Supporting Information

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SI Materials and Methods

Experimental Animals. Conditional ET_A KO mice ($ETA^{fl/fl}$) were described previously (1). $ETA^{fl/fl}$ were crossed with transgenic mice overexpressing Cre recombinase under the control of the *dbh* promoter, DBH-Cre (2), to obtain mice lacking ET_A only in SNs ($ETA^{fl/fl}$;DBH-Cre, abbreviated as SN-KO) or under the control of the *myh6* promoter, αMHC-Cre (3), to obtain mice lacking ET_A only in CMs ($ETA^{fl/fl}$;αMHC-Cre, abbreviated as CM-KO). Control littermates (Ctrl. LM) consisted of $ETA^{fl/fl}$, $ETA^{+/fl}$, $ETA^{+/+}$, and $ETA^{+/+}$;DBH-Cre littermates for SN-KO and $ETA^{fl/fl}$, $ETA^{+/fl}$, $ETA^{+/+}$, and $ETA^{+/+}$;αMHC-Cre littermates for CM-KO. All these mouse lines and Dahl salt-resistant (DR), Dahl salt-sensitive (DS), Sprague–Dawley, and Wistar rats (Charles River Laboratories) were housed under standard conditions and maintained on commercial mouse or rat chow and water ad libitum. The environment was maintained at 22 °C with a 12-h light–12-h dark cycle. For preparation of neonatal CMs or SNs, pups were killed between days 1 and 3 postnatal. Male DR and DS (9 wk of age; mean weight, 200 g; M&B) were fed on a high-salt diet [8% (vol/vol) NaCl] for 40 d. Treated DS rats received darusentan (30 mg/kg/d; DS + DA in the drinking water (was changed twice the day) with onset of the diet. Water and drug intake was controlled on a daily basis.

Transverse Aortic Constriction. Transverse aortic constriction (TAC) was performed in 9- to 10-wk-old male SN-KO and CM-KO and control littermates from the same strain as described previously (4, 5). Briefly, the animals were anesthetized with ketamine (120 mg/kg, i.p.) and xylazine (15 mg/kg, i.p.). The mice were orally intubated with 20-gauge tube and ventilated (Harvard Apparatus) at 120 breaths/min (0.2-mL tidal volume). The aortic constriction was created via a lateral thoracotomy through the second intercostal space. A suture (Prolene 7-0) was placed around the transverse aorta between the brachiocephalic and left carotid artery. The suture was ligated against a 27-gauge needle. The needle was removed, leaving a discrete stenosis. Afterward, the chest was closed. Integrity of aortic banding was confirmed by inspection of the surgical constriction and by visualization of marked differences in the caliber of the right and left carotid arteries in TAC (6). A sham procedure in which the thoracic aorta was not banded was also performed. Mice were killed from about 7 wk after surgery.

Echocardiography. For measurements of chamber dimensions and calculation of fractional shortening or ejection fraction, we used a Tracing software from VisualSonics on the parasternal long axis via the following formula: $100 \times [(LVEDV - LVESV)/LVEDV)]$, where LVEDV is left ventricular end-diastolic volume and LVESD is left ventricular end-systolic volume. To quantify the stenosis after TAC surgery, echocardiographic data were achieved at heart rates between 450 and 550 beats/min, and the evaluation was performed with short-acting isoflurane anesthesia. Arterial blood pressure of awake animals and left ventricular/intraventricular pressure were recorded as previously described in detail (7). Left ventricular end-diastolic pressure (LVEDP), left ventricular contractility [$(+)dP/dt_{max}$], and left ventricular relaxation [$(-)dP/dt_{max}$] were calculated using chart software package (ADInstruments Pty. Ltd.). Transthoracic echocardiography was performed as previously described in detail (8). The investigator conducting the echocardiography was blinded to the treatment status.

Metaiodobenzylguanidine-PET. All chemicals were purchased from Sigma–Aldrich unless otherwise noted. ^{124}I was purchased from Eckert & Ziegler. Poly-(3-{dibutyl[2-(3-and-4-vinylphenyl)ethyl] stannyl}benzylguanidinium acetate)-codivinylbenzene, a tin precursor anchored to a polystyrene matrix, was obtained from Molecular Insight Pharmaceuticals. High-performance liquid chromatography gamma was performed using an Agilent 1100 HPLC equipped with a Raytest (Germany) Model Gabi radioisotope detector. Analytical reversed-phase HPLC (RP-HPLC) analyses were performed using a Chromolith Performance RP-18 endcapped column (3 × 100 mm). The column was eluted in a linear gradient (0–100%) of acetonitrile [0.1% trifluoroacetic acid (TFA)] in water (0.1% TFA) for 5 min; flow rate of 2 mL/min; and room temperature absorbance $\lambda = 214$ nm. [^{124}I]Metaiodobenzylguanidine (MIBG) synthesis was conducted as follows: 3.2 mg of the resin was swollen for 5 min in 50 μL ethanol, and 25 μL of water was added; 120 MBq [^{124}I] in a volume of 35 μL was added, followed by 25 μL of a solution of hydrogen peroxide in acetic acid. The pH value of the reaction mixture was pH 4. The reaction mixture was stirred at a temperature of 85 °C for 20 min. At this time, HPLC analysis revealed a complete conversion, and 20 μL of a saturated solution of methionine in water was added to quench the reaction. The resin was separated by filtration over a sterile filter and washed with 0.9% NaCl. The mixture was neutralized with 35 μL of 1 M NaOH, and the pH was adjusted to 7.4 with phosphate buffer. Radio-HPLC revealed the purity (>95%) of the product. Mice were measured 7 wk after TAC surgery 1 h after i.v. injection of 10.73 ± 0.69 MBeq of [^{124}I]-MIBG. The anesthetized animals [2% (vol/vol) sevoflurane; Abbott] were placed in an Inveon small animal PET scanner (Siemens). Region of interest (ROI) analysis of the acquired images was performed using Siemens imaging software. The resulting image data were normalized to the administered activity to parameterize the microPET images in terms of %ID/g (percentage of injected dose per gram of heart corrected for radioactive decay to the time of injection). The uptake profiles were fitted to a one-compartment model (realized with the PMOD software) to estimate the influx rate constant (K_1, milliliters of medium per grams of cells per min), and the volume of distribution (V_d).

Heart Tissue Preparation. Mice were killed by cervical dislocation, and hearts were removed and weighed promptly. Relative heart weight was calculated as ratio to body weight. The left ventricles were transversely dissected, and parts of the left ventricles were quickly frozen in liquid nitrogen for protein and RNA isolation and analysis. Some hearts were arrested in diastole and completely fixed for 48 h in 4% (vol/vol) paraformaldehyde dissolved in 0.1 M PBS (pH 7.4), embedded in paraffin, longitudinally cut into 5-μm sections, and stained for histomorphometry.

Primer for RNA Analysis. The primers and probes that were used are as follows—rat: *myh6* sense 5′-tgcagaagaaactgaaggaaaa-3′ and antisense 5′-gctcggcctctagctcct-3′; *myh7* sense 5′-caccaacaaccc ctacgatt-3′ and antisense 5′-agcacatcaaaggcgctatc-3′; *th* sense 5′-tctccctgaggggtacaaaa-3′ and antisense 5′-gaattttggcttcaa atgtctca-3′; mouse: *ednra* sense 5′-tgtgagcaagaaattcaaaaattg-3′ and antisense 5′-atgaggcttttggactggtg-3′; *nppa* sense 5′-cacaga tctgatggatttcaaga-3′ and antisense 5′-cctcatcttctaccggcatc-3′; *col3a1* sense 5′-tcccctggaatctgtgaatc-3′ and antisense 5′-tgagtcg aattggggagaat-3′; *myh7* sense 5′-cgcatcaaggagctcacc-3′ and antisense 5′-ctgcagccgcagtaggtt-3′; *gapdh* sense 5′-gggttcctataaa-tacggactgc-3′ and antisense 5′-ccattttgtctacgggacga-3′; *th* sense 5′-

cccaagggcttcagaagag-3′ and antisense 5′-gggcatcctcgatgagact-3′. For SERCA (*atp2a2*) we used a taqman universal PCR mastermix (Applied Biosystems).

Isolated Heart Perfusion. Wistar rats were anesthetized with thiopental (100 mg/kg, i.p.). The hearts were rapidly cut out and rinsed in ice-cold buffer, and the aorta was cannulated for perfusion according to the Langendorff method. Within one experiment, 8–12 spontaneously beating hearts were perfused simultaneously at a constant coronary flow and a constant temperature of 37.5 °C. The perfusion medium was a modified Krebs-Henseleit solution (125 mmol/L NaCl, 16.9 mmol/L $NaHCO_3$, 0.2 mmol/L Na_2HPO_4, 4.0 mmol/L KCl, 1.85 mmol/L $CaCl_2$, 1.0 mmol/L $MgCl_2$, 11 mmol/L glucose, and 0.027 mmol/L EDTA). The buffer was gassed with 95% (vol/vol) O_2 and 5% (vol/vol) CO_2, and the pH was adjusted to 7.4. Cardiac [^{3}H]-norepinephrine (NE) uptake was determined as described previously (9). Briefly, a bolus of [^{3}H]-NE (1 mL, 3 μCi, 100 pmol NE; Amersham-Buchler) was injected into the perfusion system and proportionally distributed to the hearts and blank channels. Radioactivity was measured in the effluent. The amount of [^{3}H]-NE extracted by the hearts (uptake) was expressed as the percentage of radioactivity measured in the blank channels. Overflow of endogenous NE was evoked by electrical field stimulation as described previously (10). Briefly, isolated perfused hearts were stimulated with bipolar platinum electrodes in the presence of atropine (1 μmol/L) to prevent vagal activation (S1; 1 min, 4 Hz, 5 V). To distinguish between exocytotic and net release of NE, a second stimulation was performed in the presence of the NE reuptake inhibitor desipramine (DMI). Endogenous NE in the coronary venous effluate was determined by HPLC and electrochemical detection.

Determination of Neurohormones. The tissue concentration of NE in mice was determined by a radioenzymatic assay as previously described (10). Plasma concentrations of the stable N-terminal part (amino acids 1–98) of the prohormone form of atrial natriuretic peptide (proANP) were determined in venous blood samples taken from the femoral vein. ProANP was determined using enzyme immunoassays according to the manufacturer's instructions (Biomedica). For determination of NE tissue levels in rats, the left ventricle was rinsed in ice-cold 0.9% saline and frozen in liquid nitrogen until HPLC and electrochemical detection were performed as previously described in detail (11).

Histology. H&E and Massoń trichrome stainings were performed as previously described (12). Cardiomyocyte size was assessed on H&E-stained sections by using National Institutes of Health Image J software (http://rsb.info.nih.gov/ij/). More than 80 randomly chosen cardiomyocytes from each group were analyzed to measure cross-sectional cardiomyocyte area. To quantify cardiac fibrosis, 20 trichrome-stained sections (magnification 20×) from the left ventricle were randomly selected, and morphometric analysis using Image J was performed. Photographs where acquired with an Olympus SZH zoom stereo dissection scope with an Optronics DEI-750 CCD digital camera. All data were analyzed by a single observer blinded to the mouse genotypes.

Survival Analysis. DSs were treated as described above (DS, DS + DA). Ten rats per group were monitored, and deaths were recorded every day. Survival was described by standard Kaplan–Meier analysis.

Isolation of CMs. Rat CMs were isolated from newborn Sprague–Dawley rats as previously described (13). After isolation, CMs were maintained in DMEM containing 10% (vol/vol) FCS, 2% (vol/vol) antibiotics (penicillin/streptomycin), and 2 mM L-Gln. For the assessment of CM hypertrophy, cells were cultured for 3–5 d and then starved (0% FCS) for 24 h and stimulated with 10 nM ET1 for 18–24 h. Murine CMs were isolated from neonatal $ETA^{fl/fl}$ mice as described previously (14). Pups were decapitated, and ventricular tissue was carefully dissected and dissociated in 0.41 mg/mL collagenase (Sigma) and 0.30 mg/mL pancreatin (Sigma) in Ads buffer (116 mM NaCl, 20 mM Hepes, 0.8 mM NaH_2PO_4, 1 g/liter glucose, 5.4 mM KCl, and 0.8 mM $MgSO_4$, pH 7.35). CMs were plated on laminin/poly-L-lysine–coated plates, and cells were treated with adenovirus expressing Cre-recombinase (a kind gift by Oliver Müller, University Hospital, Heidelberg, Germany) for 12 h with 12 multiplicity of infection (MOI). For CM-SN coculture, neonatal rat SNs were plated 1 d after preparation of CMs. After 3 d, medium was changed to serum-free medium for 24 h. For hypertrophic experiments, cells were then treated with 10 nM ET1 for 18–24 h before measurement of fixation.

Plasmids and Reagents. CMs were transduced with adenoviruses harboring FLAG-HDAC4 (Ad-HDAC4), and FLAG-HDAC5 (Ad-HDAC5) were infected for 24 h as described previously (13), starved for 4 h, and stimulated with 10 nM ET1 for 12 h. Adenovirus harboring Cre recombinase was a kind gift by Oliver Müller and was generated according to the manufacturer's instructions (ViraPower Adenoviral Expression System; Invitrogen). Prazosin (αARi; Sigma) and propranolol (βARi; Sigma) were used at a concentration of 1 μM. Darusentan (kind gift of Klaus Muenter, Knoll AG, Mannheim, Germany) was used at a concentration of 1 μM.

Adenovirus Production. A luciferase reporter construct harboring three binding sites for the myocyte enhancer factor 2 [Ad-3xmyocyte enhancer factor 2 (MEF2)-Luc] was obtained from Seven Hills Bioreagents. After generation, the adenoviruses were amplified and purified with the Adeno-X Purification Kit (BD), and its infectious units per microliter were determined with the Adeno-X Rapid Titer Kit (BD).

RNA Quantification. Total RNA was isolated from ventricular tissue or from cell culture experiments by using TRIzol (Invitrogen). Total RNA was digested with DNase, and cDNA synthesis of 500 ng of RNA was carried out by using a SuperScript first-strand synthesis system for RT-PCR (Invitrogen). Quantitative real-time PCR (qPCR) was performed with Universal ProbeLibrary (Roche) by using TaqMan Universal PCR Mastermix (Applied Biosystems) and detection on a 7500 Fast Cycler (Applied Biosystems) as described previously (5).

Coimmunoprecipitation. Cells were harvested in Tris (50 mM, pH 7.4), NaCl (150–900 mM), EDTA (1 mM), and Triton X-100 (1%) supplemented with protease inhibitors (Complete; Roche Diagnostics) and PMSF (1 mM). Cells were further disrupted by passage through a 25-gauge needle, and cell debris were removed by centrifugation. FLAG-tagged proteins were immunoprecipitated with M2-agarose conjugate (Sigma-Aldrich) and thoroughly washed with lysis buffer. Bound proteins were resolved by SDS/PAGE, transferred to PVDF membranes, and immunoblotted as indicated with either a monoclonal anti-FLAG antibody (M2; Sigma-Aldrich) or a polyclonal anti–14-3-3 antibody (Abcam). Proteins were visualized with a chemiluminescence system (Santa Cruz Biotechnology).

Western Blot Analysis. Cultured cells were harvested in Tris (50 mM, pH 7.4), NaCl (150 mM), EDTA (1 mM), and Triton X-100 (1%) supplemented with protease inhibitors (Complete; Roche) and PMSF (1 mM). The cells were further disrupted by passage through a 25-gauge needle, and cell debris were removed by centrifugation. Proteins were resolved by SDS/PAGE, transferred to PVDF membranes, and immunoblotted as indicated in the figures with tyrosine hydroxylase (TH) antibody (rabbit; Santa Cruz), ET_A antibody (rabbit; Santa Cruz) or GAPDH (mouse; Santa Cruz),

followed by the appropriate secondary HRP-labeled antibodies and visualized with a chemiluminescence system (Santa Cruz).

[3H]-Leucine Incorporation. Protein synthesis was examined by analysis of [3H]-leucine incorporation. CMs and SN-CM cocultures were maintained for 4 d in DMEM containing 10% (vol/vol) FCS, 2% (vol/vol) antibiotics (penicillin/streptomycin), and 2 mM L-Gln, starved (0% FCS) for 24 h, and stimulated with 10 nM ET1. Cultures were treated with inhibitors 1 h before stimulation. After stimulation, 3 μL Ci of [3H]-leucine was added, and the cells were cultured for an additional 18 h. After two washes with ice-cold PBS, neonatal rat ventricular myocytes (NRVMs) were precipitated with 10% (vol/vol) trichloroacetic acid for 30 min at 4 °C. The precipitates were then solubilized in 0.2 N NaOH for more than 4 h. Radioactivity was measured in a liquid scintillation counter.

MEF2 Reporter Assay. CMs were transduced with Ad-3xMEF2-Luc (10 MOI) construct, expressing three copies of an MEF2 binding site to detect endogenous MEF2 activity. CMs were infected 4 h before washing and adding SN to avoid infection of neuronal cells. After 24-h culturing, cells were starved for 24 h. After treatment with 10 nM ET1 for 24 h, cells were harvested, and luciferase levels were determined.

Indirect Immunofluorescence. For immunofluorescence analysis, cocultures and monocultures were grown on laminin-coated coverslips (BD Bioscience), fixed in paraformaldehyde [4% (vol/vol)], permeabilized in 0.3% Triton X-100, and blocked in PBS containing goat serum [5% (vol/vol)]. Primary antibodies against FLAG (monoclonal rabbit; Sigma), α-actinin (monoclonal mouse; Sigma), and TH (polyclonal rabbit; Cell Signaling) were used at a dilution of 1:200. Secondary antibodies conjugated to either fluorescein or Texas red were used at a dilution of 1:200. Images were captured at a magnification of 20–60× with an Olympus BX-51 fluorescence microscope.

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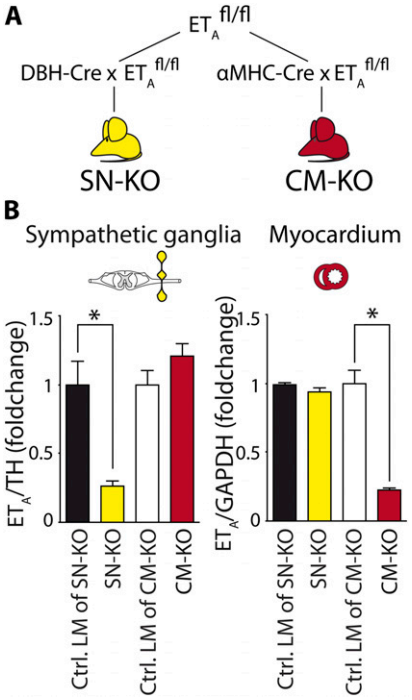

Fig. S1. Generation of pre- and postsynaptic ET_A KO. (*A*) Breeding scheme to obtain presynaptic (SN, sympathetic neuron specific) and postsynaptic (CM, cardiomyocyte specific) KO of the ET_A (αMHC, myosin heavy chain; DBH, dopamin β hydroxylase). (*B*) Transcript levels in the stellate ganglion and the left ventricle of the indicated genes were detected by quantitative real-time PCR (n = 5–10 per group). Values indicate relative expression level normalized to control littermates (without Cre expression or floxed allele) of each strain.

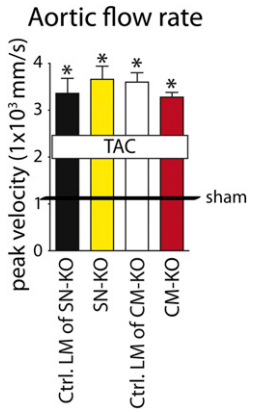

Fig. S2. Quantification of aortic stenosis. We quantified the aortic stenosis of TAC and sham-operated animals. Values indicate the flow rate within the stenosis. No significant difference was found between the groups. Values are presented as mean ± SEM; *$P < 0.05$ sham vs. TAC ($n \geq 5$ per group).

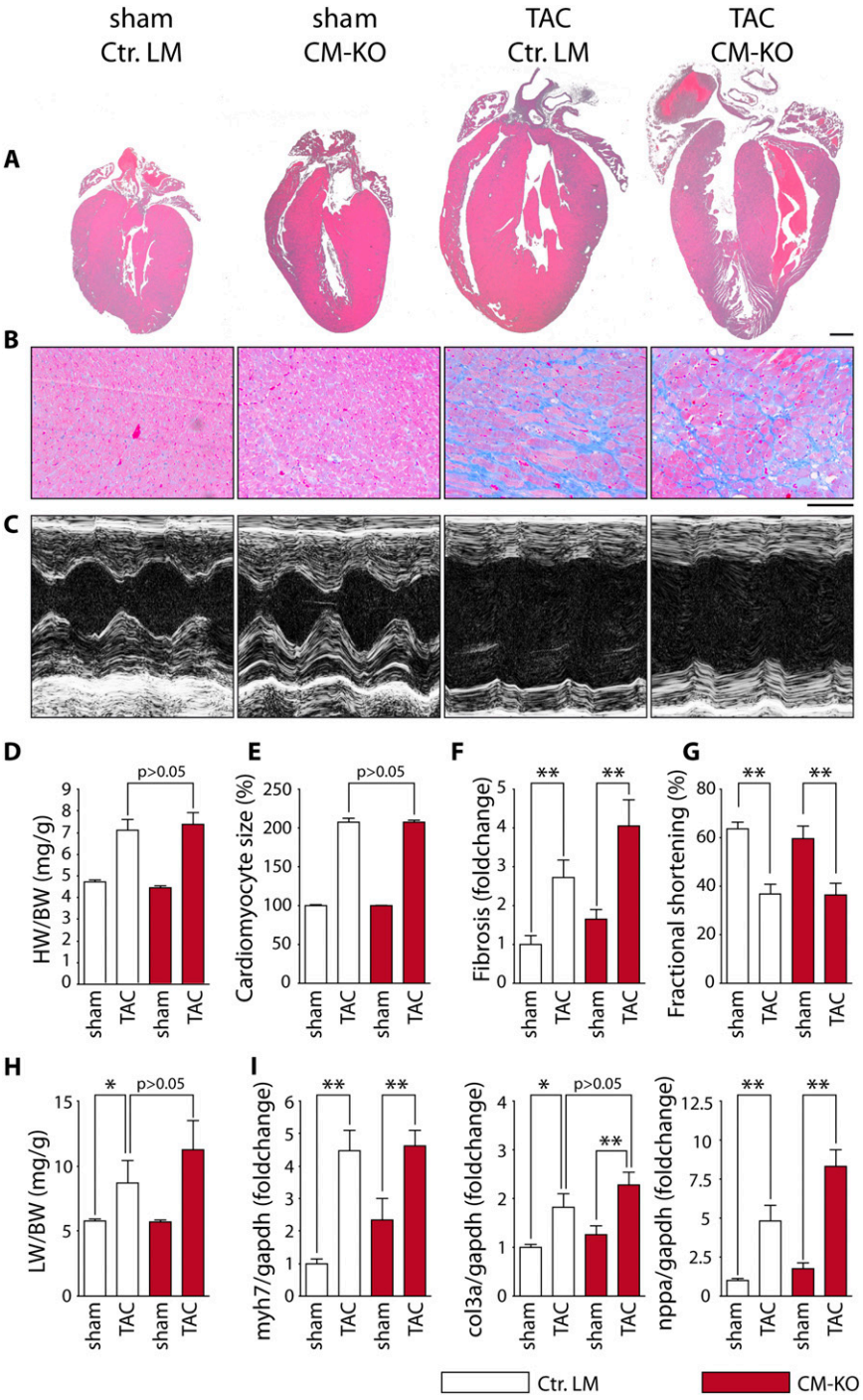

Fig. S3. Cardiomyocyte ET_A is not involved in pathological cardiac remodeling. (*A*) Representative four-chamber view of CM-KO 7 wk after sham or TAC surgery. (Scale bar, 1 mm.) (*B*) Representative trichrome-stained sections, shown as indicated. (Scale bar, 100 μm.) (*C*) Representative transthoracic echocardiography of the left ventricle (M-Mode, midventricular). Groups are shown as indicated. (*D*) Heart weight/body weight ratios of control littermates and KO ($n = 7–11$ per group). (*E*) Quantification of CM size (>80 cells per sample from more than five fields of view; $n = 3$). (*F*) Quantification of fibrosis normalized to control littermates ($n \geq 3$ per group). (*G*) Fractional shortening of CM-KO and control littermates 3 wk after TAC ($n = 6–11$ per group). (*H*) Lung weight/body weight ratios of control littermates and CM-KO animals 7 wk after TAC surgery ($n = 7–11$ per group). (*I*) Transcript levels in hearts from CM-KO 7 wk after TAC surgery from genes as indicated, normalized to control littermates ($n = 4–8$ per group). Values presented as mean ± SEM. $*P < 0.05$; $**P < 0.01$.

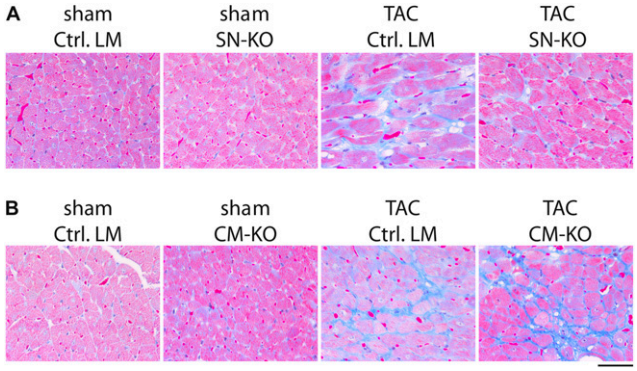

Fig. S4. Reduced cardiac fibrosis in SN-KO after TAC. Representative trichrome-stained sections from SN-KO (*A*) and CM-KO (*B*), shown as indicated. (Scale bar, 50 μm.)

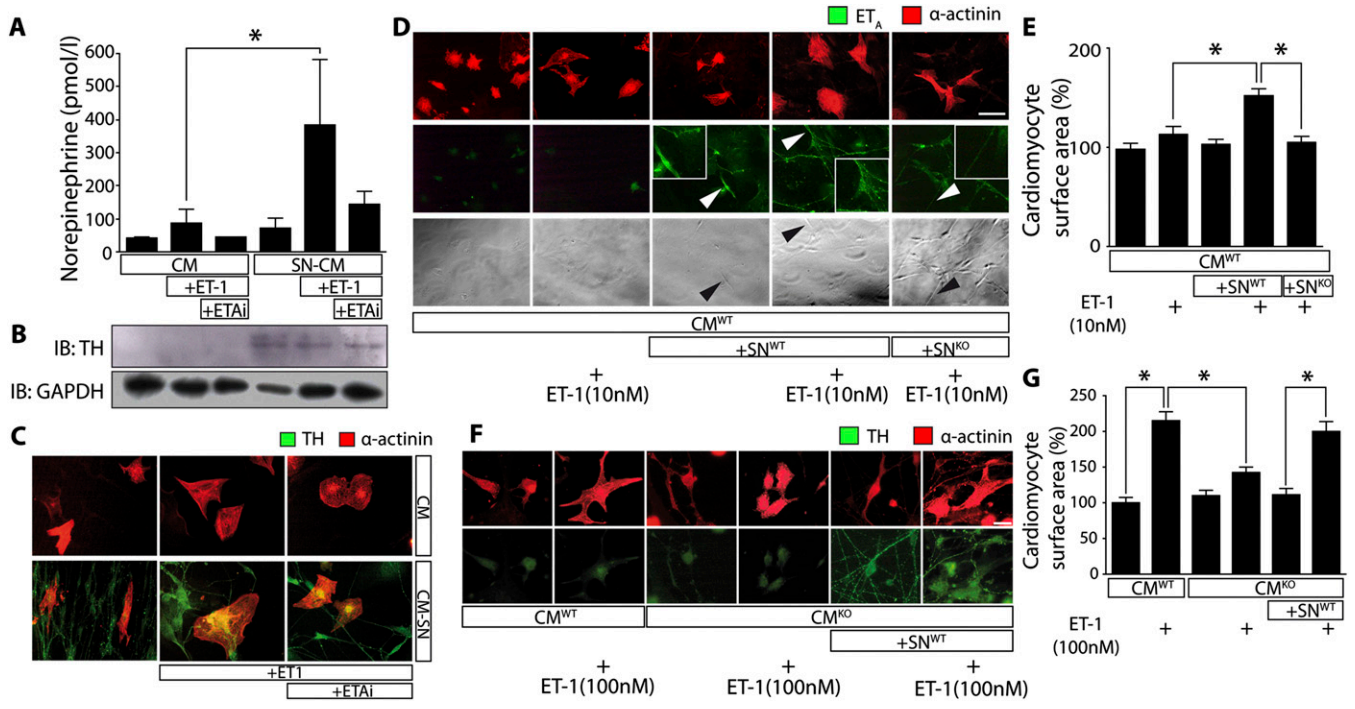

Fig. S5. Sympathetic neurons regulate cardiomyocyte hypertrophy through sympathetic ET$_A$. (*A*) Treatment with 10 nM ET1 for 1 h leads to an increase in NE release into the cell culture medium of sympathetic neuron-cardiomyocyte (SN-CM) coculture, which was attenuated by the pretreatment with darusentan (1 μM; ET$_A$i). (*B*) Western blot analysis of the SN-specific marker tyrosine hydroxylase (TH) confirmed the presence of SNs in CM-SN coculture. (*C*) Representative image in CM surface area after 24-h ET1 treatment (10 nM). (*D*) Neonatal rat ventricular myocytes (NRVMs, CM^{WT}) were cocultured with SNs from ET$_A^{fl/fl}$ mice and treated with Ad-Cre (SN^{KO}). (*Top*) Representative α-actinin stained CMs. (*Middle*) Anti-ET$_A$ immunocytochemistry, confirming reduced ET$_A$ expression (magnification in white rectangle). Please also note the higher expression of ET$_A$ in SNs in relation to CMs. Gray-scaled panels show transmitted light images to visualize the presence of sympathetic neurons. (White bar, 50 μm.) Cultures were treated as indicated (ET1 10 nM, 18 h). (*E*) Cell surface area was quantified (n = 50–70 cells). Values are shown as mean ± SEM. *P < 0.05 as indicated. (*F*) CMs from ETA$^{fl/fl}$ mice were cocultured with WT rat SNs (SN^{WT}). For ex vivo KO of ET$_A$, cells were transduced with Ad-Cre (CM^{KO}) and then treated with a high dosage (100 nM) of ET1 for 24 h as indicated; 100 nM ET1 resulted in substantial CM hypertrophy even in CM monoculture, which was attenuated in CM^{KO} but gained again by the addition of SN^{WT}. Immunocytochemistry was performed using antibodies recognizing sarcomeric α-actinin (red; *Upper*) and TH (green; *Lower*). (*G*) CM cell surface area was then quantified (n = 24–40 cells per group). *P < 0.05 as indicated. Values are presented as mean ± SEM.

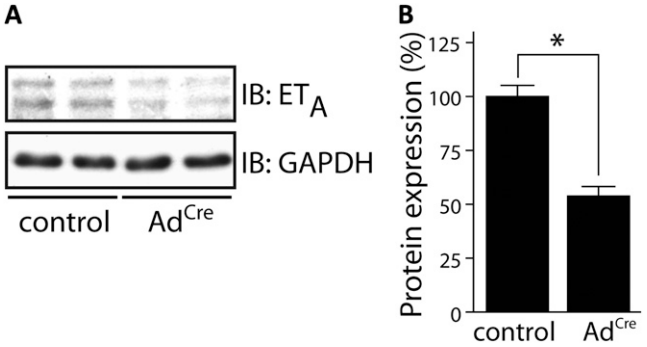

Fig. S6. In vitro deletion of ET_A in NMVM from ET_A floxed mice. (*A* and *B*) Protein expression was detected by Western blot analysis 5 d after Ad-Cre transduction ($n = 3$ per group). IB, immunoblot.

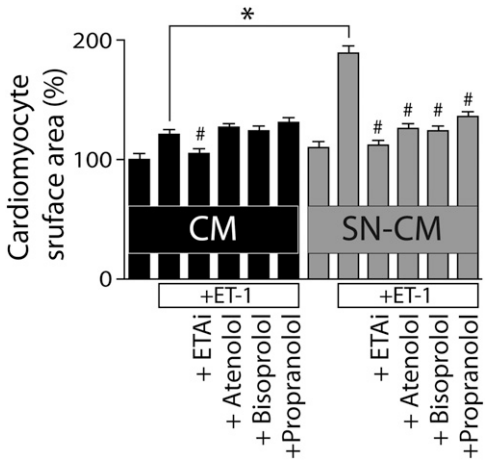

Fig. S7. Selective β1-adrenergic inhibition blocks exaggerated cardiomyocyte hypertrophy. CM cell surface area was quantified in CM and SN-CM cultures after treatment with ET1 and pretreatment with selective (bisoprolol) and nonselective inhibitors of β1-adrenergic receptors. $^{\#}P < 0.05$ vs. ET1-treated; $^{*}P < 0.05$ ET1-treated SN-CM vs. CM ($n = 100–150$ per group) (ET1: 10 nM 24 h; 1 μM propranolol; 1 μM bisoprolol; 10 μM atenolol added 1 h before ET1 treatment).

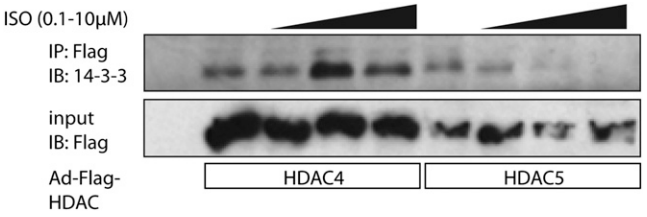

Fig. S8. β-adrenergic activation leads to increased 14-3-3 binding to HDAC4 but reduced 14-3-3 binding to HDAC5. (*A* and *B*) Coimmunoprecipitation assay using cellular extracts from NRVMs expressing FLAG-HDAC4 and FLAG-HDAC5. The FLAG-tagged proteins were immunprecipitated with an antibody against FLAG. Associated endogenous 14-3-3 was detected with an antibody against 14-3-3. The experiment was performed with and without stimulation, with isoproteronol as indicated. IB, immunoblot; IP, immunoprecipitation; ISO, isoproteronol.

Table S1. Summary of clinical endothelin receptor antagonist trials

Time of treatment	Date	Acronym	Reference	Drug	Selective for	Design	Patients number	Treated with adrenergic receptor antagonists	Beneficial effects?	Result
24 wk	2004	EARTH	(1)	D	ET_A	Randomized, double-blind, placebo-controlled	642	73%	No	No beneficial effects on LVESV (MRI), changes in LVEF, neurohormonal measurement, 6-min walk test, quality of life, NYHA class, global assessment, and composite clinical status.
3 wk	2002	HEAT	(2)	D	ET_A	Randomized, double-blind, placebo controlled	157	46%	Yes	Positive for cardiac index after 3 wk, no change in pulmonary wedge, pulmonary arterial pressure, pulmonary vascular resistance, right arterial pressure, heart rate, mean artery pressure and plasma catecholamines.
2 wk	1998		(3)	B	ET_A and ET_B	Randomized, double-blind, placebo controlled	36	0%	Yes	After 2 wk: improved cardiac output, reduced systemic and pulmonary vascular resistance; no change in heart rate.
3 wk	2001		(4)	D	ET_A	Randomized, placebo controlled	21	33%	Yes	Improvement of flow-mediated vasodilatations (FMD).
26 wk	2005	Reach-1	(5)	B	ET_A and ET_B	Randomized, double-blind, placebo-controlled	370	14–26%	No/short term; yes for 176 patients in the long-term follow-up	Termination because of safety concerns. 176 patients were followed up long term (26 wk): 26% improved in NYHA class (compared with 19% placebo); 28% showed a decline in NYHA class (compared with 43% placebo) $P = 0.045$. No change in systolic pressure, heart rate or QT time. No change when all patients are included into the analysis, suggesting a biphasic response with early worsening and later improvement in bosentan-treated patients.
3 wk	2004		(6)	D	ET_A	Randomized, double-blind, placebo-controlled	23	34%	Yes	Significant benefits in hemodynamic variables, mean pulmonary artery pressure, and heart rate. A significant reduction in mean arterial blood pressure, BNP decreased significantly in patients with darusentan, whereas big-endothelin remained unchanged. Pro-ANP tended to decrease in the active treatment group, but did not reach statistical significance.
6 h	2004		(7)	T	ET_A and ET_B	Randomized, double-blind, placebo-controlled	130	17–31%	Yes	Increase in cardiac index, decrease in BNP levels, positive trend in patients subjective dyspnea score, reduce urine output in high dosage.
6 h	2003		(8)	T	ET_A and ET_B	Randomized, double-blind, placebo-controlled	292	56%	Yes	Improvement in hemodynamics, improved cardiac index, in tendency improved dyspnea, decreased risk of clinical worsening
up to 72 h	2003	RITZ-4	(9)	T	ET_A and ET_B	Randomized, double-blind, placebo-controlled	193	71–79%	No	No change in death, worsening HF, recurrent ischemia or new myocardial infarction
up to 24 h	2003	Ritz-5	(10)	T	ET_A and ET_B	Randomized, double-blind, placebo-controlled	84	45–60%	No	No difference in SO (2), death, recurrent pulmonary edema, mechanical ventilation, myocardial infarction during the first 24 h.

In studies in which patients did not receive or only a small percentage received antiadrenergic therapies, readouts tended to be positive. B, bosentan; D, darusentan; T, tezosentan.

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