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Cardiac-specific overexpression of caveolin-3 preserves t-tubular $I_{\text{Ca}}$ during heart failure in mice

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1 | INTRODUCTION
Excitation–contraction coupling (ECC) in cardiac myocytes is initiated by the action potential, which activates sarcolemmal L-type $\text{Ca}^{2+}$ channels (LTCCs). The consequent $\text{Ca}^{2+}$ influx ($I_{\text{Ca}}$) triggers $\text{Ca}^{2+}$ release from adjacent sarcoplasmic reticulum (SR) via $\text{Ca}^{2+}$ release channels (ryanodine receptors; RyRs). This $\text{Ca}^{2+}$-induced $\text{Ca}^{2+}$ release (Fabiato, 1985) produces local increases of cytosolic [Ca$^{2+}$] ($\text{Ca}^{2+}$ sparks'; Cheng, Lederer, & Cannell, 1993) that summate to form the cytosolic $\text{Ca}^{2+}$ transient, leading to contraction. In ventricular myocytes, $I_{\text{Ca}}$, and thus RyR activation, occurs predominantly at specialized invaginations of the sarcolemma called t-tubules (Cannell, Cheng, & Lederer, 1994; Kawai, Hussain, & Orchard, 1999; Lindner, 1957). This arrangement achieves near-synchronous $\text{Ca}^{2+}$ release (Cheng, Cannell, & Lederer, 1994), and thus contraction, throughout the cell. Relaxation occurs as cytosolic [Ca$^{2+}$] decreases, mainly due to re-uptake into the SR, but also by removal from the cell via Na$^+$–Ca$^{2+}$ exchange (Negretti, O'Neill, & Eisner, 1993).

Caveolin-3 (Cav-3) is a cholesterol-binding protein that has been implicated in the genesis of t-tubules (Parton, Way, Zorzi, & Stang, 1997) and in the localization of LTCC regulatory proteins and $I_{\text{Ca}}$ to the t-tubules (Balijepalli, Foell, Hall, Hell, & Kamp, 2006; Bryant et al., 2014). Cav-3 knock-out (KO) leads to cellular hypertrophy, t-tubule disorganization and decreased t-tubular $I_{\text{Ca}}$ density (Bryant et al., 2014; Parton et al., 1997; Stang et al., 1997; Zorzi et al., 1997). Caveolin-3 (Cav-3) is an 18 kDa protein that has been implicated in t-tubule formation and function in cardiac ventricular myocytes. During cardiac hypertrophy and failure, Cav-3 expression decreases, t-tubule structure is disrupted and excitation–contraction coupling (ECC) is impaired. Previous work has suggested that Cav-3 overexpression (OE) is cardio-protective, but the effect of Cav-3 OE on these cellular changes is unknown. We therefore investigated whether Cav-3 OE in mice is protective against the cellular effects of pressure overload induced by 8 weeks’ transverse aortic constriction (TAC). Cav-3 OE mice developed cardiac dilatation, decreased stroke volume and ejection fraction, and hypertrophy and pulmonary congestion in response to TAC. These changes were accompanied by cellular hypertrophy, a decrease in t-tubule regularity and density, and impaired local $\text{Ca}^{2+}$ release at the t-tubules. However, the extent of cardiac and cellular hypertrophy was reduced in Cav-3 OE compared to WT mice, and t-tubular $I_{\text{Ca}}$ density was maintained. These data suggest that Cav-3 OE helps prevent hypertrophy and loss of t-tubular $I_{\text{Ca}}$ following TAC, but that other factors disrupt local $\text{Ca}^{2+}$ release.
METHODS

Animals and surgical procedures

Echocardiography

Ethical approval

Myocyte isolation and detubulation

New Findings

What is the central question of this study?

What is the cellular basis of the protection conferred on the heart by overexpression of caveolin-3 (Cav-3 OE) against many of the features of heart failure normally observed in vivo?

What is the main finding and its importance?

Cav-3 overexpression has little effect in normal ventricular myocytes but reduces cellular hypertrophy and preserves t-tubular \( I_{Ca} \) but not local t-tubular \( Ca^{2+} \) release, in heart failure induced by pressure overload in mice. Thus Cav-3 overexpression provides specific but limited protection following induction of heart failure, although other factors disrupt \( Ca^{2+} \) release.

2 METHODS

2.1 Ethical approval

All animal procedures were approved by the Animal Welfare and Ethics Review Board of the University of Bristol (14/6/2016) and conducted in accordance with UK legislation (Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 incorporating European Directive 2010/63/EU); the study also complies with the ethical principles under which Experimental Physiology operates.

2.2 Animals and surgical procedures

Adult (12 week) male homozygous cardiac-specific Cav-3 OE mice, produced as described previously (Tsutsumi et al., 2008), were used. Data from these mice, bred at the University of Bristol, are compared with data obtained from wild-type (WT) littermates that had undergone either sham operation or TAC that resulted in HF. The WT data have been published previously (Bryant et al., 2018a) so that only mean data, rather than original records, are shown for the WT group, for comparison with the OE data. However, the surgical and experimental procedures were the same, and performed contemporaneously, for the WT and Cav-3 OE groups, and data were obtained using the same techniques and protocols in each group, as described below. Surgery was performed at 12 weeks of age and myocyte isolations at 20 weeks of age. Mice were kept in a temperature-controlled, enriched environment with \textit{ad libitum} access to food and water.

Eight weeks of TAC was used to produce pressure overload, since this has previously been shown to result in cardiac hypertrophy and failure (Bryant et al., 2018a; Rockman et al., 1991; Tachibana, Naga Prasad, Lefkowitz, Koch, & Rockman, 2005). Briefly, animals were anaesthetized with ketamine (75 mg kg\(^{-1}\) i.P., Zoetis UK Limited, London, UK) and medetomidine (1 mg kg\(^{-1}\) i.P., Orion Corp., FI-02200 Espoo, Finland) and given buprenorphine (0.05 mg kg\(^{-1}\) s.c., Reckitt Benkiser Health Care (UK) Ltd, Hull, UK) for pain relief; the surgical plane of anaesthesia was monitored using the limb withdrawal reflex. The aortic arch was exposed via a medial sternal thoracotomy and a silk ligature (6-0) placed between the innominate and left carotid arteries and tied round a 27G needle (0.4 mm OD). Sham animals underwent the same operation but without placement of the banding suture. Animals were maintained post-operatively for 8 weeks before use.

2.3 Echocardiography

In vivo cardiac structure and function were monitored using echocardiography. Animals were anaesthetized (isoflurane 1–3%, Merial Animal Health Ltd, Harlow, UK), heart rate was monitored, and measurements of contractile performance made from M-mode images acquired from the parasternal short axis view using a Vevo 3100 (Fujifilm VisualSonics Inc., Toronto, Ontario, Canada) and MX550D transducer.

2.4 Myocyte isolation and detubulation

Animals were killed by cervical dislocation and ventricular myocytes isolated using standard enzymatic digestion via Langendorff perfusion as described previously (Bryant et al., 2014) and used on the day of isolation. Detubulation (DT), the physical and functional uncoupling of the t-tubules from the surface membrane, was achieved using formamide-induced osmotic shock as described previously (Brette & Orchard, 2003; Brette, Komukai, & Orchard, 2002; Kawai et al., 1999); comparison of membrane capacitance and currents in intact and detubulated myocytes enables the distribution of membrane currents and current density between the t-tubule and surface membranes to be determined.
2.5 Imaging and analysis of t-tubule structure

Cell width and length were measured from brightfield images of isolated myocytes used for electrophysiology. Cell volume was calculated from these measurements as described previously (Boyett, Frampton, & Kirby, 1991).

Surface and t-tubular cell membranes were labelled by incubating cells with 5 μmol l⁻¹ di-8-ANEPPS for 10 min. Image volumes were obtained using an LSM 880 confocal microscope (Zeiss, Carl Zeiss AG, Oberkochen, Germany) in Airyscan ‘super-resolution’ mode, with a 1.2 NA, x40 water immersion objective, sampled at 40 nm in-plane and 180 nm along the optical axis. Airyscan uses a 32-channel photomultiplier tube detector that collects a pinhole-plane image at every scan position, thus improving spatial resolution. In super-resolution mode, linear deconvolution provides further improvement to achieve spatial resolution that is 1.7x that of a conventional confocal microscope. The regularity of t-tubule staining was quantified by applying a 1.2 NA, x40 water immersion objective, sampled at 40 nm in-plane and 180 nm along the optical axis. Airyscan uses a 32-channel photomultiplier tube detector that collects a pinhole-plane image at every scan position, thus improving spatial resolution. In super-resolution mode, linear deconvolution provides further improvement to achieve spatial resolution that is 1.7x that of a conventional confocal microscope.

2.6 Western blotting

Following myocyte isolation, aliquots were pelleted by centrifugation, the supernatant removed and the cell pellet snap frozen in liquid nitrogen and stored at −80°C. Once all samples had been acquired, the pellets were processed simultaneously by thawing directly into lysis buffer containing 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 1% (v/v) sodium deoxycholate, 0.1% (v/v) SDS, 1 mM phenylmethylsulfonyl fluoride, complete protease inhibitors, 8 μg ml⁻¹ calpain inhibitor I, 8 μg ml⁻¹ calpain inhibitor II, 50 mM sodium fluoride, 1 mM sodium orthovanadate, and 16 mM sodium pyrophosphate, homogenized by pipetting and incubated on ice for 15 min. After centrifugation at 13,000 g 4°C for 15 min the supernatants were collected, protein concentrations estimated using the Pierce BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA) and adjustments made to allow for equal protein loading on SDS-PAGE. Ten-microgram samples of the myocyte lysates were run on 4–15% gradient SDS-PAGE gels and transferred onto Immobilon-P membrane. Blots were probed with antibodies against Cav-3 (BD Biosciences, San Jose, CA, USA, cat. no. 610420, dilution 1:5000), junctophilin-2 (JPH-2; Thermo Fisher Scientific, Waltham, MA, USA, cat. no. 40–5300, dilution 1:500) and gyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Sigma-Aldrich, St Louis, MO, USA, cat. no. G9545, dilution 1:100,000). Protein bands were visualized and images captured using horseradish peroxidase-conjugated secondary antibodies (Promega, Madison, WI, USA; cat. no. W4011, a-rabbit HRP, dilution 1:10,000 and cat. no. W4021, a-mouse HRP, dilution 1:10,000), chemiluminescence and a G:BOX Chemi XT4 imaging system (Syngene, Cambridge, UK). Gels were first probed with the antibody to Cav-3 or JPH-2, then stripped using a commercial stripping solution (Restore™ western blot stripping buffer, Thermo Fisher Scientific) and re-probed with the loading control antibody to GAPDH, before being stripped and re-probed for JPH-2 or Cav-3. The density of the bands was measured using ImageJ and normalized to GAPDH.

2.7 I_Ca recording

Myocytes were placed in a chamber mounted on a Nikon Diaphot inverted microscope. Membrane currents and cell capacitance were recorded using the whole-cell patch-clamp technique, using an Axopatch 200B, Digidata 1322A A/D converter and pClamp 10 (Molecular Devices, LLC, San Jose, CA, USA). Pipette resistance was typically 1.5–3 MΩ when filled with pipette solution (see below), and pipette capacitance and series resistance were compensated by ~70% to optimize the measurement of membrane current. Currents were activated from a holding potential of −80 mV by step depolarization to −40 mV for 200 ms (to inactivate the sodium current) followed by steps to potentials between −50 and +80 mV for 500 ms, before repolarization to the holding potential, at a frequency of 0.2 Hz. Absolute I_Ca amplitude (pA) in intact myocytes was measured as the difference between peak inward current and current at the end of the depolarizing pulse; absolute I_Ca in the t-tubular and surface membranes was calculated from measurements of I_Ca and membrane capacitance in intact and detubulated myocytes with correction for incomplete detubulation as described previously (Bryant et al., 2015). I_Ca was normalized to cell capacitance (pF; an index of membrane area) to calculate I_Ca density (pA pF⁻¹). I_Ca density in the t-tubular membrane was calculated from the loss of membrane current and capacitance following DT; I_Ca density in the surface membrane was calculated from currents measured in DT myocytes with correction for incomplete detubulation as described previously (Bryant et al., 2015). DT efficiency was not significantly different between cell types.

2.8 Measurement of SR Ca2+ release

Intracellular Ca2+ and membrane potential were recorded simultaneously along single t-tubules as described previously (Bryant et al., 2015). Briefly, myocytes were loaded with the Ca2+ indicator Fluo-4/AM (5 μmol l⁻¹ for 25 mins; Thermo Fisher Scientific) and the voltage sensitive dye di-4-AN(F)EPPTEA (0.5–1 μg ml⁻¹ for 15 min; kindly supplied by Dr Leslie Loew; Yan et al., 2012). Cells were imaged using a Zeiss LSM 880 (see above) with the confocal pinhole set to 1 Airy unit. Line-scan images along a selected t-tubule were recorded at wavelengths between 518 and 560 nm for Ca2+, and 590 and 700 nm for voltage, at a rate of 0.51 ms/line, with an excitation wavelength of 514 nm. A negative deflection in di-4-AN(F)EPPTEA fluorescence...
was used to determine the time of the AP upstroke, and the latency from the AP upstroke to the initial (>-5 SD above average pre-stimulus value) and maximum rate of rise of Ca²⁺ was determined at each point along the Fluo-4 line-scan image. The SD of latencies for each cell was used as a measure of the heterogeneity of release. Whole-cell Ca²⁺ transients were obtained using line-scans along the long axis of cells loaded with Fluo-4/AM only. Cells were field-stimulated at 0.2 Hz at 1.5 x threshold using parallel Pt electrodes.

2.9 | Solutions

The standard superfusate for electrophysiology and imaging experiments contained (in mM): 133 NaCl, 5 KCl, 1 MgSO₄, 1 CaCl₂, 1 Na₂HPO₄, 10 d-glucose, 10 HEPES, pH 7.4 (NaOH). During electrophysiological recordings, KCl was replaced with CsCl to inhibit K⁺ currents and the pipette solution contained (in mM): 110 CsCl, 20 TEACI, 0.5 MgCl₂, 5 MgATP, 5 BAPTA, 10 HEPES, 0.4 GTP-Tris, pH 7.2 (CsOH). For some experiments, cells were incubated in C3SD peptide (Pepceuticals Limited, Enderby, UK, 1 μM in 0.1 mM Ca²⁺) for 1 h at room temperature before use. C3SD was designed to mimic the ‘scaffolding domain’ of Cav-3 and thus to interfere with Cav-3 binding to its endogenous partners and, as used in the present study, has previously been shown to inhibit Cav-3-dependent signalling in cardiac myocytes (Bryant et al., 2018b). All experiments were performed at room temperature.

2.10 | Data presentation

Data are expressed as mean ± SD (of N animals for in vivo data and of n cells from N animals (n/N) for cellular measurements). Data normality was assessed using the Shapiro-Wilk test and subsequent testing was performed using Student’s t test or the Mann–Whitney U test, one-way ANOVA, or the Kruskal–Wallis test, as appropriate. Iₒ density–voltage relationship curves were analysed using repeated measures (RM) ANOVA with voltage and intervention as factors. Single myocyte properties including those elicited by a step depolarization were tested using propagation of errors from the source measurements (Bryant et al., 2015). The limit of statistical confidence was taken as P < 0.05, and is denoted by * between treatments (e.g. sham vs. TAC) for a given phenotype and by † between phenotypes (WT vs. Cav-3 OE).

3 | RESULTS

3.1 | The effect of TAC on cardiac structure and function

Cardiac structure and function were assessed in vivo using echocardiography; exemplar records from Cav-3 OE mice are shown in Figure 2a; WT data have been shown previously (see Methods). There was no significant difference between sham WT and Cav-3 OE mice for diastolic or systolic left ventricular volume (Figure 1b,c), stroke volume or ejection fraction (Figure 1d,e), cardiac output (WT: 19.3 ± 4.2 ml min⁻¹, N = 7; Cav-3 OE: 19.5 ± 2.8 ml min⁻¹, N = 6; P = 0.92; not shown) or left ventricular mass (WT: 150.3 ± 24.8 mg, N = 7; Cav-3 OE: 141.3 ± 21.2 mg, N = 6; P = 0.50; not shown). Following TAC, diastolic and systolic left ventricular volume increased, and ejection fraction decreased, in both WT and Cav-3 OE mice (Figure 1b,c,e), while stroke volume (Figure 1d) and thus cardiac output (WT: 16.3 ± 6.1 ml min⁻¹, N = 7, P = 0.30; Cav-3 OE: 11.5 ± 3.3 ml min⁻¹, N = 6, P < 0.01) decreased significantly in Cav-3 OE, but not WT mice. However, none of the measurements of cardiac function were significantly different between WT and Cav-3 OE mice following TAC. Thus, it appears that Cav-3 OE has little effect on in vivo cardiac function either in sham animals or following TAC. However, although TAC increased left ventricular mass in both types of mouse (to 339.6 ± 33.9 mg in WT, N = 7, P < 0.001; and to 271.6 ± 24.7 mg in Cav-3 OE, N = 6, P < 0.001), left ventricular mass was significantly smaller (P < 0.01) in Cav-3 OE than in WT mice following TAC. Heart rate was not significantly different between groups (WT: sham 446.9 ± 7.3 bpm, N = 7; TAC 444.7 ± 20.0 bpm, N = 7, P = 0.92; Cav-3 OE: sham 447 ± 27 bpm, N = 6; TAC 453 ± 54 bpm, N = 6, P = 0.81).

Consistent with the changes observed in vivo, following TAC both WT and Cav-3 OE mice showed a significant increase in wet heart weight (WT: sham 210 ± 34 mg, N = 7, TAC 401 ± 77 mg, N = 8, P < 0.001; Cav-3 OE: sham 227 ± 50 mg, N = 6, TAC 362 ± 35 mg, N = 6, P < 0.001) and wet lung weight (WT: sham 146 ± 22 mg, N = 7, TAC 345 ± 127 mg, N = 8, P < 0.01; Cav-3 OE: sham 173 ± 19 mg, N = 6, TAC 427 ± 134 mg, N = 6, P < 0.001), but no change in body weight (WT: sham 28.5 ± 1.5 g, N = 7, TAC 28.3 ± 2.6 g, N = 8, P = 0.86; Cav-3 OE: sham 27.4 ± 1.9 g, N = 6; TAC 26.6 ± 4.6 g, N = 6, P = 0.68) or tibia length (WT: sham 20.4 ± 0.6 mm, N = 7; TAC 19.8 ± 0.7 mm, N = 8, P = 0.10; Cav-3 OE: sham 19.8 ± 0.9 mm, N = 6; TAC 19.6 ± 0.9 mm, N = 6, P = 0.62). Thus, in both WT and Cav-3 OE mice, TAC resulted in cardiac hypertrophy, indicated by a significant increase in heart weight:tibia length ratio (HW:TL; Figure 1f), and pulmonary congestion, a symptom of congestive heart failure, indicated by a significant increase in lung weight:tibia length ratio (LW:TL; Figure 1g); however, HW:TL following TAC was significantly smaller in Cav-3 OE than in WT mice (Figure 1f). These data suggest, therefore, that Cav-3 OE per se has little effect on cardiac structure or function, but reduces the hypertrophy observed following TAC. The magnitude of these changes was similar to that reported previously in Cav-3 OE mice following TAC (Horikawa et al., 2011, and see below).

3.2 | The effect of TAC on cell morphology

Figure 2a shows exemplar confocal images of isolated Cav-3 OE myocytes stained with di-8-ANEPPS, showing an increase in cell width, suggesting cellular hypertrophy, and disruption of t-tubule structure following TAC. Mean data show that in both WT and Cav-3 OE myocytes, TAC increased cell length (WT: sham 166.1 ± 16.7 μm,
FIGURE 1  *In vivo* cardiac function and morphology. (a) Exemplar left ventricular M-mode echocardiograms from Cav-3 OE mice that had undergone either a sham (left panel) or TAC (right panel) operation. Horizontal scale bar: 100 ms; vertical scale bar: 4 mm. (b–e) Measurements of left ventricular function obtained by echocardiography from sham and TAC WT (left) and Cav-3 OE (right) mice. (b) Diastolic volume ($\mu$l). (c) Systolic volume ($\mu$l). (d) Stroke volume ($\mu$l). (e) Ejection fraction (%). (f) Heart weight to tibia length ratio (HW:TL, mg/mm). (g) Lung weight to tibia length ratio (LW:TL, mg/mm). N = 6 sham and 6 TAC Cav-3 OE mice, and 7 sham and 7 TAC WT mice. ***P < 0.001 between treatments for a given phenotype (WT or Cav-3 OE); †P < 0.05 between phenotypes for a given treatment (sham or TAC). The WT data have been published previously (Bryant et al., 2018a)

n/N = 41/10, TAC 192.0 ± 57.9 $\mu$m, n/N = 21/5, P < 0.01; Cav-3 OE: sham 149.3 ± 16.1 $\mu$m, n/N = 19/5, TAC 178.3 ± 15.6 $\mu$m, n/N = 22/5, P < 0.001) and width (WT: sham 35.2 ± 5.3 $\mu$m, n/N = 41/10, TAC 43.1 ± 3.9 $\mu$m, n/N = 21/5, P < 0.001; Cav-3 OE: sham 36.7 ± 4.5 $\mu$m, n/N = 19/5, TAC 40.5 ± 5.0 $\mu$m, n/N = 22/5, P < 0.05), and thus calculated cell volume (Figure 2b). Although Cav-3 OE had no effect on cell volume in the absence of TAC, it was significantly smaller in Cav-3 OE than in WT myocytes following TAC (Figure 2b). Cell capacitance (a function of membrane area) also increased significantly in both WT and Cav-3 OE myocytes (Figure 2c), consistent with the development of cellular hypertrophy in response to pressure overload.

Analysis of t-tubule structure using 2D FFT showed a significant decrease in t-tubule regularity in both WT and Cav-3 OE myocytes following TAC ($P_1/P_0$, Figure 2d). Since $P_1/P_0$ depends, inter alia, on t-tubule density and orientation, further detailed analysis in 3D was performed, which showed that this reduction in regularity was due, at least in part, to a significant decrease in t-tubule density (Figure 2e) and changes in tubule orientation, with a decrease in the fraction...
of transverse (0–15°) tubules (Figure 2f). However, the changes in t-tubule structure were not significantly different between WT and Cav-3 OE myocytes.

To ensure that Cav-3 expression was increased in OE mice, we used western blotting. Figure 3a shows exemplar blots (left) and corresponding mean densitometric analysis (right) showing that Cav-3 expression was increased ∼2-fold in OE compared to WT myocytes. Cav-3 expression was not significantly altered following TAC in Cav-3 OE myocytes, so that its expression level remained higher than in sham and TAC WT myocytes. Since t-tubule structure was altered following TAC in Cav-3 OE myocytes, we also investigated the expression of JPH-2, which has been implicated in t-tubule and dyad formation. Figure 3b shows exemplar blots (left) and corresponding mean densitometric analysis (right) showing that JPH-2 was not significantly altered by Cav-3 OE but decreased following TAC in both WT and Cav-3 OE myocytes.

In summary, Cav-3 OE appears to have little effect on cardiac and cell morphology, and in vivo cardiac function, and the response to TAC was qualitatively similar in WT and Cav-3 OE mice; however, both heart weight and cell volume were significantly smaller in Cav-3 OE mice than in WT, following TAC.

3.3 | ICa distribution and regulation

To determine the distribution of ICa between the surface and t-tubular membranes, ICa was measured in intact and DT myocytes. Figure 4 shows exemplar records of ICa recorded at 0 mV from intact (Figure 4a, top) and DT (Figure 4b, top) myocytes isolated from sham and TAC Cav-3 OE hearts, with the corresponding mean current density–voltage relationships shown below. Absolute ICa and ICa density were not significantly different in WT and Cav-3 OE myocytes (Figure 4c,d). However, the increase in cell capacitance caused by TAC in WT mice
(Figure 2c) occurred with little change in absolute $I_{\text{Ca}}$ density at the surface membrane following TAC, whereas WT mice show no change at the cell surface and a decrease in t-tubular $I_{\text{Ca}}$ density in response to TAC.

We have previously shown that incubating cells with C3SD, which mimics the scaffolding domain of Cav-3 and has no effect on cell capacitance (Bryant et al., 2014), decreases $I_{\text{Ca}}$ density in intact WT control myocytes (Bryant et al., 2018a; Kong et al., 2017), and that this effect is lost following Cav-3 KO and in TAC-induced HF (Bryant et al., 2018a). To investigate whether changes in this regulatory pathway might underlie the different $I_{\text{Ca}}$ distributions observed following TAC in OE mice, we determined the response to C3SD in myocytes from sham and TAC Cav-3 OE mice. Figure 4e,g show that in sham Cav-3 OE myocytes $I_{\text{Ca}}$ density was increased by pre-treatment with C3SD, as reported previously in myocytes from unoperated Cav-3 OE mice (Kong et al., 2017) but in contrast to the decrease of $I_{\text{Ca}}$ density observed in sham WT myocytes in response to C3SD (Bryant et al., 2018a; Figure 4g). However, C3SD had no effect on $I_{\text{Ca}}$ density in Cav-3 OE myocytes following TAC (Figure 4f,h); this loss of response to C3SD following TAC is similar to that reported in WT myocytes following TAC (Bryant et al., 2018a; Figure 4h). This suggests that this regulatory pathway is lost in both cell types and is not, therefore, responsible for the different distribution of $I_{\text{Ca}}$ density observed in OE and WT myocytes following TAC.
Comparison with HF in WT myocytes

C3SD P < 0.01, TAC P = 0.4, interaction P < 0.9. Two-way repeated measures ANOVA: mV difference at 0 mV in intact Cav-3 OE myocytes, and at the cell surface and t-tubules (obtained as described in Methods), compared with previously published data (Bryant et al., 2018a) from WT mice. **P < 0.01, ***P < 0.001 between treatments for a given phenotype (WT or Cav-3 OE); ††P < 0.01, †††P < 0.001 between phenotypes for a given treatment (sham or TAC). (c,d) Absolute I_{Ca} (c) and I_{Ca} density (d) at 0 mV in intact Cav-3 OE myocytes, and at the cell surface and t-tubules (obtained as described in Methods), compared with previously published data (Bryant et al., 2018a) from WT mice, in sham (left) and in mice in HF following TAC (right). Figure 6 shows the ratio of the data from Cav-3 OE mice to those from WT mice, in sham (left) and in mice in HF following TAC (right).

3.4 | Ca^{2+} release following TAC

To determine whether the preservation of t-tubular I_{Ca} in Cav-3 OE myocytes helps to maintain Ca^{2+} release, we investigated the latency and heterogeneity of Ca^{2+} release along a single t-tubule from the time of membrane depolarization (Figure 5a), which showed that Cav-3 OE had no significant effect on latency, nor did it affect the increase in latency of both the initial and maximum rate of rise of Ca^{2+} observed following TAC (Figure 5b, top), suggesting that TAC-induced impairment of local Ca^{2+} release is unaffected by Cav-3 OE. However, Cav-3 OE caused a significant decrease in the heterogeneity of Ca^{2+} release compared to WT myocytes, and although heterogeneity increased following TAC in both cell types, it remained smaller in Cav-3 OE myocytes (Figure 5b, bottom) suggesting more uniform Ca^{2+} release along the t-tubules following Cav-3 OE. However, TAC had little effect on the early (release) phase of the whole cell Ca^{2+} transient, which was not significantly different in WT and Cav-3 OE myocytes and showed no change in either time to peak or amplitude (Figure 5c).

3.5 | Comparison with HF in WT myocytes

Figure 6 shows the ratio of the data from Cav-3 OE mice to those from WT mice, in sham (left) and in mice in HF following TAC (right). These data show that Cav-3 OE had little effect on measurements from sham hearts and myocytes, consistent with previous work showing little effect of Cav-3 OE on cardiac morphology or function in the absence of TAC (Horikawa et al., 2011; Kong et al., 2017; Markandeya et al., 2015). However, following TAC, HW:TL and cell volume were...
**FIGURE 5** Local Ca\textsuperscript{2+} release and systolic Ca\textsuperscript{2+} transient. (a) Line-scan images of the rising phase of the Ca\textsuperscript{2+} transient and (below) the associated average t-tubular di-4-AN(F)EPPTEA signal in representative sham (top) and TAC (bottom) Cav-3 OE myocytes. Horizontal scale bar: 10 ms; vertical scale bar: 5 μm. The time of the AP upstroke, initial rise of Ca\textsuperscript{2+} and maximum rate of rise of Ca\textsuperscript{2+} have been marked in yellow, red and blue, respectively. (b) Mean latency and heterogeneity of SR Ca\textsuperscript{2+} release in sham (n/N = 28/5) and TAC (n/N = 18/4) Cav-3 OE myocytes compared with WT sham (n/N = 43/12) and TAC (n/N = 12/3) myocytes. (c) Whole cell Ca\textsuperscript{2+} transient amplitude (F/F\textsubscript{0}) and time to peak (ms) measured from Ca\textsuperscript{2+} transients of sham (n/N = 25/5) and TAC (n/N = 22/4) Cav-3 OE myocytes compared with WT sham (n/N = 53/13) and TAC (n/N = 14/3) myocytes. *P < 0.05, **P < 0.01, ***P < 0.001 between treatments for a given phenotype (WT or Cav-3 OE); †P < 0.05, ††P < 0.01 between phenotypes for a given treatment (sham or TAC). The WT data have been published previously (Bryant et al., 2018a).”

significantly smaller in OE than in WT, consistent with previous work (Horikawa et al., 2011; Markandeya et al., 2015). In addition, I\textsubscript{Ca} amplitude and density were greater in intact OE than in WT myocytes, as a result of a small decrease at the cell surface but a large increase at the t-tubules. Thus, the decrease in t-tubular I\textsubscript{Ca} normally observed following TAC (Bryant et al., 2018a) is prevented by Cav-3 overexpression, and although Cav-3 OE exerts an anti-hypertrophic effect and increases I\textsubscript{Ca}, these effects only become apparent following TAC.

4 | DISCUSSION

The present data show that TAC caused qualitatively similar changes in Cav-3 OE mice to those reported previously in WT mice (Bryant et al., 2018a): cardiac hypertrophy and failure, with disrupted cell structure and function. However, the cardiac and cellular hypertrophy associated with TAC were smaller in OE animals and t-tubular I\textsubscript{Ca} density was maintained, although I\textsubscript{Ca} density at the surface membrane decreased; this contrasts with the decrease in t-tubular I\textsubscript{Ca} density with no change at the cell surface observed in WT myocytes. Thus Cav-3 OE appears to confer limited but specific protection against the effects of TAC.

4.1 | Cardiac structure and function

Echocardiography showed that Cav-3 OE had little effect on cardiac structure or function in sham animals (Figure 1). Following TAC, diastolic and systolic left ventricular volume and left ventricular mass increased, and post mortem measurements also showed an increase
in HW:TL and LW:TL in Cav-3 OE mice. However, although cardiac function was not significantly different in WT and Cav-3 OE mice following TAC, the hypertrophic response to TAC was smaller in Cav-3 OE than in WT mice. These changes are similar to those reported previously following 4 weeks TAC in Cav-3 OE mice (Horikawa et al., 2011) and suggest that Cav-3 OE has maintained anti-hypertrophic effects following TAC, consistent with the idea that Cav-3 inhibits the hypertrophic p42/44 mitogen-activated protein kinase (Woodman et al., 2002) and calmodulin-dependent calcineurin/nuclear factor of activated T cells (Markandeya et al., 2015) pathways.

Following TAC, cardiac function was not significantly different, and there were similar decreases in ejection fraction, in WT and Cav-3 OE mice. However, stroke volume and cardiac output decreased significantly in Cav-3 OE but not in WT mice following TAC. This implies that an increase in heart size in WT (cf. the larger scatter in Figure 1b) compared to Cav-3 OE mice helped to maintain stroke volume and thus cardiac output despite similar decreases in ejection fraction. Thus, the anti-hypertrophic effect of Cav-3 OE may impair the ability of the heart to maintain cardiac output.

Previous work has shown that 4 weeks’ TAC caused only a small (not significant) decrease in cardiac function in Cav-3 OE mice, but a significant decrease in WT mice (Horikawa et al., 2011; Markandeya et al., 2015). The contrast with the current work may be due to the longer (8 weeks) exposure to TAC in the present study. Taken together these data suggest that, while the anti-hypertrophic effect of Cav-3 is maintained, the deleterious effect on cardiac function, while slowed in onset, can still occur in Cav-3 OE mice following TAC. However, the observation that Cav-3 OE has little effect on either the size or the function of the heart in the absence of TAC (Figure 6; Horikawa et al., 2011; Markandeya et al., 2015) suggests that Cav-3 expression is normally sufficient to inhibit hypertrophy and enable normal ECC.

4.2 | Cell structure

In the absence of TAC, Cav-3 OE had little effect on cell size or structure, as reported previously (Kong et al., 2017), but cells were larger and t-tubule structure was disrupted following TAC, as in WT myocytes (Bryant et al., 2018a). However, the increase in cell volume...
following TAC was significantly smaller in OE than in WT myocytes, providing a mechanism for the reduced hypertrophy observed in the whole heart and consistent with the suggestion that caveolin inhibits hypertrophic pathways (Galbiati et al., 1998; Markandeya et al., 2015; Woodman et al., 2002). Knockout and loss-of-function mutations of Cav-3 are associated with hypertrophic cardiomyopathy, further supporting a role for Cav-3 as an inhibitor of cardiac hypertrophic signalling pathways (Hayashi et al., 2004; Woodman et al., 2002).

4.3 | Cell function

Cav-3 OE had little effect on the function of myocytes from sham hearts. However, the distribution of I_{Ca} was markedly different in OE and WT myocytes following TAC. TAC-induced heart failure in WT mice is associated with a decrease in t-tubular I_{Ca} density due to cellular hypertrophy with no change in absolute current, and no change in I_{Ca} density at the cell surface (Bryant et al., 2018a). In contrast, in OE myocytes, there was no change in t-tubular I_{Ca} density following TAC, because absolute current increased proportionally with membrane area, but I_{Ca} density at the cell surface decreased, since absolute I_{Ca} was unchanged despite cellular hypertrophy. Thus, following TAC, Cav-3 OE maintains I_{Ca} density at the t-tubular membrane but not at the cell surface.

Previous work has shown that chronically decreasing Cav-3 expression, via either KO or TAC, leads to decreased t-tubular I_{Ca} density as a result of an increase in membrane area (Bryant et al., 2018a), whereas acute disruption of Cav-3 activity using C3SD peptide decreases I_{Ca} with no change of capacitance (Bryant et al., 2018a; Kong et al., 2017). However, in agreement with previous work (Kong et al., 2017), the present data show that C3SD increases I_{Ca} in OE myocytes although, as in WT myocytes, this regulation was lost following TAC.

These data suggest that Cav-3 alters I_{Ca} density by (at least) two mechanisms. The first is by altering cell growth. It has previously been suggested that Cav-3 is anti-hypertrophic (Woodman et al., 2002), consistent with the observations in vivo, and in KO and WT TAC myocytes. Previous work has shown that hypertrophic pathways can be activated by Ca\^{2+} entry via LTCCs (Gao et al., 2012), although LTCCs localized to caveolae do not appear to contribute to hypertrophic signalling in mouse ventricular myocytes (Correll et al., 2017); thus Cav-3 may inhibit hypertrophic signalling by altering the distribution of LTCCs, and thus I_{Ca}. The present study suggests that Cav-3 OE does not augment the anti-hypertrophic effect of basal Cav-3 levels in sham myocytes, since cell volume and capacitance in the absence of TAC are similar in WT and OE myocytes. However, Cav-3 OE does appear to be anti-hypertrophic following TAC, when OE myocytes are smaller than those from WT. Thus, it appears that Cav-3 levels are normally sufficient to inhibit hypertrophy, and only become insufficient to do so following TAC, either because Cav-3 levels decrease and/or because of stimulation of hypertrophic pathways. This anti-hypertrophic effect of Cav-3 following TAC will help to maintain I_{Ca} density. However, the observation that absolute t-tubular I_{Ca} increases suggests additional effects of Cav-3 OE, in particular modulation of acute signalling pathways. Previous work suggests that Cav-3 helps localize I_{Ca} to the t-tubules, and C3SD decreases t-tubular I_{Ca} in WT myocytes, although this effect is lost following TAC (Bryant et al., 2018a). It has been suggested that Cav-3-dependent stimulation of I_{Ca} is due to co-localization of LTCCs with components of the protein kinase A pathway (Bryant et al., 2014). However, recent work has shown LTCC clustering leading to co-operative gating (Ghosh et al., 2018); if Cav-3 plays a role in this process, loss of Cav-3 activity would be expected to decrease I_{Ca}. The present study shows that C3SD increases I_{Ca} in sham Cav-3 OE myocytes, suggesting that the levels of Cav-3 expression achieved in OE myocytes may inhibit these Cav-3 dependent pathways. Such inhibition could occur as the result of autoinhibition or because there is abnormally located Cav-3 in OE myocytes which competes with normally localized proteins. Reducing Cav-3 expression, activity or regulation, via TAC or C3SD, may relieve this inhibitory effect to produce the increases in I_{Ca} observed in these conditions; the loss of effect of C3SD on I_{Ca} following TAC is also indicative of such loss of Cav-3 dependent regulation.

In contrast to t-tubular I_{Ca}, I_{Ca} density at the cell surface decreased following TAC in Cav-3 OE mice, due to cellular hypertrophy with little change in absolute I_{Ca}. Thus, following TAC, in OE myocytes t-tubular I_{Ca} appears to be maintained at the expense of I_{Ca} at the surface membrane, whereas in WT myocytes t-tubular I_{Ca} density decreases with no change at the cell surface. This suggests that changes in Cav-3 expression occur predominantly at the t-tubules, where it competes with that at the surface membrane to bind the proteins which localize I_{Ca}.

Regardless of the mechanism underlying the maintenance of t-tubular I_{Ca}, the latency and heterogeneity of local Ca\^{2+} release at the t-tubule still increased following TAC which, with the disruption of t-tubule morphology also observed following TAC, would be expected to desynchronize Ca\^{2+} release and thus impair contraction. However, the whole cell Ca\^{2+} transient showed little change, suggesting that it is dominated by other factors and is not responsible for the impaired cardiac performance observed following TAC. However, the Ca\^{2+} transient was monitored at a low stimulation frequency (0.2 Hz) compared with the mouse's normal heart rate, to enable comparison with I_{Ca}, which was recorded at this frequency to allow recovery from inactivation between voltage clamp pulses. It remains possible, therefore, that changes in local Ca\^{2+} release may decrease Ca\^{2+} transient amplitude, and thus impair cardiac performance, at physiological frequencies. However, the impairment occurred in the presence of increased heart size, so that reduced stroke volume and ejection fraction do not necessarily imply reduced contractility because myocyte contraction will have to overcome the higher wall tension that will result from the increase in heart size (law of Laplace).

The lack of effect of Cav-3 OE on the latency of Ca\^{2+} release despite the recovery of t-tubular I_{Ca} suggests that the decrease in I_{Ca} is not the primary cause of disruption of Ca\^{2+} release following TAC. This may be because of redundancy in the Ca\^{2+}-induced Ca\^{2+} release process (Cannell, Berlin, & Lederer, 1987) in mice, and/or because the LTCCs, and thus I_{Ca}, which are regulated by Cav-3 are predominantly extra- dyadic (Glukhov et al., 2015; Sanchez-Alonso et al., 2016), which could also explain why similar decreases in I_{Ca} amplitude produced by Ca\^{2+} channel blockers, which will affect all Ca\^{2+} channels, inhibit release (Bryant et al., 2015). The TAC-induced disruption of Ca\^{2+} release may,
Therefore, be due predominantly to disruption of the dyad (Louch et al., 2013), consistent with the observed decrease in JPH-2 and with recent work showing dispersion of RyR clusters in rat myocytes in HF (Kolstad et al., 2018), so that RyR dispersion and loss of t-tubular \( I_{\text{Ca}} \) may have summative effects that impair \( \text{Ca}^{2+} \) release along t-tubules. Interestingly, however, Cav-3 OE decreased the heterogeneity of \( \text{Ca}^{2+} \) release in both sham and TAC myocytes, suggesting that Cav-3 may increase the uniformity of t-tubular \( \text{Ca}^{2+} \) release by altering the distribution of \( I_{\text{Ca}} \) or the response to \( I_{\text{Ca}} \) along the t-tubule.

5 | SUMMARY

These data show that Cav-3 OE alone has little effect on the structure or function of either the whole heart or ventricular myocytes. However, following TAC, Cav-3 OE is anti-hypertrophic and helps to maintain t-tubular \( I_{\text{Ca}} \); this is not secondary to the smaller increase in t-tubular membrane area in OE myocytes, because absolute \( I_{\text{Ca}} \) increases. This attenuation of the TAC phenotype by Cav-3 OE is consistent with previous work suggesting that Cav-3 plays a role in the local regulation of \( I_{\text{Ca}} \) and is anti-hypertrophic, but it remains unclear whether these effects are direct or secondary.

COMPETING INTERESTS

None declared

AUTHOR CONTRIBUTIONS

All laboratory experiments were performed at the University of Bristol. S.M.B., C.H.T.K., D.M.R., H.H.P., A.F.J., M.B.C. and C.H.O. conceived the study and designed the project methods. S.M.B., C.H.T.K. and J.J.W. contributed to acquisition, analysis or interpretation of data and S.M.B., C.H.T.K., J.J.W., D.M.R., H.H.P., A.F.J., M.B.C. and C.H.O. contributed to the drafting of the manuscript and its revision. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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