Cardiovascular Research (2013) **97**, 240–250
doi:10.1093/cvr/cvs320

# Cardiac sympathetic neurons provide trophic signal to the heart via β2-adrenoceptor-dependent regulation of proteolysis

**Tania Zaglia[1,2], Giulia Milan[2], Mauro Franzoso[1,2], Enrico Bertaggia[2], Nicola Pianca[2], Eleonora Piasentini[2], Vanessa A. Voltarelli[3], David Chiavegato[2], Patricia C. Brum[3], David J. Glass[4], Stefano Schiaffino[2,5], Marco Sandri[1,2,5], and Marco Mongillo[1,2,5]***

[1]Department of Biomedical Sciences, University of Padova, Viale G. Colombo 3, Padova 35121, Italy; [2]Venetian Institute of Molecular Medicine (VIMM), University of Padova, Via Orus 2, Padova 35129, Italy; [3]Affiliation-School of Physical Education and Sport, University of São Paulo, 65 Butantã, São Paulo 05508-900, Brazil; [4]Muscle Diseases, Novartis Institutes for Biomedical Research, 100 Technology Square, Cambridge, MA 02139, USA; and [5]CNR Institute of Neuroscience, Viale G. Colombo 3, Padova 35121, Italy

*Received 4 July 2012; revised 30 September 2012; accepted 15 October 2012; online publish-ahead-of-print 22 October 2012*

**Time for primary review: 33 days**

**Aims** Increased cardiac sympathetic neuron (SN) activity has been associated with pathologies such as heart failure and hypertrophy, suggesting that cardiac innervation regulates cardiomyocyte trophism. Whether continuous input from the SNs is required for the maintenance of the cardiomyocyte size has not been determined thus far.

**Methods and results** To address the role of cardiac innervation in cardiomyocyte size regulation, we monitored the effect of pharmacological sympathetic denervation in mice on cardiac structure, function, and signalling from 24 h to 30 days in the absence of other pathological stimuli. SN ablation caused an immediate reduction in the cardiomyocyte size with minimal consequences on the resting contractile function. Atrophic remodelling was mediated by the ubiquitin–proteasome system through FOXO-dependent early induction of the muscle-specific E3 ubiquitin ligases Atrogin-1/MAFbx and MuRF1, which was followed by activation of the autophagy–lysosome system. MuRF1 was found to be determinant in denervation atrophy as remodelling did not develop in denervated MuRF1 knock-out (KO) hearts. These effects were caused by decreased basal stimulation of cardiomyocyte β2-adrenoceptor (AR), as atrophy was prevented by treatment of denervated mice with the β2-AR agonist clenbuterol. Consistent with these data, we also observed that β2-AR KO mice showed cardiac atrophy at rest.

**Conclusion** Cardiac SNs are strong regulators of the cardiomyocyte size via β2-AR-dependent repression of proteolysis, demonstrating that the neuro-cardiac axis operates constitutively for the determination of the physiological cardiomyocyte size. These results are of great clinical relevance given the role of β-AR in cardiovascular diseases and their modulation in therapy.

**Keywords** β-adreoceptors • Ubiquitin–proteasome system • Atrophy • Sympathetic nervous system • Cardiac remodelling

## 1. Introduction

The heart adapts its mass in response to various stimuli including mechanical, metabolic, and neuro/hormonal signals, all of which cause changes in the relative rates of protein synthesis and degradation.[1] Protein degradation occurs either via autophagy–lysosome system, mainly involved in organelle removal, or through the ubiquitin–proteasome system (UPS).[2] The UPS relies on the activity of three enzymes: E1, a ubiquitin-activating enzyme; E2, mediating ubiquitin conjugation; and E3, a ubiquitin ligase enzyme.[2,3] Atrogin-1/MAFbx and MuRF1 are muscle-specific ubiquitin ligases[4,5] whose activity contributes to cell size regulation both in skeletal and cardiac muscles. Consistent with their role in mediating proteolysis, over-expression of cardiac atrogin-1/MAFbx or MuRF1 has been shown to prevent surgically or pharmacologically induced cardiomyocyte hypertrophy,[6–8] while genetic ablation of MuRF1 increases the propensity to develop cardiac hypertrophy upon pressure overload.[9] Conversely, it has been demonstrated that MuRF1 is up-regulated

* Corresponding author. Tel: +39 0497923229; fax: +39 0497923250, Email: marco.mongillo@unipd.it

during regression of cardiac hypertrophy as well as in nutrient deprivation- and dexamethasone-induced atrophy.[10,11]

Cardiac atrophy following food deprivation or mechanical unloading is characterized both by increased proteolysis and decreased protein synthesis.[10,12] In skeletal muscle, similar changes in the relative rates of protein synthesis and degradation[13,14] leading to atrophy have been shown to depend on the loss of neuro/hormonal signals upon denervation, which resulted in increased MuRF1-mediated degradation of myosin heavy chain [15] and myosin-binding proteins.[16]

The heart is densely innervated by sympathetic neurons (SNs) that, by releasing norepinephrine (NE) and activating β-adrenergic receptors (β-ARs), operate physiologically as a short-term enhancer of the heart rate, conduction velocity, and cardiac contractility to match blood flow requirements of peripheral organs.[17] β-Adrenergic stimulation has also longer-lasting effects on cellular metabolism, survival and on the modulation of cardiomyocyte growth. Both mice lacking the pre-synaptic α2-ARs, that are characterized by a higher basal sympathetic outflow,[18,19] and NGF-overexpressing mice, which show sympathetic hyper-innervation, develop cardiac hypertrophy.[20] Systemic administration of formoterol and salmeterol, two β2-adrenoceptor (AR) agonists, elicits cardiac muscle hypertrophy,[21] and β-AR activation prevents left ventricular (LV) atrophy experimentally induced by cardiac haemodynamic unloading.[22,23] Conversely, mice with total β-AR deficiency,[24] as well as rabbits chronically treated with β-AR blockers[25] have reduced the cardiac mass. Interestingly, a link between β2-AR-dependent signalling and expression of atrogin-1/MAFbx and MuRF1 has recently been proposed.[26]

Taken together, these data suggest that cardiac SN activity may constitutively tune the physiological cardiac mass, by modulating the cellular mechanisms of protein synthesis and degradation, but this hypothesis has not been investigated directly thus far.

# 2. Methods

## 2.1 Mice

The following animal models were used in this study: CD1, C57BL/6J, and FVB WT mice (Charles River, Milan, Italy), MuRF1 knock-out (KO) and β2-AR KO mice, P1 Sprague-Dawley neonatal rats. All experimental procedures were performed according to the European Commission guidelines and have been approved by the local ethical committee and the relevant Italian authority (Ministero della Salute, Ufficio VI), in compliance of Italian Animal Welfare Law (Law n 116/1992 and subsequent modifications), and complying with the Directive 2010/63/EU of the European Parliament.

## 2.2 Pharmacological sympathectomy

Pharmacological sympathectomy was obtained with 6-hydroxy-dopamine (6-OH-DOPA) (100 mg/kg, ip) (Tocris Bioscience, R&D Systems, Inc., Minneapolis, MN, USA).[39] The drug was administered at days $t = 0$, $t = 2$, and $t = 7$. Animals were sacrificed 2, 4, 8, and 30 days after the first 6-OH-DOPA injection using excess anaesthesia. Control mice were treated with the vehicle (0.9% NaCl and 0.1% ascorbic acid).

## 2.3 Echocardiography

Echocardiography was performed both in control and 30-day-denervated mice under anaesthesia during constant monitoring of temperature, respiratory rate, and ECG, using a Vevo 2100™ (VisualSonics, Toronto, Canada) system equipped with a 30 MHz transducer. For more details, see Supplementary material online.

## 2.4 Tissue samples and immunofluorescence analysis

For histological and immunofluorescence analyses, hearts were harvested from mice and either frozen directly in liquid nitrogen or fixed in 1% paraformaldehyde (Sigma). More details and the primary antibodies used in this are described in the Supplementary material online Section and *Table S1*, respectively.

## 2.5 RT–qPCR analysis

Total RNA was prepared using the SV Total RNA Isolation System according to the manufacturer's protocol; for details, see Supplementary material online. Primers are listed in Supplementary material online, *Table S2*.

## 2.6 Western blotting

Total ventricular extracts from both the right and the left ventricles were obtained as previously described[41] and protein concentration was determined by the BCA assay (Pierce). SDS–PAGE was performed on 3–8 and 4–12% gradient gels (Invitrogen) loading from 10 to 60 μg protein/lane. Extracts transferred onto PVDF (Invitrogen) were processed with the primary antibodies described in Supplementary material online, *Table S1* either 1 h at room temperature or over night at 4°C. The blots were then incubated with secondary antibodies conjugated to horseradish peroxidase and the reactivity was revealed by enhanced chemiluminescence (Pierce).

## 2.7 Cardiomyocyte culture

Details of the procedure are described in the Supplementary material online.

## 2.8 Statistical analysis

All data are expressed as the mean ± SEM. Comparison between the experimental groups was made by using the non-paired Student's *t*-test and ANOVA tests followed by Bonferroni correction, with $P < 0.05$ being considered statistically significant.

# 3. Results

## 3.1 Pharmacological ablation of the cardiac sympathetic nervous system causes reduction in cardiomyocyte size

Cardiac sympathetic nerve terminals, as identified by immunoreactivity to tyrosine hydroxylase, DOPA β-hydroxylase, and synapsin I, are interspersed among the cardiomyocytes in all regions of the adult mouse heart, with a higher density in the right ventricle (RV) and the subepicardium of both the LV and the RV (Supplementary material online, *Figure S1*). To obtain cardiac sympathetic denervation, we used 6-OH-DOPA, which induces peripheral autonomic neuron degeneration without affecting central adrenergic neurons,[27] with only minimal consequences on resting blood pressure (BP) and cardiac haemodynamics.[28,29] Such treatment ablated ~90% of cardiac sympathetic nerve terminals already 24 h after the first 6-OH-DOPA injection, and caused complete and persistent denervation after 8 and 30 days (*Figure 1A* and *C*). In accord with the removal of cardiac sympathetic efferents, the chronotropic response to parasympathetic antagonism elicited by atropine administration was significantly reduced in denervated mice, when compared with vehicle-treated controls (*Figure 1B*).

Denervated hearts were characterized by a significant reduction in size, as demonstrated by the decrease in the heart weight/body weight

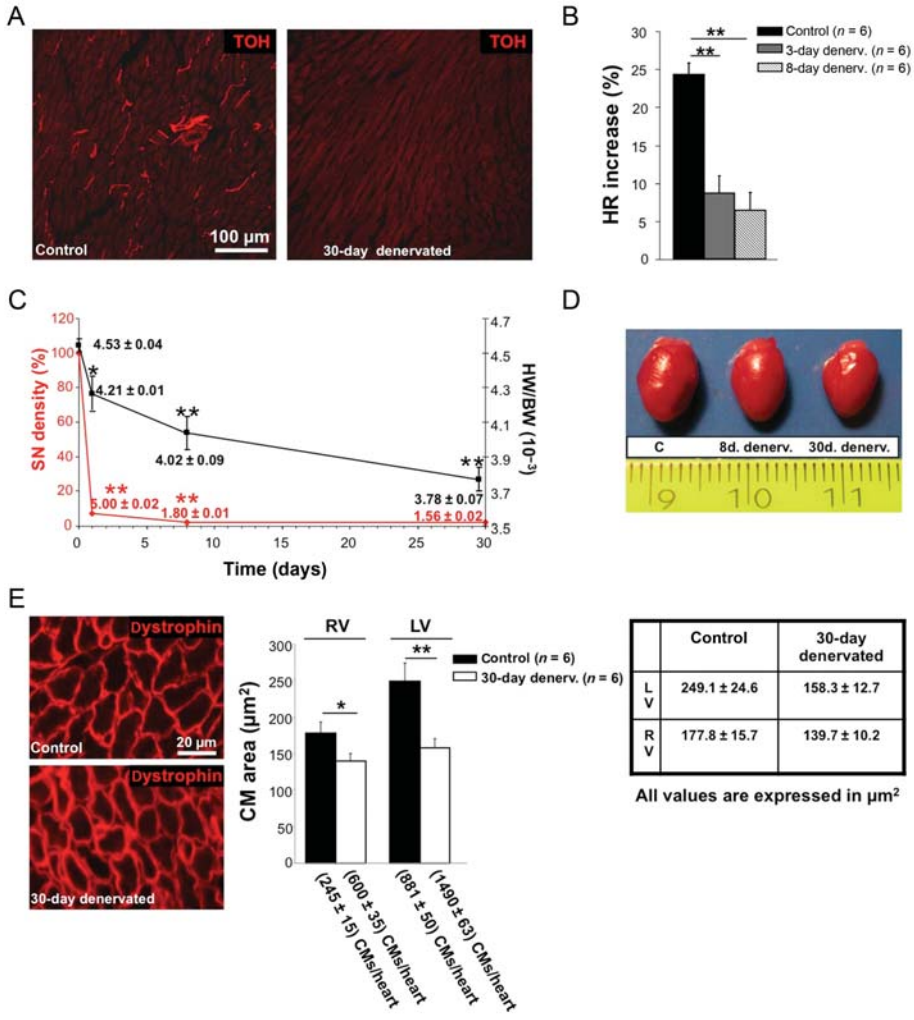



**Figure 1** Cardiac sympathetic nervous system ablation induces heart atrophy. (*A*) Confocal immunofluorescence analysis of ventricular cryosection from control and 30-day denervated hearts stained with an antibody against tyrosine hydroxylase (TOH, red signal). Bar: 100 μm. (*B*) Evaluation of heart rate (HR) changes upon atropine injection in control, 3- and 8-day denervated mice (**$P < 0.01$; $n = 6$ mice for each group). (*C*) Evaluation of sympathetic neuron (SN) density and heart weight/body weight (HW/BW) in vehicle-treated control and 1-, 8-, and 30-day-denervated mice. Error bars indicate SEM (**$P < 0.01$; $n = 16$ mice for each group). (*D*) Hearts from adult vehicle-treated control, 8- and 30-day-denervated mice. (*E*) Immunofluorescence analysis of cryosections from vehicle-treated and 30-day-denervated mice stained with an antibody to dystrophin (left panel) and morphometric evaluation of the cardiomyocyte cross-sectional area (right panels). Error bars indicate SEM (** $P < 0.01$; $n = 6$ mice for each group). The number of CMs (mean ± SD) evaluated for each heart is shown on the graph.

ratio (HW/BW), that started rapidly 24 h after treatment, and progressed with time, reaching a 15% decrease at Day 30 (*Figure 1C* and *D*). This was confirmed by a significant reduction in the cross-sectional area of ventricular cardiomyocytes, which was already evident 8 days (data not shown) upon denervation and maximal at Day 30 (*Figure 1E*). The denervated myocardium had normal histology (*Figure 2A*), with no signs of infiltration, cardiomyocyte necrosis, or interstitial fibrosis (*Figure 2B*), that retained normal sarcomeric organization (*Figure 2C*). The assessment of cardiac function by echocardiography suggested that atrophic remodelling in the denervated hearts was not dependent on reduced haemodynamic stress, even when the degree of atrophic remodelling was maximal in this model (e.g. 30 days), as the hearts of 6-OH-DOPA-treated mice had unchanged contractility [fractional shortening (FS) denervated: 31.17 ± 1.37% vs. control: 29.64 ± 1.50%] and only a moderate decrease of the ejection fraction (EF), when compared with vehicle-treated controls (EF, denervated: 67.53 ± 2.97% vs. control: 76.33 ± 2.36%) (Supplementary material online, *Figure S2*). Furthermore, the assessment of myocardial strain using speckle tracking imaging demonstrated that both circumferential and radial strain were unchanged in the denervated mice (circumferential strain: denervated: −15.89 ± 2.58 vs. controls: −14.43 ± 1.92; radial strain: denervated −27.9 ± 5 vs. controls: −27.8 ± 8.1). In line with the functional measurements, we did not detect changes in the expression level of foetal genes associated to cardiac hypertrophy and unloading atrophy (alpha-skeletal actin and atrial natriuretic factor),[30] with the exception of β-MHC, which was increased in 30-day-denervated hearts, when compared with controls (*Figure 2D–F*). Nutrient deprivation is a well-accepted activator of atrophic remodelling in both cardiac and skeletal muscles.[31] In the

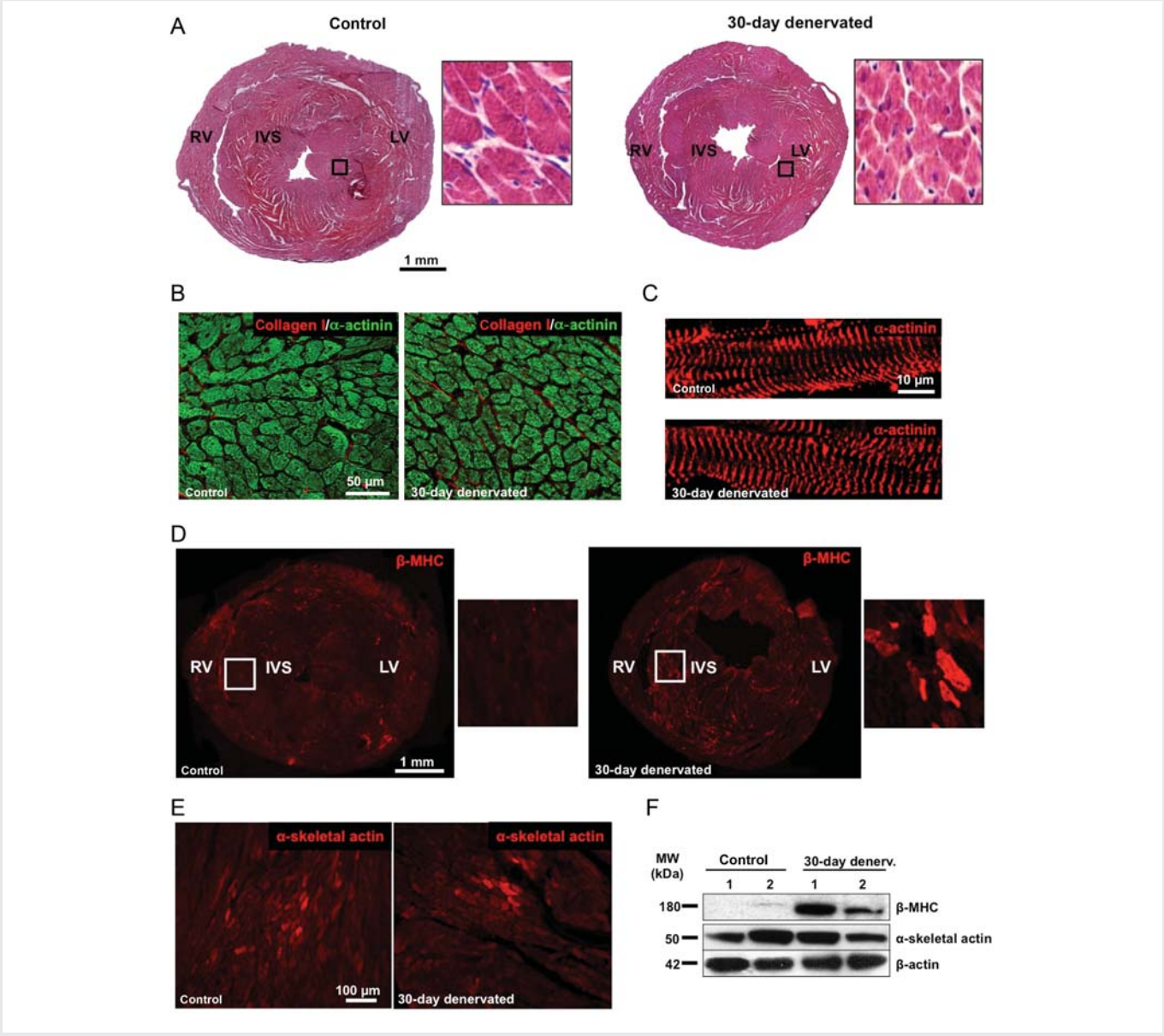



**Figure 2** Denervated hearts do not show histological alterations. (*A*) Haematoxilin–eosin of heart cryosections from vehicle-treated and 30-day-denervated mice. Bar: 1 mm. RV, right ventricle; IVS, interventricular septum; LV, left ventricle. (*B*) Confocal immunofluorescence analysis of ventricular cryosections from vehicle-treated control and 30-day-denervated mice co-stained with antibodies to sarcomeric actinin (green signal) and collagen I (red signal), showing no signs of myocardial interstitial fibrosis upon 6-OH-DOPA treatment. Bar: 50 μm. (*C*) Immunofluorescence analysis of ventricular cryosections from vehicle-treated control and 30-day-denervated mice stained with an antibody specific for sarcomeric actinin, showing no sarcomeric disorganization in denervated hearts. Bar: 10 μm. (*D* and *E*) Immunofluorescence analysis of ventricular cryosections from vehicle-treated and 30-day-denervated mice stained with antibodies specific for β-myosin heavy chain (β-MHC) (*D*) and skeletal actin (*E*). Bars: (*D*) 1 mm; (*E*) 100 μm. (*F*) Western blot analysis on total ventricular extracts from control and 30-day-denervated hearts. MW, molecular weight.

first 3 days of 6-OH-DOPA treatment, the mice underwent a modest and transient BW reduction (~10% at Day 3), which is completely recovered from the eighth day after treatment onwards (Supplementary material online, *Figure S3A*). To determine whether metabolic changes could be responsible for denervation atrophy, mice underwent caloric restriction (4 g of normal chow food/day)[32,33] for 3 (group II) or 7 days (group I), followed by free food access causing a 10–15% reduction in BW. Consistent with previous reports, we observed a significant reduction in cardiac weight (HW/BW, 8-day diet: 0.0041 ± 0.0002 vs. control: 0.0045 ± 0.0002) during the diet. However, at difference with the denervated mice, the heart size returned identical to controls after normal diet was re-established (HW/BW, 30-day diet: 0.0046 ± 0.0003 vs. C: 0.0045 ± 0.0002) (Supplementary material online, *Figure S3B–D*).

Taken together, these results suggest that atrophic remodelling upon cardiac denervation does not depend on haemodynamic unloading or metabolic deprivation, but suggests that sympathetic innervation attends to an independent mechanism that provides constitutive trophic signal to cardiomyocytes, and therefore regulating their physiologic size.

## 3.2 Atrophic remodelling in denervated hearts is mediated by FOXO (*Forkhead box protein O*)-dependent up-regulation of the ubiquitin ligases atrogin-1/MAFbx and MuRF1

Based on the evidence that the cardiomyocyte size is regulated by the relative rates of protein synthesis and degradation, we sought to determine whether the removal of sympathetic input to the heart influenced the regulation of proteostasis. We, therefore, assessed the expression level of the main genes controlling the UPS, namely the ubiquitin ligases MuRF-1 and atrogin-1/MAFbx, and the autophagy-related genes LC3, Beclin, p62, Bnip3, and cathepsin L. Atrogin1/MAFbx and MuRF1 were induced at the transcript level 24 h after 6-OH-DOPA treatment and progressively decreased 8 and 30 days upon heart denervation (*Figure 3A*). Based on the evidence that in skeletal muscle activation of the autophagy programme follows UPS activation,[34] we sought to determine whether autophagy was activated in denervated hearts. No significant changes in the autophagy-related genes were detected at Days 8 and 30 (*Figure 3B*). However, in 30-day-denervated hearts, there was a significant increase in the protein level of the low molecular weight LC3II band (*Figure 3C*), a marker of autophagosome activation, and decreased protein levels of p62, the best-known mammalian autophagy-specific substrate, both molecular events associated to activation of the autophagy–lysosome system. In addition, Beclin1 and Bnip3 appeared significantly down-regulated in both 8- and 30-day-denervated hearts (*Figure 3C*), which also supports activation of autophagy. These data indicate that denervation increased UPS-dependent proteolysis, which was subsequently accompanied by activation of the autophagy–lysosome system. As the transcriptional regulation of atrogin-1/MAFbx and MuRF1 is modulated by the FOXO family of transcription factors and inhibitor of nuclear factor kappa-B kinase subunit β (IkKβ)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathways,[35,36] we assessed whether these pathways were activated following denervation. In denervated hearts, there was a progressive decrease in the phosphorylation level of FOXO1 which started already 24 h after denervation and persisted for the time of the analysis (*Figure 4A*). Moreover, the protein level of the phosphorylated isoform of IkKβ was decreased 24 h after 6-OH-DOPA treatment, but returned to levels comparable with controls at Days 8 and 30 (*Figure 4B*). These results suggest that ablation of sympathetic efferents to the heart leads to up-regulation of the ubiquitin ligases atrogin-1/MAFbx and MuRF-1 through a FOXO-dependent pathway. We next investigated signalling dependent on the serine–threonine kinase Akt, which is a known regulator of FOXO transcription factors, as well as being central to the modulation of protein synthesis. Western blot analyses demonstrated a decrease in phospho-Akt in denervated hearts which started already 24 h after denervation (*Figure 4C*) and persisted at 8 and 30 days (*Figure 4D*). In addition, denervated hearts showed a significant reduction in the phosphorylated isoform of the ribosomal protein S6, as well as the p-S6/S6 ratio, when compared with vehicle-treated controls (*Figure 4C* and *D*). These data suggest that upon ablation of cardiac SNs, a decrease in the activated form of Akt leads to rapid up-regulation of the muscle-specific ubiquitin ligases Atrogin-1/MAFbx and MuRF-1, through activation of FOXO transcription factors, initiating cardiac atrophic remodelling.

## 3.3 MURF1 knock-out mice are protected from denervation-induced cardiac atrophy

The ubiquitin ligase MuRF1 has previously been shown to control degradation of sarcomeric proteins.[9] To address the role of MuRF1 in denervation-induced cardiac atrophy, we denervated MuRF1 KO mice (MuRF1−/−, in the C57BL6 background), which have been previously demonstrated to have normal cardiac structure and function.[9] MuRF1−/− mice examined 30 days after 6-OH-DOPA treatment were compared with MuRF1 littermate controls (MuRF1+/+) as well as to vehicle-treated MuRF1−/−. Denervation, which in MuRF1+/+ controls induced cardiac atrophy to a degree comparable with that of CD1 mice (HW/BW, denervated: 0.0041 ± 0.0001 vs. vehicle-treated controls: 0.0048 ± 0.0004) (*Figure 5A–C*) did not cause atrophic remodelling in MuRF1−/− hearts, as demonstrated by unchanged HW/BW and cardiomyocyte cross-sectional area (HW/BW, denervated: 0.0053 ± 0.0002 vs. vehicle-treated controls: 0.0052 ± 0.0002) (*Figure 5A–C*). These results indicate that genetic ablation of MuRF1 protects the heart from denervation atrophy, supporting the conclusion that MuRF1 is a central regulator in such remodelling process.

## 3.4 Noradrenaline modulates MuRF1 expression through β2-AR signalling both *in vivo* and in cultured cardiomyocytes

At least two different neurotransmitters released by cardiac SNs, namely noradrenaline (NE) and Neuropeptide Y (NPY), have been shown to have a role in regulating cardiomyocyte trophism *in vitro*.[37,38] To identify the sympathetic neurotransmitter involved in the regulation of MuRF1 expression, we evaluated changes in the expression level of this ubiquitin ligase in neonatal cardiomyocytes treated with beta-adrenergic stimuli. We assessed MuRF1 levels in cells treated with a non-selective adrenergic agonist, NE, and upon treatment with the beta2 selective agonist clenbuterol (CL), or with NPY. Serum starvation caused a significant increase in cardiomyocyte MuRF1 expression, which was prevented both by NE and CL treatment, whereas NPY treatment did not have effects (*Figure 6A*). Based on these results and the effects of CL in skeletal muscle,[39] we hypothesized that stimulation of β2-AR would prevent cardiac atrophic remodelling following denervation. Denervated mice were administered CL, delivered at constant concentration by subcutaneous osmotic minipumps, and such treatment prevented both the decrease in HW/BW (denervated: 0.00380 ± 0.00007, denervated + CL: 0.00420 ± 0.00012, CL: 0.0046 ± 0.00012 vs. control: 0.00450 ± 0.00004) and the reduction in the cardiomyocyte cross-sectional area (*Figure 6B* and *C*). These results support that β2-AR signalling has a role in the determination of the physiological myocardial mass. To further validate the notion that β2-AR signalling has a major role in the determination of the physiological myocardial mass, we examined the heart size in β2-AR KO mice. As expected, β2-AR KO mice had decreased HW/BW and cardiomyocyte cross-sectional area, at baseline when compared with littermate controls (HW/BW, β2-AR KO: 0.0030 ± 0.0001 vs. control: 0.0038 ± 0.0001; CM cross-sectional area, β2-AR KO: 252.20 ± 25.01 $\mu m^2$ vs. control: 306.94 ± 26.29 $\mu m^2$) (*Figure 6D*). Akin to denervated hearts, β2-AR KO mice did not show impairment of cardiac contractility (FS: β2-AR KO: 45 ± 1% vs. control: 48 ± 2%), or significant changes in tail-cuff BP (β2-AR KO: 109 ± 6 mmHg vs. control: 109 ± 1 mmHg) and heart

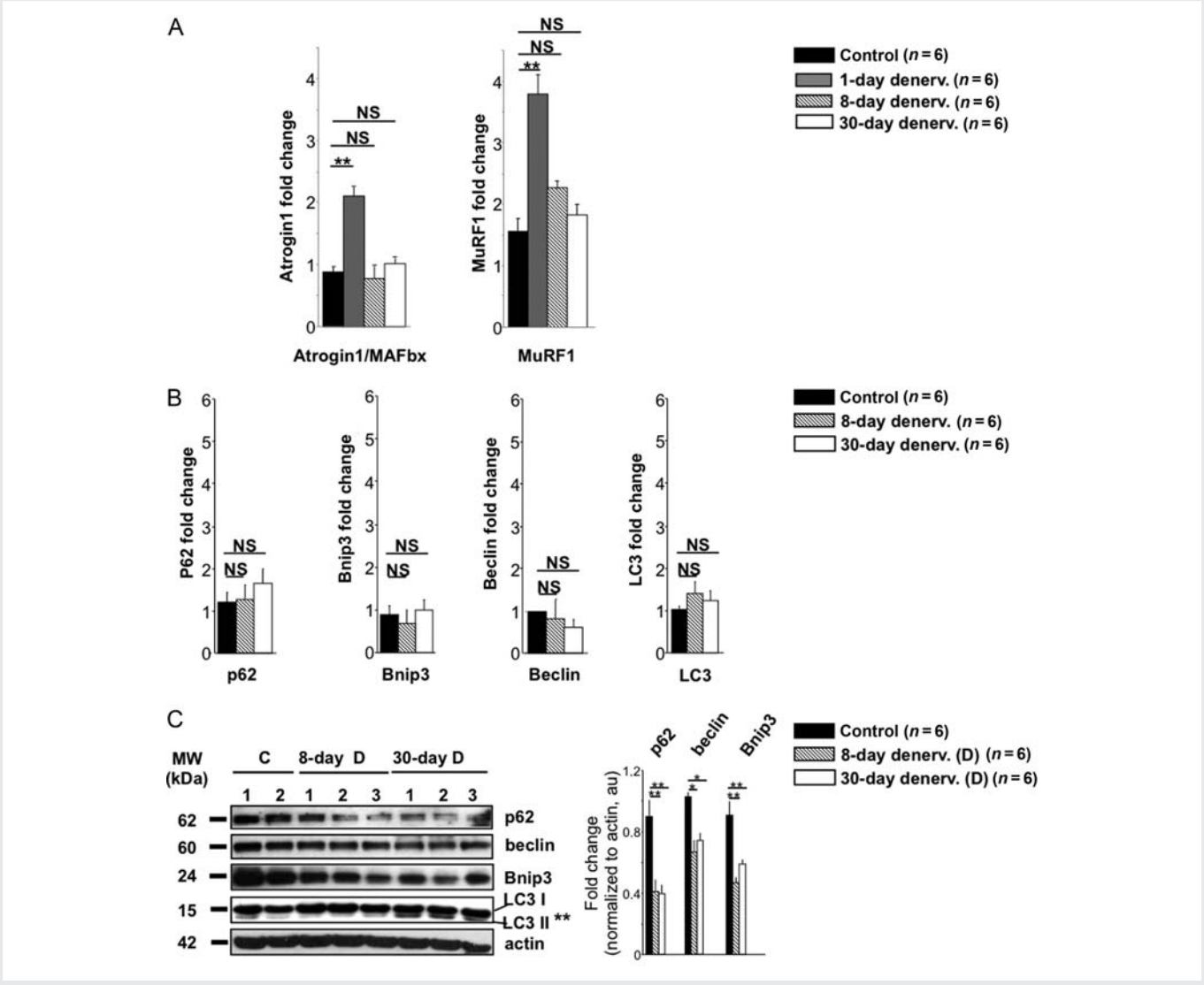



**Figure 3** Denervation-induced cardiac atrophy is mediated by activation of the ubiquitine proteasome and the autophagy–lysosome systems. (*A*) Atrogin-1/MAFbx and MURF1 gene expression detected by RT–qPCR in total ventricular extracts from vehicle-treated control, 1-, 8-, and 30-day-denervated mice. Error bars indicate SEM (**$P < 0.01$; NS, no significant; $n = 6$ mice for each group). (*B*) Autophagy-related gene expression was detected by RT–qPCR in total ventricular extracts from vehicle-treated, 8- and 30-day-denervated mice. Error bars indicate SEM (**$P < 0.01$; NS, not significant; $n = 6$ mice for each group). (*C*) Western blot analysis on total ventricular extracts from vehicle-treated control, 8- and 30-day-denervated hearts. MW, molecular weight. The densitometric analysis is reported. Error bars indicate SEM (*$0.01 < P < 0.05$, **$P < 0.01$; NS, not significant; $n = 6$ mice for each group).

rate (HR: β2-AR KO: 523 ± 30 b.p.m. vs. control: 502 ± 4 b.p.m.) (*Figure 6E–G*). Altogether, these results suggest that constitutive β2-AR signalling regulates the cardiac mass by controlling UPS activity through repression of MuRF1 expression.

# 4. Discussion

The heart has the ability to adjust its performance to the perfusional demand of peripheral tissues, through both acute control of contractility and reversible changes in the cardiac mass.[17,40] Although it is well-accepted that the autonomic nervous system tunes contractile function on a beat-to-beat basis,[41] it is not clear whether the regulation of the physiological cardiomyocyte size relies on continuous trophic input from the cardiac neurons.

Mechanical, metabolic, and neuro/hormonal signals have all been implicated in the modulation of cardiomyocyte growth in exercise- or disease-induced cardiac hypertrophy.[42] Conversely, termination of these signals upon relief of the pro-hypertrophic stimulus (e.g. mechanical unloading or removal of aortic banding) has been shown to induce 'reverse' remodelling.[43,44] Such plasticity in the cardiomyocyte size requires mechanisms that control the relative rates of protein synthesis and degradation, the latter depending mainly on proteasome activity and autophagy.[45,46] Here, we show that ablation of sympathetic efferents to the heart offsets the proteolytic machinery by up-regulating the muscle-specific ubiquitin ligases MuRF1 and atrogin-1/MAFbx, with subsequent activation of the autophagy–lysosome system, and reduces Akt-S6K signalling, suggesting reduced protein synthesis. The combined effect of such processes is the

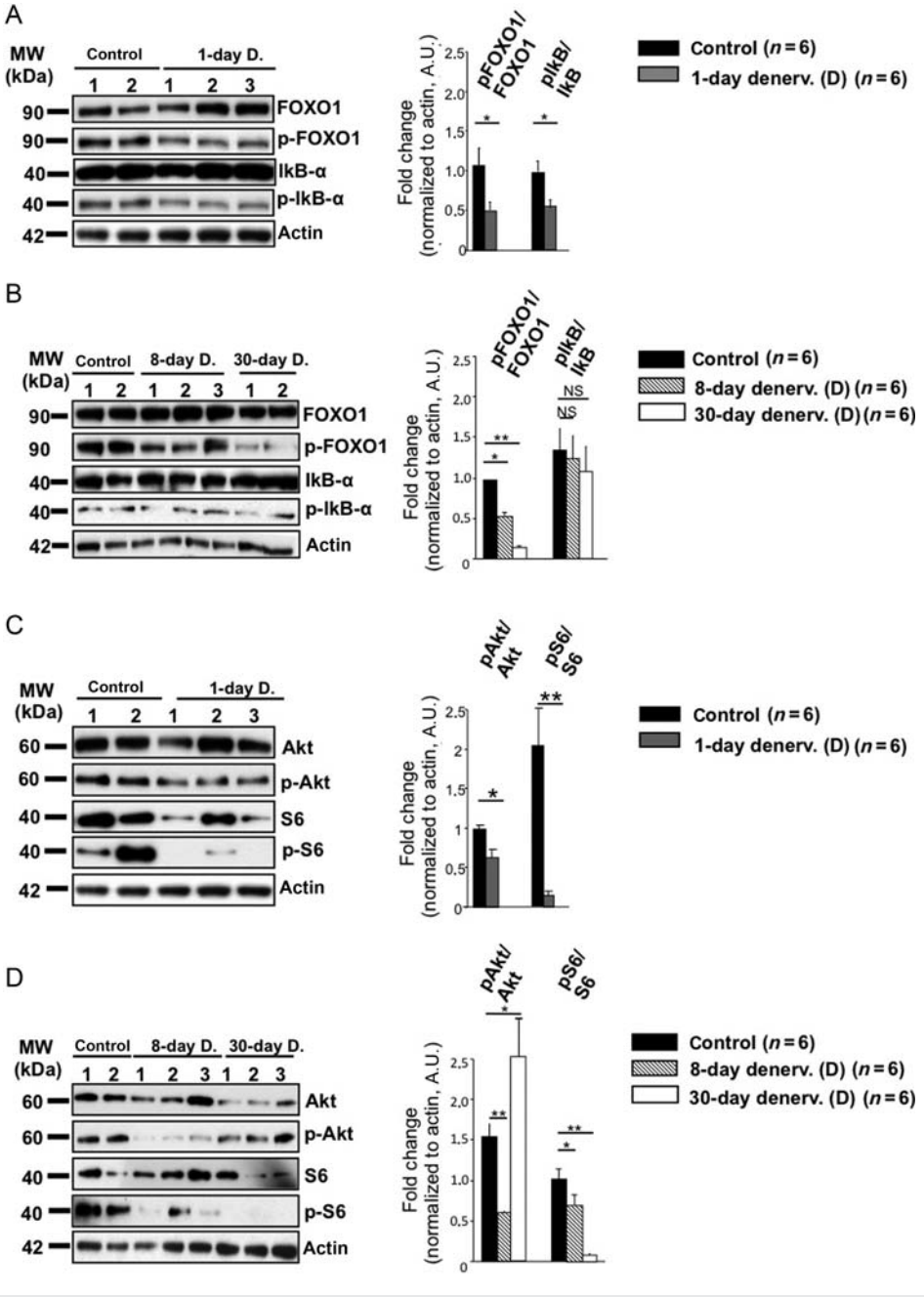
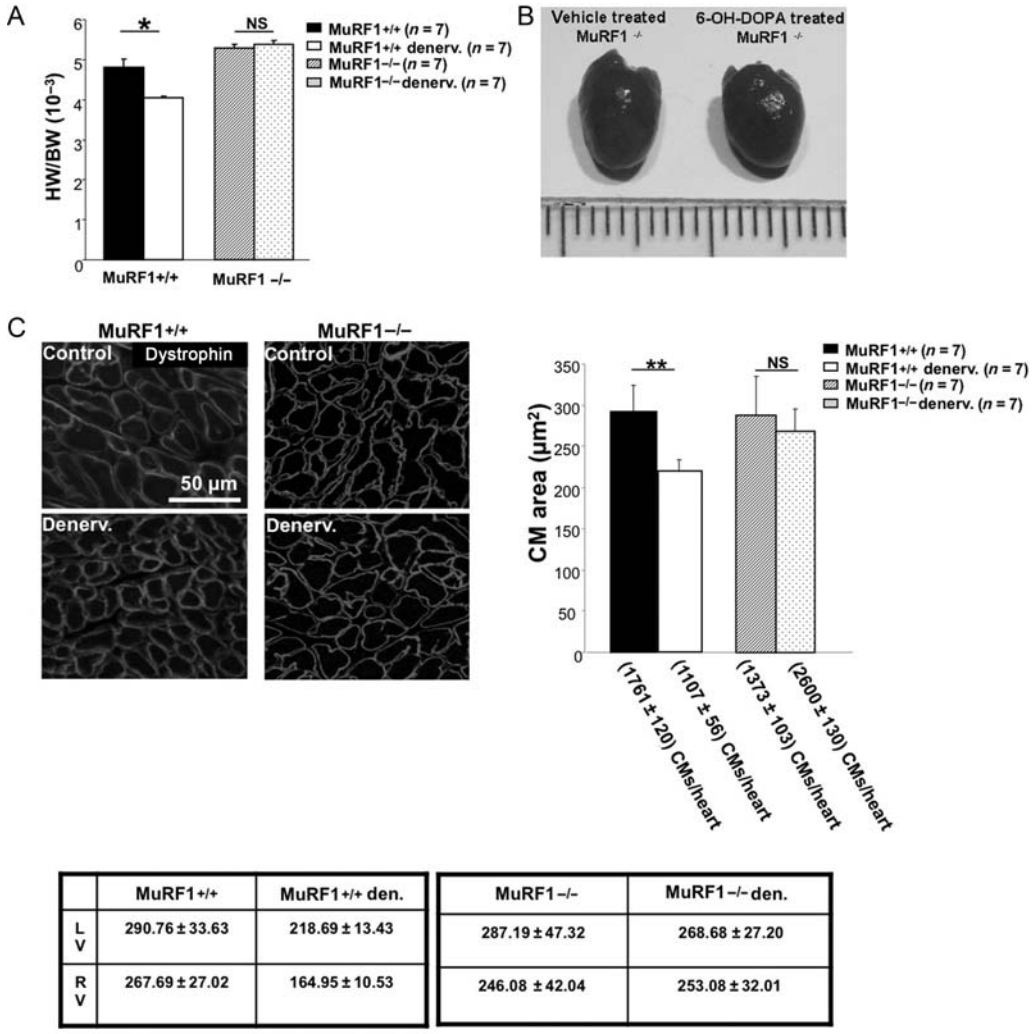



**Figure 4** UPS activation in denervated hearts is mediated by FOXO-dependent pathway. (*A*–*D*) Western blot analysis of total ventricular extracts from vehicle-treated, 1-, 8-, and 30-day-denervated hearts. MW, molecular weight. The densitometric analysis is reported. Error bars indicate SEM (*$0.01 < P < 0.05$, **$P < 0.01$; NS, no significant; $n = 6$ mice for each group).

induction of atrophic remodelling in cardiomyocytes, which become smaller than in normally innervated hearts, in the context of a structurally and functionally normal myocardium. Induction of the atrogenes depends on the reduction of constitutive adrenergic input to the β2-AR, with subsequent decrease in PI3K/Akt signalling that allows activation of the FOXO family of transcription factors and of their target genes MuRF1 and atrogin1/MAFbx. Therefore, our data indicate that the physiological cardiomyocyte size relies on constitutive activation of β2-AR signalling by neuronally released NE that negatively regulates the proteolytic machinery. Consistently, chronic administration of the β2-AR agonist CL prevented atrophic remodelling in denervated hearts, and mice lacking β2-ARs had smaller hearts in the absence of significant impairment in cardiac function. Furthermore, denervation atrophy did not develop in MuRF1 KO mice, indicating that among the main cardiac ubiquitin ligases, MuRF1 is dominant in determining the cardiomyocyte mass, a view consistent with the observation that MuRF1 targets include constituents of the sarcomere, which is responsible for nearly half of cardiac cell volume.[4] These results are in line with a recent report showing that acute β2-AR stimulation down-regulates MuRF1 and atrogin-1/MAFbx




| | MuRF1+/+ | MuRF1+/+ den. | MuRF1−/− | MuRF1−/− den. |
|---|---|---|---|---|
| LV | 290.76 ± 33.63 | 218.69 ± 13.43 | 287.19 ± 47.32 | 268.68 ± 27.20 |
| RV | 267.69 ± 27.02 | 164.95 ± 10.53 | 246.08 ± 42.04 | 253.08 ± 32.01 |

**Figure 5** MuRF1 knock-out (MurF1−/−) mice do not develop denervation-induced cardiac atrophy. (*A*) Evaluation of the heart weight/body weight (HW/BW) ratio in vehicle-treated control and 30-day-denervated MuRF1+/+ mice and vehicle-treated and 30-day-denervated MuRF1−/− mice. Error bars indicate SEM (*$0.01 < P < 0.05$; $n = 7$ mice for each group). (*B*) Hearts from vehicle-treated and denervated MuRF1−/− mice. (*C*) Immunofluorescence analysis of ventricular cryosections from vehicle-treated and 30-day-denervated MuRF1+/+ mice and vehicle-treated and 30-day-denervated MuRF1−/− mice stained with an antibody against dystrophin (left panel). Bar: 50 μm. Morphometric evaluation of the cardiomyocyte cross-sectional area is shown in the right panel. RV, right ventricle; LV, left ventricle. Error bars indicate SEM (*$0.01 < P < 0.05$, **$P < 0.01$; $n = 6$ mice for each group). The number of LV CMs (mean ± SD) evaluated for each heart is reported at the bottom of the graph.

genes,[26] and with those described in skeletal muscle, which undergoes atrophy upon sympathetic denervation, a process mediated by MuRF1 induction and antagonized by β2-AR stimulation.[39]

Although the mechanisms leading to cardiac hypertrophy have extensively been studied, using a variety of pharmacological, surgical, and genetic approaches, the mediators of cardiac atrophy are much less clarified. Atrophic remodelling of the heart has mainly been investigated in response to changes in metabolic and mechanical signals, for instance in models of caloric restriction and haemodynamic unloading [heterotopic heart transplantation (hht)], respectively.[32,47] These conditions mediate cardiac atrophy through up-regulation of ubiquitin ligases. In the case of caloric restriction atrophy, the AMPK pathway activates atrogenes responding to changes in the metabolic intake. In unloading atrophy, the ubiquitin ligases are activated by extreme haemodynamic unloading, with a nearly complete loss of contractility[48] and a degree of atrophic remodelling incompatible with cardiac function. The results of this study add to the picture the autonomic neuro-endocrine axis of cardiac size regulation, whereby the trophic effect of β2-AR-dependent signalling finely tunes cardiomyocyte protein balance within a more limited range. In fact, denervated hearts developed a much smaller atrophic remodelling even in long-term observation (HW/BW decrease, denervated: 15% vs. hht: 45%), in the absence of significant changes in cardiac function.

Further investigation will be needed to determine whether in the normal heart persistent activation of β2-AR signalling is obtained by an increase in the resting NE levels in the myocardium, or by repeated transient bursts of NE discharge. In the second model, receptor stimulation is obtained with repeated sympathetic discharge during locomotor activity, postural changes, or environmental stimulation.

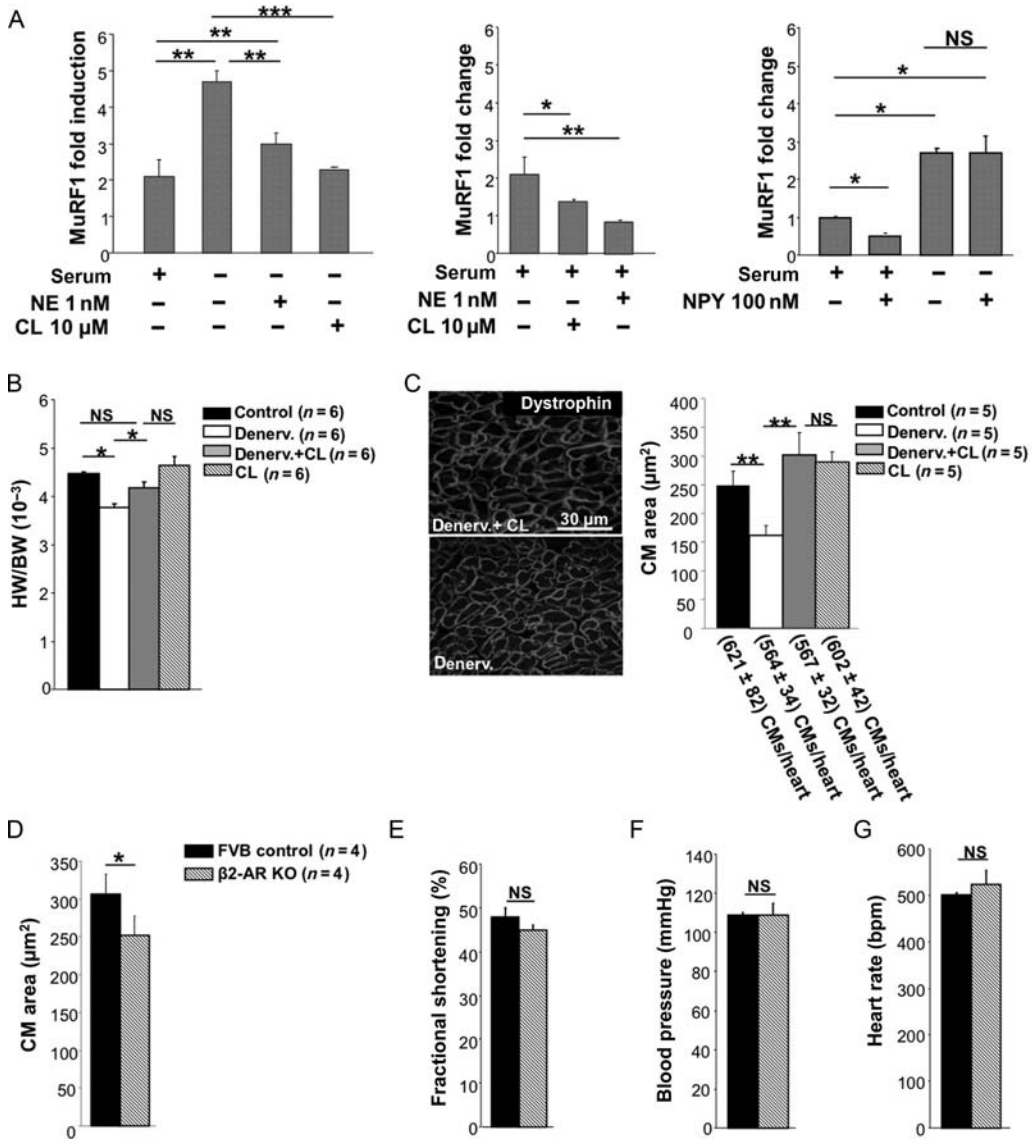



**Figure 6** β2-adrenoceptor (β2-AR) stimulation modulates cardiomyocyte trophism. (*A*) RT–qPCR analysis in extracts from rat neonatal cultured cardiomyocytes. Error bars indicate SEM (***$P < 0.001$; **$P < 0.01$; NS, no significant; $n = 6$, for each group). NE, norepinephrine; CL, clenbuterol; NPY, Neuropeptide Y. (*B*) Evaluation of heart weight/body weight (HW/BW) in vehicle-treated control, denervated, denervated + CL- and CL-treated mice. Error bars indicate SEM (*$P < 0.05$; $n = 6$, for each group). (*C*) Immunofluorescence analysis of ventricular cryosections from 30-day-denervated and 30-day-denervated + CL-treated mice stained with an antibody against dystrophin (left panel). Bar: 30 μm. Morphometric evaluation of the cardiomyocyte cross-sectional area of vehicle-treated control, denervated, denervated + CL- and CL-treated mice (right panel). Error bars indicate SEM (*$0.01 < P < 0.05$; NS, not significant; $n = 5$ mice for each group). The number of LV CMs (mean ± SD) evaluated for each heart is reported. (*D*) Morphometric evaluation of the cardiomyocyte cross-sectional area β2-adrenoceptor (β2-AR) KO mice and relative littermate controls. Error bars indicate SEM (*$P < 0.05$; number of animals = 4, for each group). (e.g.) Evaluation of fractional shortening (*E*), blood pressure (*F*), and heart rate (*G*) in vehicle-treated control and β2-AR KO mice. Error bars indicate SEM (*$P < 0.05$; NS, not significant; number of animals = 6 for each group).

Consistent with this model, we observed circadian variation in MuRF1 gene transcription with the lowest level detected during the dark period[29,49] (and Dyar, in preparation), when bursts of sympathetic activity are more frequent.[50]

Interestingly, β2-ARs are capable of independently control different signalling pathways through both Gs or β-arrestin-dependent pathways.[51] The reduction in Akt and ERK (Supplementary material online, *Figure S4*) pathways occurring in the denervated hearts in the absence of impaired contractile function suggests the intriguing hypothesis that the continuously active mechanism of transcriptional regulation of β2-AR on the atrogenes might be mediated by β-arrestin 2.[52–55]

## 5. Conclusions

Our results demonstrate that the sympathetic nervous system constitutively regulates the physiological cardiomyocyte size, through β2-AR-dependent modulation of protein degradation by the UPS.

This mechanism underlines the importance of regulated proteolysis in cardiac cell physiology, and expands our current knowledge on cardiac cell and tissue homoeostasis. Furthermore, the identification of a previously unrecognized function of the cardiac sympathetic innervation is of great clinical relevance given that prolonged activation of β-ARs is associated to hypertrophy and failure, and β-AR modulation is central in the treatment of these conditions.

# Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

## Acknowledgements

We are grateful to Dr Ken Dyar and Prof. Tullio Pozzan for helpful discussion, Dr Paola Pesce and D.C. for echocardiography analyses, Prof. Antoon Moorman for anti-βMHC antibody, Lino Bigon, Giulia Carotta, Valentina Mazzariol, and Dr. Anne Picard for technical assistance.

**Conflict of interest:** none declared.

## Funding

This work was supported by: University of Padova (Progetto di Ricerca di Ateneo 2010 to M.M. CPDA0977 and Progetto Giovani Studiosi 2010 to T.Z. GRIC101133), the Telethon Foundation, Grant n. GGP11224 to M.M.; Fondation Leducq and the Italian Ministry of Education (MiUR) to MS; Grants-Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, 2008/56483-1 and 2010/50048-1) to P.C.B.

## References

1. Baskin KK, Taegtmeyer H. AMP-activated protein kinase regulates E3 ligases in rodent heart. *Circ Res* 2011;**109**:1153–1161.
2. Willis MS, Townley-Tilson WHD, Kang EY, Homeister JW, Patterson C. Sent to destroy: the ubiquitin proteasome system regulates cell signaling and protein quality control in cardiovascular development and disease. *Circ Res* 2010;**106**: 463–478.
3. Wang X, Robbins J. Heart failure and protein quality control. *Circ Res* 2006;**99**: 1315–1328.
4. Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA *et al*. Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* 2001;**294**:1704–1708.
5. Gomes MD, Lecker SH, Jagoe RT, Navon A, Goldberg AL. Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. *Proc Natl Acad Sci USA* 2001;**98**:14440–14445.
6. Li H-H, Kedar V, Zhang C, McDonough H, Arya R, Wang D-Z *et al*. Atrogin-1/muscle atrophy F-box inhibits calcineurin-dependent cardiac hypertrophy by participating in an SCF ubiquitin ligase complex. *J Clin Invest* 2004;**114**:1058–1071.
7. Li H-H, Willis MS, Lockyer P, Miller N, McDonough H, Glass DJ *et al*. Atrogin-1 inhibits Akt-dependent cardiac hypertrophy in mice via ubiquitin-dependent coactivation of Forkhead proteins. *J Clin Invest* 2007;**117**:3211–3223.
8. Arya R, Kedar V, Hwang JR, McDonough H, Li H-H, Taylor J *et al*. Muscle ring finger protein-1 inhibits PKC{epsilon} activation and prevents cardiomyocyte hypertrophy. *J Cell Biol* 2004;**167**:1147–1159.
9. Willis MS, Ike C, Li L, Wang D-Z, Glass DJ, Patterson C. Muscle ring finger 1, but not muscle ring finger 2, regulates cardiac hypertrophy *in vivo*. *Circ Res* 2007;**100**:456–459.
10. Preedy VR, Smith DM, Kearney NF, Sugden PH. Rates of protein turnover *in vivo* and *in vitro* in ventricular muscle of hearts from fed and starved rats. *Biochem J* 1984;**222**: 395–400.
11. Samarel AM, Parmacek MS, Magid NM, Decker RS, Lesch M. Protein synthesis and degradation during starvation-induced cardiac atrophy in rabbits. *Circ Res* 1987;**60**: 933–941.
12. Klein I, Hong C, Schreiber SS. Cardiac atrophy in the heterotopically transplanted rat heart: *in vitro* protein synthesis. *J Mol Cell Cardiol* 1990;**22**:461–468.
13. Sacheck JM, Hyatt J-PK, Raffaello A, Jagoe RT, Roy RR, Edgerton VR *et al*. Rapid disuse and denervation atrophy involve transcriptional changes similar to those of muscle wasting during systemic diseases. *FASEB J* 2007;**21**:140–155.
14. Wing SS, Haas AL, Goldberg AL. Increase in ubiquitin-protein conjugates concomitant with the increase in proteolysis in rat skeletal muscle during starvation and atrophy denervation. *Biochem J* 1995;**307**:639–645.
15. Clarke BA, Drujan D, Willis MS, Murphy LO, Corpina RA, Burova E *et al*. The E3 Ligase MuRF1 degrades myosin heavy chain protein in dexamethasone-treated skeletal muscle. *Cell Metab* 2007;**6**:376–385.
16. Cohen S, Brault JJ, Gygi SP, Glass DJ, Valenzuela DM, Gartner C *et al*. During muscle atrophy, thick, but not thin, filament components are degraded by MuRF1-dependent ubiquitylation. *J Cell Biol* 2009;**185**:1083–1095.
17. Sampson KJ, Kass RS. Molecular mechanisms of adrenergic stimulation in the heart. *Heart Rhythm* 2010;**7**:1151–1153.
18. Altman JD, Trendelenburg AU, MacMillan L, Bernstein D, Limbird L, Starke K *et al*. Abnormal regulation of the sympathetic nervous system in alpha2A-adrenergic receptor knockout mice. *Mol Pharmacol* 1999;**56**:154–161.
19. Hein L, Altman JD, Kobilka BK. Two functionally distinct alpha2-adrenergic receptors regulate sympathetic neurotransmission. *Nature* 1999;**402**:181–184.
20. Kiriazis H, Du XJ, Feng X, Hotchkin E, Marshall T, Finch S *et al*. Preserved left ventricular structure and function in mice with cardiac sympathetic hyperinnervation. *Am J Physiol Heart Circ Physiol* 2005;**289**:H1359–H1365.
21. Ryall JG, Sillence MN, Lynch GS. Systemic administration of beta2-adrenoceptor agonists, formoterol and salmeterol, elicit skeletal muscle hypertrophy in rats at micromolar doses. *Br J Pharmacol* 2006;**147**:587–595.
22. Tevaearai HT, Eckhart AD, Walton GB, Keys JR, Wilson K, Koch WJ. Myocardial gene transfer and overexpression of beta2-adrenergic receptors potentiates the functional recovery of unloaded failing hearts. *Circulation* 2002;**106**:124–129.
23. Birks EJ, Tansley PD, Hardy J, George RS, Bowles CT, Burke M *et al*. Left ventricular assist device and drug therapy for the reversal of heart failure. *N Engl J Med* 2006;**355**: 1873–1884.
24. Lee S, Grafweg S, Schneider T, Jimenez M, Giacobino JP, Ghanem A *et al*. Total beta-adrenoceptor deficiency results in cardiac hypotrophy and negative inotropy. *Physiol Res* 2010;**59**:679–689.
25. Nayler WG, Slade A, Vaughan Williams EM, Yepez CE. Effect of prolonged beta-adrenoceptor blockade on heart weight and ultrastructure in young rabbits. *Br J Pharmacol* 1980;**68**:363–371.
26. Zhang W, Yano N, Deng M, Mao Q, Shaw SK, Tseng Y-T. β-Adrenergic receptor-PI3K signaling crosstalk in mouse heart: elucidation of immediate downstream signaling cascades. *PLoS One* 2011;**6**:e26581.
27. Finch L, Haeusler G, Thoenen H. A comparison of the effects of chemical sympathectomy by 6-hydroxydopamine in newborn and adult rats. *Br J Pharmacol* 1973;**47**: 249–260.
28. Daffonchio A, Franzelli C, Di Rienzo M, Castiglioni P, Ramirez AJ, Parati G *et al*. Effect of sympathectomy on blood pressure variability in the conscious rat. *J Hypertens Suppl* 1991;**9**:S70–S71.
29. Makino M, Hayashi H, Takezawa H, Hirai M, Saito H, Ebihara S. Circadian rhythms of cardiovascular functions are modulated by the baroreflex and the autonomic nervous system in the rat. *Circulation* 1997;**96**:1667–1674.
30. Depre C, Davies PJ, Taegtmeyer H. Transcriptional adaptation of the heart to mechanical unloading. *Am J Cardiol* 1999;**83**:58H–63H.
31. Jagoe RT, Goldberg AL. What do we really know about the ubiquitin-proteasome pathway in muscle atrophy? *Curr Opin Clin Nutr Metab Care* 2001;**4**:183–190.
32. Gruber C, Nink N, Nikam S, Magdowski G, Kripp G, Voswinckel R *et al*. Myocardial remodelling in left ventricular atrophy induced by caloric restriction. *J Anat* 2012;**220**: 179–185.
33. Kruger M, Moser M, Ussar S, Thievessen I, Luber CA, Forner F *et al*. SILAC mouse for quantitative proteomics uncovers kindlin-3 as an essential factor for red blood cell function. *Cell* 2008;**134**:353–364.
34. Sandri M. Autophagy in health and disease. 3. Involvement of autophagy in muscle atrophy. *Am J Physiol Cell Physiol* 2010;**298**:C1291–C1297.
35. Skurk C, Izumiya Y, Maatz H, Razeghi P, Shiojima I, Sandri M *et al*. The FOXO3a transcription factor regulates cardiac myocyte size downstream of AKT signaling. *J Biol Chem* 2005;**280**:20814–20823.
36. Cai D, Frantz JD, Tawa NE, Melendez PA, Oh B-C, Lidov HGW *et al*. IKKbeta/NF-kappaB activation causes severe muscle wasting in mice. *Cell* 2004;**119**:285–298.
37. Nicholl SM, Bell D, Spiers Jp, McDermott BJ. Neuropeptide Y Y(1) receptor regulates protein turnover and constitutive gene expression in hypertrophying cardiomyocytes. *Eur J Pharmacol* 2002;**441**:23–34.
38. Deng XF, Rokosh DG, Simpson PC. Autonomous and growth factor-induced hypertrophy in cultured neonatal mouse cardiac myocytes. Comparison with rat. *Circ Res* 2000;**87**:781–788.
39. Goncalves DA, Silveira WA, Lira EC, Graca FA, Paula-Gomes S, Zanon NM *et al*. Clenbuterol suppresses proteasomal and lysosomal proteolysis and atrophy-related genes in denervated rat soleus muscles independently of Akt. *Am J Physiol Endocrinol Metab* 2012;**302**:E123–E133.
40. Triposkiadis F, Karayannis G, Giamouzis G, Skoularigis J, Louridas G, Butler J. The sympathetic nervous system in heart failure physiology, pathophysiology, and clinical implications. *J Am Coll Cardiol* 2009;**54**:1747–1762.
41. Wang W, Zhu W, Wang S, Yang D, Crow MT, Xiao RP *et al*. Sustained beta1-adrenergic stimulation modulates cardiac contractility by Ca2+/calmodulin kinase signaling pathway. *Circ Res* 2004;**95**:798–806.
42. Schluter KD, Millar BC, McDermott BJ, Piper HM. Regulation of protein synthesis and degradation in adult ventricular cardiomyocytes. *Am J Physiol* 1995;**269**:C1347–C1355.

43. Levin HR, Oz MC, Chen JM, Packer M, Rose EA, Burkhoff D. Reversal of chronic ventricular dilation in patients with end-stage cardiomyopathy by prolonged mechanical unloading. *Circulation* 1995;**91**:2717–2720.
44. Razeghi P, Sharma S, Ying J, Li Y-P, Stepkowski S, Reid MB *et al.* Atrophic remodeling of the heart *in vivo* simultaneously activates pathways of protein synthesis and degradation. *Circulation* 2003;**108**:2536–2541.
45. Kundu M, Thompson CB. Macroautophagy versus mitochondrial autophagy: a question of fate? *Cell Death Differ* 2005;**12**(Suppl 2):1484–1489.
46. Zheng Q, Li J, Wang X. Interplay between the ubiquitin-proteasome system and autophagy in proteinopathies. *Int J Physiol Pathophysiol Pharmacol* 2009;**1**:127–142.
47. Sharma S, Ying J, Razeghi P, Stepkowski S, Taegtmeyer H. Atrophic remodeling of the transplanted rat heart. *Cardiology* 2006;**105**:128–136.
48. Razeghi P, Volpini KC, Wang M-E, Youker KA, Stepkowski S, Taegtmeyer H. Mechanical unloading of the heart activates the calpain system. *J Mol Cell Cardiol* 2007;**42**:449–452.
49. Oosting J, Struijker-Boudier HA, Janssen BJ. Autonomic control of ultradian and circadian rhythms of blood pressure, heart rate, and baroreflex sensitivity in spontaneously hypertensive rats. *J Hypertens* 1997;**15**:401–410.
50. Wang N, Yang G, Jia Z, Zhang H, Aoyagi T, Soodvilai S *et al.* Vascular PPARgamma controls circadian variation in blood pressure and heart rate through Bmal1. *Cell Metab* 2008;**8**:482–491.
51. Nobles KN, Xiao K, Ahn S, Shukla AK, Lam CM, Rajagopal S *et al.*. Distinct phosphorylation sites on the beta(2)-adrenergic receptor establish a barcode that encodes differential functions of beta-arrestin. *Sci Signal* 2011;**4**:ra51.
52. Shenoy SK, Drake MT, Nelson CD, Houtz DA, Xiao K, Madabushi S *et al.* beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. *J Biol Chem* 2006;**281**:1261–1273.
53. Drake MT, Shenoy SK, Lefkowitz RJ. Trafficking of G protein-coupled receptors. *Circ Res* 2006;**99**:570–582.
54. Zhu WZ, Zheng M, Koch WJ, Lefkowitz RJ, Kobilka BK, Xiao RP. Dual modulation of cell survival and cell death by beta(2)-adrenergic signaling in adult mouse cardiac myocytes. *Proc Natl Acad Sci USA* 2001;**98**:1607–1612.
55. Chesley A, Lundberg MS, Asai T, Xiao RP, Ohtani S, Lakatta EG *et al.* The beta(2)-adrenergic receptor delivers an antiapoptotic signal to cardiac myocytes through G(i)-dependent coupling to phosphatidylinositol 3′-kinase. *Circ Res* 2000;**87**:1172–1179.