J Physiol 595.12 (2017) pp 3919–3930

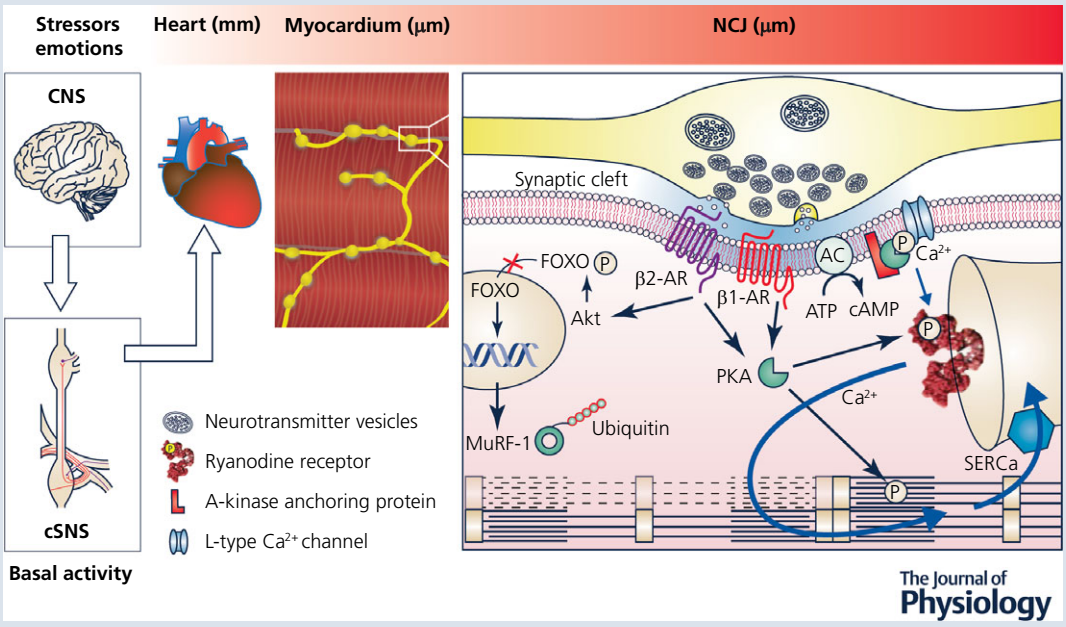
The Journal of Physiology

TOPICAL REVIEW

Cardiac sympathetic innervation, from a different point of (re)view

Tania Zaglia[1,2,3] and Marco Mongillo[2,3,4]

[1] *Department of Cardiac, Thoracic and Vascular Sciences, via Giustiniani 2, 35128, University of Padova, Padova, Italy*
[2] *Department of Biomedical Sciences, via Ugo Bassi 58/B, 35131, University of Padova, Padova, Italy*
[3] *Venetian Institute of Molecular Medicine, via G.Orus, 2, 35129, Padova, Italy*
[4] *CNR institute of Neurosciences, viale Colombo 3, 35133 Padova, Italy*



Abstract The audience of basic and clinical scientists is familiar with the notion that the sympathetic nervous system controls heart function during stresses. However, evidence indicates that the neurogenic control of the heart spans from the maintenance of housekeeping functions in resting conditions to the recruitment of maximal performance, in the fight-or-flight responses, across a whole range of intermediate states. To perform such sophisticated functions, sympathetic ganglia integrate both peripheral and central inputs, and transmit information to the heart via 'motor' neurons, directly interacting with target cardiomyocytes. To date, the dynamics and mode of communication between these two cell types, which determine how neuronal

Tania Zaglia, PhD, is a junior group leader at the VIMM, Padova. Her research focuses on the role of cardiac sympathetic innervation in the regulation of cardiomyocyte structure through modulation of intracellular proteostasis. She has discovered how cardiac sympathetic innervation regulates cardiac mass, and identified how alterations in cardiac proteostasis lead to cardiomyopathy. Her research uses *in vitro* and *in vivo* models, with various techniques from cellular biochemistry and signalling, to organ physiology. To train her cardiac sympathetic neurons, she works out at the gym. **Marco Mongillo**, MD, PhD, coordinates the Molecular Cardiology lab at the VIMM, and his main research interests are centred on the intercellular communication of sympathetic neurons with their myocardial cellular targets. He developed and uses novel tools to explore second messenger signalling, using confocal and multiphoton microscopy, electrophysiology and optogenetics, both in cellular models and *in vivo*. To avoid experimental biases, he refrains from exercise.

DOI: 10.1113/JP273120

information is adequately translated into the wide spectrum of cardiac responses, are still blurry. By combining the anatomical and structural information brought to light by recent imaging technologies and the functional evidence in cellular systems, we focus on the interface between neurons and cardiomyocytes, and advocate the existence of a specific 'neuro-cardiac junction', where sympathetic neurotransmission occurs in a 'quasi-synaptic' way. The properties of such junctional-type communication fit well with those of the physiological responses elicited by the cardiac sympathetic nervous system, and explain its ability to tune heart function with precision, specificity and elevated temporal resolution.

(Received 9 November 2016; accepted after revision 23 February 2017; first published online 27 February 2017)
Corresponding authors M. Mongillo: CNR institute of Neurosciences, viale Colombo 3, 35133 Padova, Italy. Email: marco.mongillo@unipd.it; T. Zaglia: Department of Cardiac, Thoracic and Vascular Sciences, via Giustiniani 2, 35128, University of Padua, Padova, Italy. Email: tania.zaglia@unipd.it

Abstract figure legend The cardiac sympathetic nervous system continuously controls heart function, by integrating inputs from environmental and intrinsic stimuli, including stresses and emotions (CNS, top box), relayed to the heart through the postganglionic effector neurons which originate from the sympathetic ganglia chain (cSNS, bottom box). The heart is densely innervated by sympathetic neurons, whose processes lay on CMs, establishing multiple discrete neuro-cardiac contacts in correspondence to neurotransmitter releasing sites (middle box, enlarged). Recent work reviewed here supports the concept that the specific molecular organization of the neuro-cardiac junctions (right box) allows signal transmission between neurons and cardiac effector cells to operate in a *quasi-synaptic* behaviour.

Abbreviations AR, adrenoceptors; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; EM, electron microscopy; EPSP, excitatory postsynaptic potential; HCN4, hyperpolarization activated cyclic nucleotide gated potassium channel 4; HR, heart rate; HRV, heart rate variability; MuRF1, muscle ring finger protein-1; NCX, sodium–calcium exchanger; NA, noradrenaline; NMJ, neuro-muscular junction; NPY, neuropeptide Y; PKA, protein kinase A; PLN, phospholamban; RyR2, ryanodine receptors 2; SAN, sino-atrial node; SN, sympathetic neurons; SNS, sympathetic nervous system.

Introduction

The autonomic nervous system continuously controls heart performance, through the cardiac sympathetic and parasympathetic neurons (SNs and PSNs), to match blood output with the perfusional demand of the organism, both during daily activities and in response to stressors (Bers & Despa, 2009). To perform such housekeeping functions, cervical and thoracic sympathetic ganglia and cardiac parasympathetic ganglia receive inputs from both peripheral afferents, sensing mechanical, metabolic and chemical parameters, and efferents coming from the central nervous system, which transmits information on physiological state, behaviour and emotions (James, 1980; Armour *et al.* 1997; Samuels, 2007; Taggart *et al.* 2016). The autonomic post-ganglionic 'motor' neurons directly innervate the heart, and are coupled to the conducting (SNs and PSNs) and working cardiomyocytes (almost exclusively SNs) (Hoover *et al.* 2004), thus serving as 'end-effectors' of the multiplexed network forming the backbone of the brain–heart connections. Several comprehensive articles (for a topical collection see *The Journal of Physiology*, June 2016) have reviewed various aspects of the neurocardiology of the autonomic nervous system, including the sophisticated regulation of its 'proximal' pre-ganglionic section (Ardell & Armour, 2016; Habecker *et al.* 2016; Janig, 2016), brainstem and higher centres processing (Shivkumar & Ardell, 2016), and the role of cyclic nucleotide signalling in the modulation of sympatho-vagal balance (Li & Paterson, 2016).

We will focus here on cardiac SNs, which densely innervate all myocardial regions, are implicated in several heart diseases, and represent the main topic of our current research. Our interest stems from a question opened by the large body of evidence collected during the last few decades of neuro-cardiology research (Ardell & Armour, 2016). Remarkably, a plethora of qualitatively different inputs have been shown to influence the activity of cardiac SNs which, in turn, control accordingly heart physiology by operating at multiple levels, from the modulation of heart rate (HR) and contractility (Li *et al.* 2000; Bers, 2001; Shan *et al.* 2010), to the regulation of cardiomyocyte size and structure (Ogawa *et al.* 1992; Kanevskij *et al.* 2002; O'Connell *et al.* 2003; Zaglia *et al.* 2013; Kreipke & Birren, 2015). The dynamics and modality of communication between neurons and cardiomyocytes determine how neuronal information is conveyed to the cardiac targets. Here, we will review data on the structural and functional basis of the SN–cardiomyocyte interaction, and speculate on how the signalling dynamics of intercellular communication may allow the system to work with *wide effect range, precision* and *specificity* of responses to the diverse stimuli.

Sympathetic innervation of the heart: a network crowded with neuro-cardiac contacts

The whole mammalian heart is innervated by SNs, which enter the heart from the epicardium and extend their processes throughout the myocardial interstitium, running parallel to capillary vessels (Ieda *et al.* 2007; Kimura *et al.* 2012). Neuronal sympathetic processes display the characteristic pearl necklace morphology, with repeated enlargements (varicosities) where the neuroexocytosis apparatus localizes, releasing various neurotransmitters (mostly noradrenaline (NA), but also neuropeptide Y (NPY) and ATP) (Thaemert, 1966; Sosunov *et al.* 1997) (Fig. 1*A* and *B*). In the last few decades, the literature has convincingly shown that the heart is populated by a high number of neurons, but the extent of the innervation density has somehow remained underestimated for a long time (Zhou *et al.* 2004; Ieda *et al.* 2007; Clarke *et al.* 2010; Muhlfeld *et al.* 2010). The idea that not all cardiomyocytes are in touch with a neuronal process is a common tenet, even among basic cardiologists, physiologists and anatomists. Such under-appreciation of the coverage of the heart neuronal network is possibly the consequence of the imaging methods used to visualize SNs in the tissue, as most studies had specific objectives and used very high (electron microscopy, EM) or very low magnification (histology) methods (Baluk & Fujiwara, 1984; Choate *et al.* 1993; Zhou *et al.* 2004; Ieda *et al.* 2007; Clarke *et al.* 2010; Muhlfeld *et al.* 2010; Pauza *et al.* 2014). Recently, by meticulously quantifying the number of neurons in serial myocardial sections, stained with antibodies for neuron-specific markers, we have estimated that processes are present in the heart (Anversa *et al.* 1989; Hirsch *et al.* 2013; Franzoso *et al.* 2016; Pianca *et al.* 2016) in a proportion close to the capillary–cardiomyocyte ratio (Anversa *et al.* 1989; Hirsch *et al.* 2013; Franzoso *et al.* 2016; Pianca *et al.* 2016). By imaging the heart with the three-dimensional capabilities of two-photon microscopy, the smallest sympathetic processes can be appreciated, showing that, at least for the rodent heart, not only are all cardiomyocytes in contact with several varicosities from the same neuronal process, but also that each cardiomyocyte establishes parallel contacts, possibly with processes from different neurons (Freeman *et al.* 2014) (Fig. 1*C*). Beyond cardiomyocytes, which account for the largest mass, the heart is made of several other physically and functionally connected cell types, for the most part cardiac fibroblasts and endothelial cells (Franzoso *et al.* 2016). Thus from a 'holistic' point of view, the heart consists of a complex multicellular network, held together by an interstitial cast of extracellular matrix, and encapsulated in a dense mesh of neurons. Interestingly, all the aforementioned myocardial cells express receptors for sympathetic neurotransmitters (Queen & Ferro, 2006; Diaz-Araya *et al.* 2015), and thanks to the capillary innervation of the heart, each of these cells is within short range of a neuronal process, suggesting that cardiac SNs may control myocardial function in a cell-specific fashion.

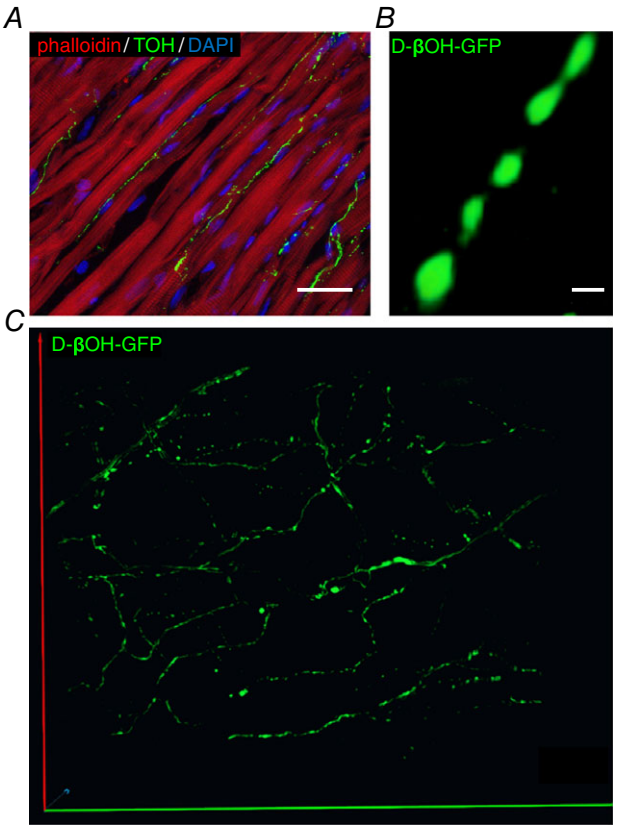


Figure 1. Two- and three-dimensional reconstructions of the sympathetic innervation of the murine myocardium
A, confocal immunofluorescence imaging of myocardial sections from normal adult mice, stained with an antibody to tyrosine hydroxylase (TOH, green) and TRITC-conjugated phalloidin (red). Nuclei are counterstained with DAPI (blue). The image is a detail from the left ventricular subepicardial region. Scale bar: 25 μm. *B*, SNs innervating the myocardium show the typical pearl-necklace morphology as shown in high-magnification confocal fluorescence images of myocardial sections from dopamine-β-hydroxylase-GFP (D-βOH-GFP) transgenic mice. Scale bar: 1 μm. *C*, three-dimensional reconstruction, at the 2-photon microscope of the sympathetic network within a portion of the left ventricular subepicardium in an adult, Langendorff-perfused D-βOH-GFP heart. A segment of 230 μm by 280 μm by 50 μm was imaged. Images in *B* and *C*, are reproduced with permission from Freeman *et al.*, *J Neurosci Methods* 2014.

How the cardiac sympathetic nervous system runs the show

The textbook view of the cardiac sympathetic nervous system (SNS) is best represented by the acute activation of neuronal NA discharge in the so-called 'fight-or-flight' reaction to both intrinsic (haemodynamic, emotional)

and extrinsic (fear, pain) stressors (Jansen *et al.* 1995). However, it is worth remembering that, even in unstressed conditions, SNs control fundamental cardiac homeostatic mechanisms, i.e. by participating to the fine-tuning of HR (Lombardi *et al.* 1996), and activating long-term β-adrenoceptor (β-AR)-dependent pathways involved in transcriptional control and cell division (Ogawa *et al.* 1992; Kanevskij *et al.* 2002; O'Connell *et al.* 2003; Zaglia *et al.* 2013; Kreipke & Birren, 2015). In addition, recent studies have revealed that both SNs and PSNs, by releasing brain-derived natriuretic factor (BDNF), tonically influence cardiac contractility and relaxation (Feng *et al.* 2015), thus indicating that both branches of the cardiac autonomic nervous system cooperate in the regulation of 'basal' heart physiology. It is remarkable, and yet unexplained, how information can be carried by the same neurons, encoded by a limited number of variables (amplitude and frequency of action potentials), and elicit responses as different as subtle or extreme modulation of HR, short-term control of inotropy (mostly mediated by β1-ARs) (Jansen *et al.* 1995) and long-term regulation of cell size (controlled by β2-ARs) (Zaglia *et al.* 2013). Here, we will present evidence showing that such multiple abilities of the cardiac SNS may be enabled by specific organization of the SN–cardiomyocyte contacts, and the peculiar signalling dynamics underlying neuro-cardiac coupling.

The fight-or-flight response

Sympathetic neuron activation, following acute stresses, elicits maximal transient activation of heart chronotropy and inotropy, an evolutionarily conserved, life-saving effect, occurring potently and instantaneously, known as 'fight-or-flight' response. The positive inotropic effects are mediated by stimulation of β-ARs, leading to an increase in cAMP and activation of PKA, which phosphorylates key Ca^{2+} handling proteins, including L-type Ca^{2+} channels, PLN and RyR2, which together increase the availability of Ca^{2+} for contraction (Rochais *et al.* 2004; Bers, 2008). The chronotropic effect depends on β-ARs (mainly β1) of sino-atrial node (SAN) cells, which regulate the pacemaker rate, mostly through actions on HCN4- and NCX-driven membrane currents dependent on cAMP and Ca^{2+} signalling (Stieber *et al.* 2003; DiFrancesco, 2010) (Fig. 2). In these situations, an increase in chronotropy and inotropy occurs simultaneously, to maximize cardiac output, and the common tenet is that myocardial β-ARs are stimulated by the increase in [NA] diffusely released by SNs throughout the myocardial interstitium (Mann *et al.* 2014). This has been the prevailing view to explain the physiology of the acute neurogenic control of heart function. However, there are some factors that encourage refinement of this, apparently linear, working model.

Neuronal NA is discharged into the myocardial interstitium, which:

(i) has a volume larger by several orders of magnitude than that of neurotransmitter vesicles (Thaemert, 1966);
(ii) is bathed by fluids in constant flow from the arterial to the venous sections of the coronary circulation;
(iii) contains a large set of catecholamine degradation enzymes (Kaludercic *et al.* 2010).

Considering all of these features, the neuro-cardiac communication system described above would be rather inefficient, and would seem to be designed to protect the interstitial compartment from the excess of NA, rather than enabling prompt and efficient communication with cardiac cells. In fact, neurons would be required to release a very high amount of NA to achieve a steady-state concentration of agonist sufficient to activate β-ARs, to the extent and for as long as necessary to elicit adequate cardiac responses. This would necessitate elevated energy expenditure for NA synthesis, release and re-uptake, making prompt NA availability upon closely repeated stimulation difficult. An alternative view, which would better fit with the physiological requirements of the system, would imply a direct interaction between the neurotransmitter releasing (neuronal varicosities) and sensing (cardiomyocyte membranes) sites, and would identify the intercellular space as the active compartment where neurotransmission takes place.

Basal effects of sympathetic neurons on sino-atrial node cells

The latitude of the effects of SN-dependent control of SAN function is very wide. In resting conditions, even during sinusal rhythm, physiological variation in the interval between consecutive beats (known as heart rate variability, HRV) can be observed, without appreciable change in catecholamine concentration in the plasma compartment (Fig. 2). While the neurogenic mechanisms regulating HRV have mostly been attributed to the PSN/vagal influence, the SNs participate with other factors (e.g. hormones, respiratory rate, haemodynamic reflexes and temperature) in the modulation of this parameter (Lombardi *et al.* 1996). Consistently, blockade of neuronal input to the heart with atropine and propranolol, as well as heart transplant, causing heart denervation, ablate such variability, resulting in a fixed HR (Ahmed *et al.* 1994; Poletto *et al.* 2011; Vanderlaan *et al.* 2012). At the molecular level, the sympathetic input accelerates SAN automaticity, as explained above, although to a reduced degree compared to the full-blown activation of the fight-or-flight reaction. These different effects could be dependent on minimal neuronal discharge of NA, which, however, would be significantly diluted upon release into

the interstitial spaces. In addition, the kinetics of NA washout and degradation, combined with the presence of both sympathetic and parasympathetic neuronal endings, would together oppose to the temporally precise control of HR on a beat-to-beat basis. The anatomy of SAN innervation, and the temporal precision of pacemaker regulation are indirect clues supporting a model whereby direct neuro-cardiac coupling underlies neurogenic control of heart chronotropism. This hypothesis is further supported by the observation that in co-cultures, direct

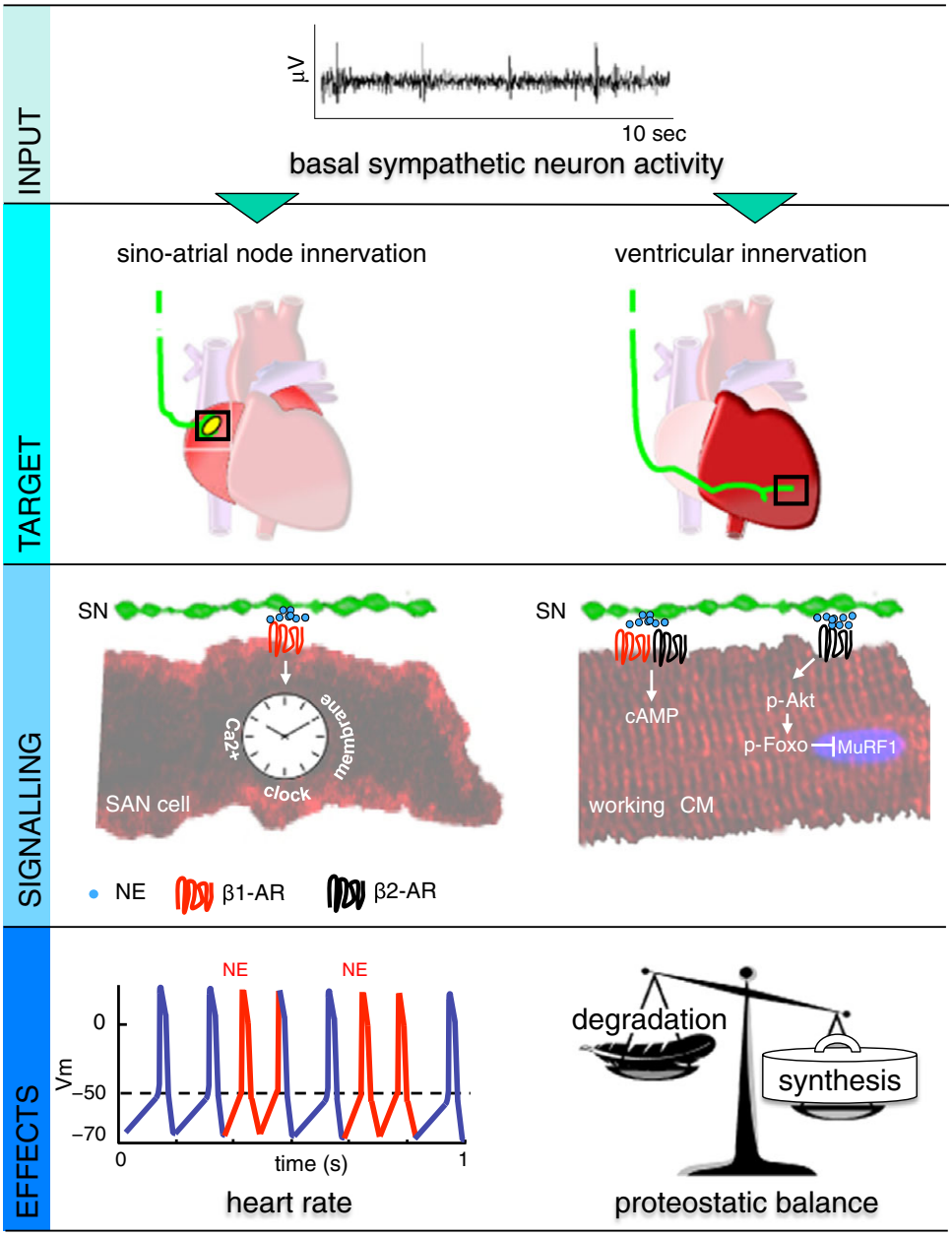


Figure 2. Effects of resting cardiac sympathetic neuron activity
Resting SN activity (INPUT) leads to basal activation of β-ARs expressed by both SAN cells and ventricular cardiomyocytes (TARGET). In the SAN, activation of β1-ARs increases automaticity by modulating the so-called Ca^{2+} and membrane clocks, as reviewed in Lakatta *et al.*, *Circ Res 2010* (SIGNALLING, left panel). On these bases, SNs innervating the SAN contribute to the non-random variation of the beat-to-beat intervals known as HRV (EFFECTS, left panel). At the level of ventricular cardiomyocytes, NA activates via β-ARs the cAMP–PKA signalling pathway, which increases Ca^{2+} availability for cell contraction. In resting conditions, SNs constitutively activate β2-ARs which, by impinging on the Akt–FOXO pathway, repress protein degradation (SIGNALLING, right panel). Thus, resting SN activity constitutively regulates the cardiomyocyte proteostatic equilibrium and is required for the maintenance of the correct cardiac mass (EFFECTS, right panel).

intercellular contact is required for neurons to modulate cardiomyocyte beating rate (Oh *et al.* 2016).

Basal effects of sympathetic neurons on ventricular cardiomyocytes

The role of SN in regulating basal HR dynamics, as well as the influence of NA in promoting, via α1-ARs, cardiomyocyte hypertrophy in the early postnatal heart growth, are well accepted notions (O'Connell *et al.* 2003). Recently, several studies, including ours, have indicated that resting activity of SNs also impacts on fully developed ventricular cardiomyocytes, constitutively and independently from the presence of acute stressors (Zaglia *et al.* 2013; Kreipke & Birren, 2015). This evidence is supported by the effect of sympathetic denervation on the control of the Akt–FOXO pathway, which transcriptionally regulates the main muscle-specific ubiquitin ligases, thus impinging on intracellular protein degradation and, in turn, on cell size. Indeed, soon after ablation of the SNs in mice, the heart underwent a significant decrease in mass and cell size (atrophic remodelling), which was prevented by treatment of the mice with the β2-AR agonist clenbuterol, and was absent in mice lacking the ubiquitin ligase MuRF1 (Zaglia *et al.* 2013). It has been shown that sympathetic processes distribute within the heart walls in a species-specific pattern, which results, in the mouse myocardium, in higher innervation density in sub-epicardial, than sub-endocardial regions. Interestingly, the transmural distribution of cardiomyocyte size correlates with innervation density, and such differences are ablated by interfering with the β2-AR–Akt–MuRF-1 signalling pathway (Hirsch *et al.* 2013; Pianca *et al.* 2016). Taken together, these results demonstrate that the sole requirement for the maintenance of cardiac structure throughout the entire life span is the presence and physiological distribution of SNs, evidently working at their resting activity level, being responsible for cardiomyocyte eutrophy. In addition, these data suggest that the neurotransmitter discharged by ventricular SNs has a limited spatial range of action, which may result from the close apposition between varicosities and target cell membranes. Together with the evidence that both β1- and β2-ARs preferentially accumulate on cardiomyocyte membrane in proximity to the neuronal varicosity (Devic *et al.* 2001; Shcherbakova *et al.* 2007), these results support the functional role of direct neuro-cardiac coupling (Fig. 3). In addition, the different activation dynamics of the two β-AR isotypes may explain how SNs control β2-AR dependent signalling in basal conditions. In fact, given that the β1-ARs have higher affinity for NA, than β2-ARs (Devic *et al.* 2001), it is unlikely that activation of trophic signalling is mediated by an increase in resting NA levels in the myocardium, as this would constitutively activate the abundant β1-AR isotype. On the other hand, when considering that β2-AR stimulation initiates long-lasting downstream effects (mediated by either cAMP–Epac, PI3K–Akt, or β-arrestin pathways) (Paula-Gomes *et al.* 2013), the constitutive control of atrogenes transcription may develop from short repeated neuronal discharges,

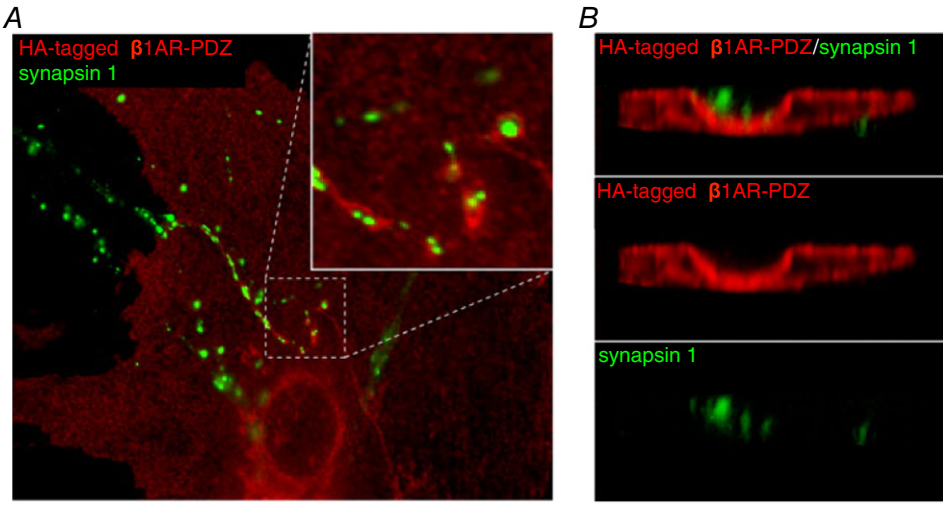


Figure 3. β1-Adrenoceptors accumulate in the portion of cardiomyocyte membrane innervated by sympathetic neurons
A and-*B*, mouse neonatal cardiomyocytes, infected with a recombinant adenovirus expressing HA-tagged β1AR-PDZ, were co-cultured with stellate ganglia neurons for 6 days. Cells were co-stained with antibodies to HA (red signal) and synapsin-1 (green signal) and analysed with confocal (*A*) and two-photon (*B*) microscope. The inset in *A* and images in *B* show evidence of the accumulation of β1-AR in the cardiomyocyte membrane portions contacted by SNs (reproduced with permission from Shcherbakova *et al.*, *J Cell Biol* 2007).

such as those occurring during normal daily activities (i.e. postural changes, movement). A tempting inference of this model is that, even if the effects are seen at different time scales, the SN activity underlying size regulation in ventricular cardiomyocytes is conceptually similar to that regulating HRV in SAN cells (Fig. 2).

A close look at the properties of cardiac hardwiring

Taken together, the examples outlined above demonstrate that, in addition to the modulation of neuronal activity, other characteristics of neuro-cardiac communication at the cell–cell interface, including receptor affinity, trafficking and intercellular signalling dynamics, allow the heart to tune to the right pitch in both resting and stressed condition. Thus, understanding how SNs communicate with their myocardial targets is determinant to the comprehension of cardiac physiology (Armour, 2003). Research from several investigators, in the last few decades, has focused on the interactions between cardiac SNs and cardiomyocytes in the mammalian heart. A large set of data, accrued using morphological and ultrastructural analyses, demonstrates that sympathetic varicosities and cardiomyocyte membranes are in close apposition, reminiscent of the specific neuromuscular contact sites of the neuromuscular junction (NMJ) in the close intermembrane distance and the expected polarization of neurotransmitter vesicles on the side facing the cardiomyocyte (Thaemert, 1966, 1969; Landis, 1976; Choate *et al.* 1993). The concept of the 'neuro-cardiac junction (NCJ)' has failed, however, to gain acceptance in cardiac physiology, mostly because the results of these studies were not conclusive, and specific molecular determinants were not identified. One of the possible explanations of such variable results is that tissue analyses have been performed in hearts from different species (amphibians *vs.* mammals) and have mostly focused on specific regions of the atria, where cardiomyocyte interaction with the neurons may differ from that of the ventricular myocardium, and thus be sustained by different membrane protein complexes. However, a significant series of *in vitro* studies showed that neurons influence the beating rate of directly innervated cardiomyocytes, and although the observations were limited to the description of the phenomenon, the concept of 'quasi-synaptic' communication between SNs and cardiomyocytes was proposed (Lloyd & Marvin, 1990; Conforti *et al.* 1991; Lockhart *et al.* 1997; Zaika *et al.* 2011). Such a definition has mostly been based on the identification of the close intercellular apposition between SNs and cardiomyocytes, indicating a putative 'cleft', which suggests that intercellular communication could rely on properties similar to conventional neuro-effector synapses, including rapidity, efficiency and target specificity of signal transmission. Recent evidence, accrued using SN–cardiomyocyte co-cultures, has increased our knowledge of molecular detail on the neuro-cardiac contact site and identified protein complexes present in neurons and myocytes at the cellular interface (Larsen *et al.* 2016). These include β-ARs in the post-junctional cardiomyocyte membrane, specific adaptor/scaffold proteins of the β-AR signalling pathway (e.g. SAP97, AKAP79), and putative cell–cell interaction molecules (e.g. cadherin, β-catenins). Some of these findings were also confirmed in *ex vivo* studies in murine myocardial sections (Shcherbakova *et al.* 2007). Although the molecular machinery that physically links the two membranes together (as in properly structured synapses/junctions) has not been fully reconstructed, it is tempting to speculate that cardiac homologues of the structural proteins serving as tethers in the NMJ may play corresponding roles in the NCJ. Interestingly, it was recently observed that synaptic neuro-effector contacts were established in co-cultures between human pluripotent stem cell-derived SNs and cardiomyocytes, but did not form when the same neuronal population was co-cultured with non-cardiac muscle cells (Oh *et al.* 2016). These data suggest that the properties discussed above are not peculiar to the rodent myocardium and indicate the target specificity of SN interactions. As previously discussed, in their journey through the myocardial interstitium, SNs encounter frequent cardiac fibroblasts. Interestingly, it has been suggested, based on *in vitro* experiments, that cardiac fibroblasts may also directly interact with SNs (neuronally differentiated PC12 cells), although our observations indicate that the dynamics of such interactions, which are labile and transient in time, are different from the stable contacts established with cardiomyocytes (Mias *et al.* 2013; Franzoso *et al.* 2014). The combination of target selectivity and the behaviour of intercellular interactions among myocardial cellular targets may reflect cell-specific functions of neuro-cardiac coupling. In summary, while morphological evidence supports the concept that neuro-effector coupling is constructed around specific interaction complexes, and suggests that neurons may use synapse-like communication, especially with cardiomyocytes, this concept has not been addressed at the functional level. In fact, the intercellular signalling dynamics, the effective concentration range of neurotransmitters, and how the properties of intercellular communication impinge on the target cell responses have not been elucidated, thus far.

Evidence and speculations around 'neuro-cardiac junctions'

The analysis of the physiological mechanisms regulated by cardiac SNs described above allows us to sketch out some of the general requirements of neuro-cardiac

communication. First, sympathetic neurotransmission must occur potently upon maximal neuronal activation, as occurs in the presence of life-threatening stimuli, in 'fight-or-flight' reaction. Second, neuronal activation needs to initiate cardiac activation almost instantaneously, to rapidly increase blood pressure, through stimulation of force and frequency of heart contractions. Third, this system has to ensure that under stress the entire heart muscle undergoes changes in inotropy at the same time. Fourth, the system must be precise enough to operate chronically, almost on a beat-to-beat basis, in the regulation of electrophysiology (e.g. HRV in SAN cells) and trophic signalling (e.g. proteostasis in ventricular cells). Finally, efficiency must be predictable, to minimize neurotransmitter disposal. Collectively, these tasks necessitate such sophisticated coordination that only an expert 'director' can orchestrate the cardiac 'symphony'. Neurotransmission has been the subject of a good number of simulation studies, based on structural/anatomical, and *in vitro* data. As previously described, the structural details, demonstrating the dense innervation of the myocardium, and the direct interaction between SN and myocardial target cells, suggest that neuro-cardiac coupling may occur at specific junctional sites. Modelling studies have considered the junctional arrangement of the neuro-cardiac interaction, characterized by a relatively narrow intercellular cleft (80–100 nm wide), in which NA is preferentially discharged, thanks to polarization of the neuronal active zone. By limiting NA diffusion to the quasi two-dimensional volume enclosed by the two membranes, the synaptic cleft allows a high [NA] to be achieved, upon release of relatively few molecules, thus potently and efficiently activating cardiac β-ARs. In numerical simulations, the time to maximal agonist concentration in the cleft has been estimated in around 50–100 μs, thus virtually abolishing the lag from NA release to cardiomyocyte β-AR activation (Šćepanović, 2011). As a consequence, the cardiac response time to neuronal activation is reduced to the kinetics of intracellular signalling in the target cardiomyocyte, and in line with this, the molecular elements of β-AR transduction cascade are tethered in proximity to the junctional site, to guarantee fast signalling activation, as described above. In addition, *in vitro* studies suggest that the composition of the postsynaptic membrane may re-arrange following neuronal stimulation, leading to internalization of activated β2-ARs, and subsequent membrane recycling, away from the neuronally innervated portion (Devic *et al.* 2001; Shcherbakova *et al.* 2007). The time to reappearance of the receptor at the junctional site might therefore represent the limiting factor for neurogenic activation of β2-AR signalling, upon repeated NA discharges. Interestingly, in such model, the postsynaptic receptor dynamics would limit the effects of elevated neuronal firing rates, thus introducing a *lowpass* filter in the β2-AR-dependent communication between SNs and myocytes. Such properties would imply that trophic inputs to cardiomyocytes depend on the neuronal firing rate only within a limited range of low frequencies, which may be in tune with that of resting neuronal activity. Collectively, evidence suggests that intercellular signalling between cardiac sympathetic motor neurons and cardiomyocytes may be channelled through elementary units, represented by the 'NCJ', where a 'quasi-synaptic' communication takes place, and whose properties fit well with those of neurogenic regulation of cardiac function.

Emergent properties of cardiac neuro-effector connectivity

Biophysical and numerical simulations tell us that in synapses with similar geometry, given the restricted

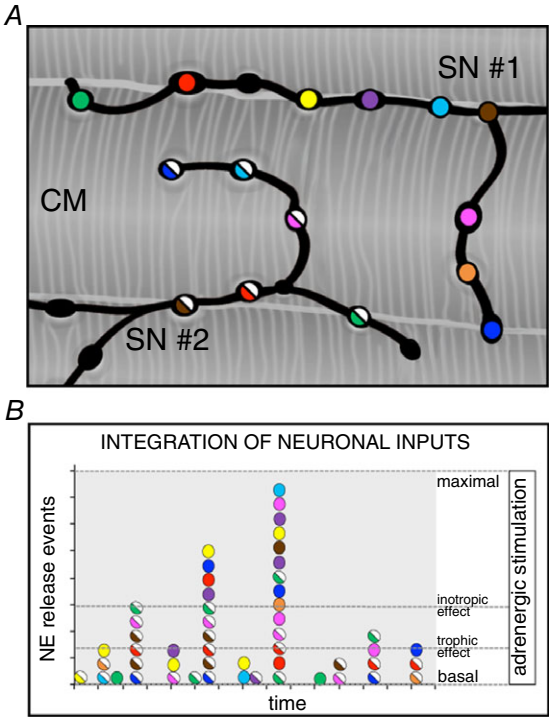


Figure 4. Integration of multiple neuronal inputs by the innervated cardiomyocyte
A, recent advancements in imaging cardiac sympathetic innervation demonstrate that each cardiomyocyte interacts with multiple contacts from the same neuronal process (each varicosity is highlighted by one colour), and may simultaneously be innervated by different neurons (two neurons in the picture are represented by filled or half-filled circles, respectively). In addition, the literature supports the notion that neuronal varicosities act as point sources of neurotransmitters, and that each varicosity may release NA independently. *B*, we thus made the hypothesis that activation of increasing number of varicosities, and recruitment of more neuronal processes, may allow grading of the responses of target cells from basal to maximal activation, across a wide range of intermediate effects.

volume of the intercellular cleft, NA rapidly reaches maximal concentration, thus behaving as a discrete system, in which only 'on' and 'off' states are possible. In addition, the evidence that the β-AR–cAMP–PKA pathway is compartmentalized in cardiomyocytes (Lefkimmiatis & Zaccolo, 2014) implies that, if sympathetic varicosities act as point sources of NA, they would only ignite a restricted number of localized β-ARs, thus limiting the downstream effects to a subset of targets within a small sub-cellular volume. Together, these features fail to explain how cardiac responses can be graded across the wide physiological range of action of the cardiac SNS, and reach sufficent potency during acute stresses. If neurons communicate with the heart through neuro-cardiac junctions, the question is therefore: '*how can they modulate whispers and shouts*?'. The system anatomy may provide elements for speculation. As discussed above, each cardiomyocyte may be contacted by multiple neurotransmitter release sites, from one or more neuronal processes (Fig. 1). As a consequence, the innervated cardiomyocyte can potentially receive NA simultaneously from multiple point sources, and integrate downstream the cumulative neuronal input at signalling level. Interestingly, *in vitro* evidence shows that each SN varicosity releases NA in response to miniature EPSPs (Brock & Cunnane, 1987). Thus, the effects of SNs on HRV, and on neuro-transcriptional coupling, controlling cardiomyocyte proteostasis and size, may be independent from cell-wide neuronal action potentials. Conventionally, the release probability of single sympathetic varicosities is considered very low, but it may be dynamically modulated by local feedback mechanisms, and other factors not yet uncovered in detail. As a consequence, the increase in NA release probability may 'recruit' active varicosities, from a single or multiple neuronal processes, signalling to the same target cell, and may therefore represent a mechanism tailored to grade effector responses up to the highest physiological limit (Fig. 4). Although the factors finely regulating the activity of single cardiac SN varicosities *in vivo*, and the mechanisms whereby higher order circuits engage post-ganglionic neurons, are unknown, these speculations add another layer of complexity to the system, and explain the sophisticated potential of neurogenic control of heart function.

Novel instruments to decode the neuro-cardiac (inter)play

While the use of transgenic murine models with targeted modification/deletion of proteins involved in neuro-cardiac coupling (e.g. β-AR, neurotrophin receptors) has clearly aided neurocardiology research, the accurate study of sympatho-cardiac communication remains a challenge, mostly for the anatomical constraints of the system (e.g. size and frailty of neuronal processes, heart beating), and the complexity of its regulation. Recently, the expression of photoactivatable optogenetic proteins in SNs or PSNs has allowed the direct and selective control of cardiac neuron function, with high temporal and spatial resolution, thus offering an appealing strategy to address neurotransmitter release kinetics and isolate local cardiac regulation *in vivo*. While these techniques have been used thus far mostly in proof-of-concept experiments, it is expected that they will provide a direct answer to the parts of our discussion which remain at the time being conceptual speculations.

Concluding envoi

The control on heart activity, orchestrated by the cardiac SNS, is without doubt one of the most comprehensive mechanisms for allowing physiological adaptation to the ever-changing internal and external environment. Here, we have interpreted existing research to support the concept that the range of such control spans broadly, from the maintenance of housekeeping functions (cardiac trophism) to the recruitment of maximal heart performance. In addition, we have reappraised the mechanisms underlying signal transmission between neurons and cardiomyocytes, and endorsed the theory that cardiac sympathetic 'synapses' mediate intercellular communication. By speculating on the implications of neuro-cardiac coupling for systems regulatory physiology, our intent is to offer a different point of view on the structural and functional organization of the cardiac SNS. Such a novel interpretation of the physiology of neurogenic heart regulation may also deepen understanding of the mechanisms of common cardiac diseases, which have been associated with SNS dysfunction, and impact on widely used cardiovascular pharmacotherapies. For many this interpretation may look unfamiliar, others may disagree with it, but we hope to have kindled interest in looking more closely at cardiac sympathetic innervation.

References

Ahmed MW, Kadish AH, Parker MA & Goldberger JJ (1994). Effect of physiologic and pharmacologic adrenergic stimulation on heart rate variability. *J Am Coll Cardiol* **24**, 1082–1090.

Anversa P, Capasso JM, Ricci R, Sonnenblick EH & Olivetti G (1989). Morphometric analysis of coronary capillaries during physiologic myocardial growth and induced cardiac hypertrophy: a review. *Int J Microcirc Clin Exp* **8**, 353–363.

Ardell JL & Armour JA (2016). Neurocardiology: structure-based function. *Compr Physiol* **6**, 1635–1653.

Armour JA (2003). *Neurocardiology – Anatomical and Functional Principles*. HeartMath Research Center, Institute of HeartMath, Publication, Boulder Creek, CA, USA.

Armour JA, Murphy DA, Yuan BX, Macdonald S & Hopkins DA (1997). Gross and microscopic anatomy of the human intrinsic cardiac nervous system. *The Anatomical Record* **247**, 289–298.

Baluk P & Fujiwara T (1984). Direct visualization by scanning electron microscopy of the preganglionic innervation and synapses on the true surfaces of neurons in the frog heart. *Neurosci Lett* **51**, 265–270.

Bers D (2001). *Excitation-Contraction Coupling and Cardiac Contractile Force*, vol. **237**. Springer Science & Business Media, Germany.

Bers DM (2008). Calcium cycling and signaling in cardiac myocytes. *Annu Rev Physiol* **70**, 23–49.

Bers DM & Despa S (2009). Na/K-ATPase – an integral player in the adrenergic fight-or-flight response. *Trends Cardiovasc Med* **19**, 111–118.

Brock JA & Cunnane TC (1987). Relationship between the nerve action potential and transmitter release from sympathetic postganglionic nerve terminals. *Nature* **326**, 605–607.

Choate JK, Klemm M & Hirst GD (1993). Sympathetic and parasympathetic neuromuscular junctions in the guinea-pig sino-atrial node. *J Auton Nerv Syst* **44**, 1–15.

Clarke GL, Bhattacherjee A, Tague SE, Hasan W & Smith PG (2010). β-Adrenoceptor blockers increase cardiac sympathetic innervation by inhibiting autoreceptor suppression of axon growth. *J Neurosci* **30**, 12446–12454.

Conforti L, Tohse N & Sperelakis N (1991). Influence of sympathetic innervation on the membrane electrical properties of neonatal rat cardiomyocytes in culture. *J Dev Physiol* **15**, 237–246.

Devic E, Xiang Y, Gould D & Kobilka B (2001). Beta-adrenergic receptor subtype-specific signaling in cardiac myocytes from β1 and β2 adrenoceptor knockout mice. *Mol Pharmacol* **60**, 577–583.

Diaz-Araya G, Vivar R, Humeres C, Boza P, Bolivar S & Munoz C (2015). Cardiac fibroblasts as sentinel cells in cardiac tissue: Receptors, signaling pathways and cellular functions. *Pharmacol Res* **101**, 30–40.

DiFrancesco D (2010). The role of the funny current in pacemaker activity. *Circ Res* **106**, 434–446.

Feng N, Huke S, Zhu G, Tocchetti CG, Shi S, Aiba T, Kaludercic N, Hoover DB, Beck SE, Mankowski JL, Tomaselli GF, Bers DM, Kass DA & Paolocci N (2015). Constitutive BDNF/TrkB signaling is required for normal cardiac contraction and relaxation. *Proc Natl Acad Sci USA* **112**, 1880–1885.

Franzoso M, Zaglia T & Mongillo M (2016). Putting together the clues of the everlasting neuro-cardiac liaison. *Biochim Biophys Acta* **1863**, 1904–1915.

Franzoso M, Zaglia T, Pianca N, Di Benedetto G, Sandre M, Gobbo V, Schiaffino S, Marin O, Lo Preiato R & Mongillo M (2014). The neuro-cardiac interaction defines an extracellular microdomain required for neurotrophic signaling. *Cardiovasc Res* **103**, S16–S16.

Freeman K, Tao W, Sun H, Soonpaa MH & Rubart M (2014). In situ three-dimensional reconstruction of mouse heart sympathetic innervation by two-photon excitation fluorescence imaging. *J Neurosci Methods* **221**, 48–61.

Habecker BA, Anderson ME, Birren SJ, Fukuda K, Herring N, Hoover DB, Kanazawa H, Paterson DJ & Ripplinger CM (2016). Molecular and cellular neurocardiology: development, and cellular and molecular adaptations to heart disease. *J Physiol* **594**, 3853–3875.

Hirsch E, Hilfiker-Kleiner D, Balligand JL, Tarone G, De Windt L, Bauersachs J, Ferdinandy P, Davidson S, Hausenloy DJ & Schulz R (2013). Interaction of the heart and its close and distant neighbours: report of the meeting of the ESC working groups myocardial function and cellular biology. *Cardiovasc Res* **99**, 595–599.

Hoover DB, Ganote CE, Ferguson SM, Blakely RD & Parsons RL (2004). Localization of cholinergic innervation in guinea pig heart by immunohistochemistry for high-affinity choline transporters. *Cardiovasc Res* **62**, 112–121.

Ieda M, Kanazawa H, Kimura K, Hattori F, Ieda Y, Taniguchi M, Lee JK, Matsumura K, Tomita Y, Miyoshi S, Shimoda K, Makino S, Sano M, Kodama I, Ogawa S & Fukuda K (2007). Sema3a maintains normal heart rhythm through sympathetic innervation patterning. *Nat Med* **13**, 604–612.

James TN (1980). Neural control of the heart in health and disease. *Adv Intern Med* **26**, 317–345.

Janig W (2016). Neurocardiology: a neurobiologist's perspective. *J Physiol* **594**, 3955–3962.

Jansen AS, Nguyen XV, Karpitskiy V, Mettenleiter TC & Loewy AD (1995). Central command neurons of the sympathetic nervous system: basis of the fight-or-flight response. *Science* **270**, 644–646.

Kaludercic N, Takimoto E, Nagayama T, Feng N, Lai EW, Bedja D, Chen K, Gabrielson KL, Blakely RD, Shih JC, Pacak K, Kass DA, Di Lisa F & Paolocci N (2010). Monoamine oxidase A-mediated enhanced catabolism of norepinephrine contributes to adverse remodeling and pump failure in hearts with pressure overload. *Circ Res* **106**, 193–202.

Kanevskij M, Taimor G, Schafer M, Piper HM & Schluter KD (2002). Neuropeptide Y modifies the hypertrophic response of adult ventricular cardiomyocytes to norepinephrine. *Cardiovasc Res* **53**, 879–887.

Kimura K, Ieda M & Fukuda K (2012). Development, maturation, and transdifferentiation of cardiac sympathetic nerves. *Circ Res* **110**, 325–336.

Kreipke RE & Birren SJ (2015). Innervating sympathetic neurons regulate heart size and the timing of cardiomyocyte cell cycle withdrawal. *J Physiol* **593**, 5057–5073.

Landis SC (1976). Rat sympathetic neurons and cardiac myocytes developing in microcultures: correlation of the fine structure of endings with neurotransmitter function in single neurons. *Proc Natl Acad Sci USA* **73**, 4220–4224.

Larsen HE, Lefkimmiatis K & Paterson DJ (2016). Sympathetic neurons are a powerful driver of myocyte function in cardiovascular disease. *Sci Rep* **6**, 38898.

Lefkimmiatis K & Zaccolo M (2014). cAMP signaling in subcellular compartments. *Pharmacol Ther* **143**, 295–304.

Li D & Paterson DJ (2016). Cyclic nucleotide regulation of cardiac sympatho-vagal responsiveness. *J Physiol* **594**, 3993–4008.

Li L, Desantiago J, Chu G, Kranias EG & Bers DM (2000). Phosphorylation of phospholamban and troponin I in beta-adrenergic-induced acceleration of cardiac relaxation. *Am J Physiol Heart Circ Physiol* **278**, H769–H779.

Lloyd TR & Marvin WJ Jr (1990). Sympathetic innervation improves the contractile performance of neonatal cardiac ventricular myocytes in culture. *J Mol Cell Cardiol* **22**, 333–342.

Lockhart ST, Turrigiano GG & Birren SJ (1997). Nerve growth factor modulates synaptic transmission between sympathetic neurons and cardiac myocytes. *J Neurosci* **17**, 9573–9582.

Lombardi F, Malliani A, Pagani M & Cerutti S (1996). Heart rate variability and its sympatho-vagal modulation. *Cardiovasc Res* **32**, 208–216.

Mann DL, Zipes DP, Libby P & Bonow RO (2014). *Braunwald's Heart Disease: a Textbook of Cardiovascular Medicine*. Elsevier Health Sciences, Amsterdam, Netherlands.

Mias C, Coatrieux C, Denis C, Genet G, Seguelas MH, Laplace N, Rouzaud-Laborde C, Calise D, Parini A, Cussac D, Pathak A, Senard JM & Gales C (2013). Cardiac fibroblasts regulate sympathetic nerve sprouting and neurocardiac synapse stability. *PLoS One* **8**, e79068.

Muhlfeld C, Papadakis T, Krasteva G, Nyengaard JR, Hahn U & Kummer W (2010). An unbiased stereological method for efficiently quantifying the innervation of the heart and other organs based on total length estimations. *J Appl Physiol (1985)* **108**, 1402–1409.

O'Connell TD, Ishizaka S, Nakamura A, Swigart PM, Rodrigo MC, Simpson GL, Cotecchia S, Rokosh DG, Grossman W, Foster E & Simpson PC (2003). The $\alpha_{1A/C}$- and α_{1B}-adrenergic receptors are required for physiological cardiac hypertrophy in the double-knockout mouse. *J Clin Invest* **111**, 1783–1791.

Ogawa S, Barnett JV, Sen L, Galper JB, Smith TW & Marsh JD (1992). Direct contact between sympathetic neurons and rat cardiac myocytes in vitro increases expression of functional calcium channels. *J Clin Invest* **89**, 1085–1093.

Oh Y, Cho GS, Li Z, Hong I, Zhu R, Kim MJ, Kim YJ, Tampakakis E, Tung L, Huganir R, Dong X, Kwon C & Lee G (2016). Functional coupling with cardiac muscle promotes maturation of hPSC-derived sympathetic neurons. *Cell Stem Cell* **19**, 95–106.

Paula-Gomes S, Goncalves DA, Baviera AM, Zanon NM, Navegantes LC & Kettelhut IC (2013). Insulin suppresses atrophy- and autophagy-related genes in heart tissue and cardiomyocytes through AKT/FOXO signaling. *Horm Metab Res* **45**, 849–855.

Pauza DH, Rysevaite K, Inokaitis H, Jokubauskas M, Pauza AG, Brack KE & Pauziene N (2014). Innervation of sinoatrial nodal cardiomyocytes in mouse. A combined approach using immunofluorescent and electron microscopy. *J Mol Cell Cardiol* **75**, 188–197.

Pianca N, Prando V, Franzoso M, Di Bona A, Campione M, Sandri M, Zaglia T & Mongillo M (2016). Sympathetic neurons that innervate the heart locally modulate cardiomyocyte trophic and electrophysiological properties. In *Cardiovascular Research*, pp. S46–S46. Oxford Univ Press, Oxford.

Poletto R, Janczak AM, Marchant-Forde RM, Marchant-Forde JN, Matthews DL, Dowell CA, Hogan DF, Freeman LJ & Lay DC Jr (2011). Identification of low and high frequency ranges for heart rate variability and blood pressure variability analyses using pharmacological autonomic blockade with atropine and propranolol in swine. *Physiol Behav* **103**, 188–196.

Queen LR & Ferro A (2006). Beta-adrenergic receptors and nitric oxide generation in the cardiovascular system. *Cell Mol Life Sci* **63**, 1070–1083.

Rochais F, Vandecasteele G, Lefebvre F, Lugnier C, Lum H, Mazet JL, Cooper DM & Fischmeister R (2004). Negative feedback exerted by cAMP-dependent protein kinase and cAMP phosphodiesterase on subsarcolemmal cAMP signals in intact cardiac myocytes: an *in vivo* study using adenovirus-mediated expression of CNG channels. *J Biol Chem* **279**, 52095–52105.

Samuels MA (2007). The brain–heart connection. *Circulation* **116**, 77–84.

Šćepanović D (2011). A model of sinoatrial node cell regulation by the autonomic nervous system. Thesis (PhD), Massachusetts Institute of Technology http://hdl.handle.net/1721.1/68457.

Shan J, Kushnir A, Betzenhauser MJ, Reiken S, Li J, Lehnart SE, Lindegger N, Mongillo M, Mohler PJ & Marks AR (2010). Phosphorylation of the ryanodine receptor mediates the cardiac fight or flight response in mice. *J Clin Invest* **120**, 4388–4398.

Shcherbakova OG, Hurt CM, Xiang Y, Dell'Acqua ML, Zhang Q, Tsien RW & Kobilka BK (2007). Organization of beta-adrenoceptor signaling compartments by sympathetic innervation of cardiac myocytes. *J Cell Biology* **176**, 521–533.

Shivkumar K & Ardell JL (2016). Cardiac autonomic control in health and disease. *J Physiol* **594**, 3851–3852.

Sosunov AA, Hassall CJ, Loesch A, Turmaine M, Feher E & Burnstock G (1997). Neuropeptide Y-immunoreactive intracardiac neurones, granule-containing cells and nerves associated with ganglia and blood vessels in rat and guinea-pig heart. *Cell Tissue Res* **289**, 445–454.

Stieber J, Herrmann S, Feil S, Loster J, Feil R, Biel M, Hofmann F & Ludwig A (2003). The hyperpolarization-activated channel HCN4 is required for the generation of pacemaker action potentials in the embryonic heart. *Proc Natl Acad Sci USA* **100**, 15235–15240.

Taggart P, Critchley H, van Duijvendoden S & Lambiase PD (2016). Significance of neuro-cardiac control mechanisms governed by higher regions of the brain. *Auton Neurosci* **199**, 54–65.

Thaemert JC (1966). Ultrastructure of cardiac muscle and nerve contiguities. *J Cell Biol* **29**, 156–162.

Thaemert JC (1969). Fine structure of neuromuscular relationships in mouse heart. *Anat Rec* **163**, 575–585.

Vanderlaan RD, Conway J, Manlhiot C, McCrindle BW & Dipchand AI (2012). Enhanced exercise performance and survival associated with evidence of autonomic reinnervation in pediatric heart transplant recipients. *Am J Transplant* **12**, 2157–2163.

Zaglia T, Milan G, Franzoso M, Bertaggia E, Pianca N, Piasentini E, Voltarelli VA, Chiavegato D, Brum PC, Glass DJ, Schiaffino S, Sandri M & Mongillo M (2013). Cardiac sympathetic neurons provide trophic signal to the heart via beta2-adrenoceptor-dependent regulation of proteolysis. *Cardiovasc Res* **97**, 240–250.

Zaika O, Zhang J & Shapiro MS (2011). Functional role of M-type (KCNQ) K^+ channels in adrenergic control of cardiomyocyte contraction rate by sympathetic neurons. *J Physiol* **589**, 2559–2568.

Zhou S, Chen LS, Miyauchi Y, Miyauchi M, Kar S, Kangavari S, Fishbein MC, Sharifi B & Chen PS (2004). Mechanisms of cardiac nerve sprouting after myocardial infarction in dogs. *Circ Res* **95**, 76–83.

Additional information

Competing interests

Nothing to declare.

Funding

This work was supported by Telethon Foundation, Grant n. GGP11224 to Marco Mongillo and the French Muscular Dystrophy Association (AFM-Téléthon; grant n. 19996) to Tania Zaglia.

Acknowledgements

We are grateful to Michael Rubart for enjoyable discussion, and Mauro Franzoso, Nicola Pianca, Valentina Prando and Giulia Borile for technical assistance and critical reading. We apologize to the many colleagues whose papers have contributed important advances in the topic of this review, but due to space limitations were not cited.