1.2}\) x \(b_\text{ind} \) mice when compared with wild-type or \(b_\text{ind} \) mice (n = 8–15 per genotype, ***P < 0.001).
or (human) Ca1.2-transgenic background, respectively, is due to combination of channel subunits from different species.

The one functional consequence that we can trace back directly and exclusively to long-term (or subacute) β2-subunit overexpression is the induction of resistance of channels towards cAMP-dependent stimulation. This finding deserves a detailed follow-up on its own, because a reduced bandwidth of—e.g. adrenergic—regulation is certainly an important aspect of heart-failure pathophysiology, and the Ca2+ channel may be a direct player here rather than just an innocent endpoint of a thoroughly altered signalling cascade.26 The findings of Miriyala et al.,15 together with our previous9 and present results provide a molecular basis to explain why individual Ca2+ channels in heart failure are hyperactive and resistant to stimulation by phosphorylation. It will be tempting to find out whether therapeutic reversal of Ca2+-channel remodelling can reconstitute adrenergic responses in heart-failure models.

The most prominent functional consequence of Ca2+-channel remodelling was observed with cardiac rhythm: CaV1.2 and double-transgenic animals revealed marked bradycardia under both anaesthetized (catheter data) and conscious (telemetry) conditions. Moreover, arrhythmic events were recorded when CaV1.2 (1/11 animals), but in particular when both transgenes were overexpressed (6/10 animals). Bradycardia was not associated with altered atrial morphology or atrio-ventricular conduction block.

Figure 6 Cardiac fibrosis in transgenic mice overexpressing CaV1.2 Ca2+-channel subunits. (A) Sirius-red staining of mid-ventricular cardiac sections from wild-type mice and mice overexpressing the pore-forming subunit CaV1.2, the auxiliary subunit β2a, or both subunits, respectively. Hearts of mice overexpressing CaV1.2 or additionally β2a (tg CaV1.2 x tgind β2a) displayed marked fibrosis. Inserts show heart slices stained with haematoxylin–eosin. (B) Interstitial fibrosis was determined by Sirius-red staining and is displayed as percentage of left ventricular cross-sectional area. Overexpression of CaV1.2 lead to significant increases in interstitial fibrous tissue (n = 8–16 per genotype, *P < 0.05, **P < 0.01). (C) mRNA expression of connective tissue growth factor (Ctgf) was quantified by qPCR. In cardiac ventricles of tg CaV1.2 and tg CaV1.2 x tgind β2a mice Ctgf expression was significantly increased (n = 8–15 per genotype, ***P < 0.001).
and may be a consequence of an altered adrenergic response in vivo. On the other hand, it is tempting to speculate that heartbeat irregularities may have been triggered by the gross alterations of single-channel activity induced by β₂ subunit overexpression leading to further enhanced Ca²⁺ influx via transgenic Caᵥ1.2/β₂a channels as a kind of last straw. The lack of pathology in the presence of β₂ subunit overexpression alone indicates that the β₂ subunit may be an essential but by itself not sufficient factor for disturbances in cardiac rhythm. It can be speculated that not only the amount of Ca²⁺-influx enhancement, but also the underlying mechanism (e.g. increased channel activity vs. increased channel activity plus increased channel density) is essential. The respective roles of single-channel behaviour vs. overall calcium load, as well as the cellular mechanism of arrhythmia need further study.

The short 4-week period of intervention is likewise an important strength and limitation of our present study. Although our previous study has demonstrated that induction of the β₂a transgene in vivo occurs and takes effect within 48 h, many pathophysiological sequelae may take more time to develop, and therefore longer treatment and observation periods deserve to be studied. On the other hand, the molecular mechanisms of alterations will be hard to identify as soon as complex haemodynamic and morphological changes ensue in an animal model. In this situation, specific molecular interference (e.g. by siRNA) may be a strategy superior to the transgenic approach. Though the bradycardic and the arrhythmic phenotype of our mouse strategy superior to the transgenic approach. Though the expression become potentiated by in vivo. Our data suggest that cardiac hypertrophy and contractile failure would require extended timeframes and/or an extent of overexpression of Caᵥ1.2 and/or β₂ subunit that are not pertinent to the human pathophysiology.

Supplementary material

Supplementary Material is available at Cardiovascular Research online.

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