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# Functional evidence for an active role of B-type natriuretic peptide in cardiac remodelling and pro-arrhythmogenicity

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**Aims** During heart failure (HF), the left ventricle (LV) releases B-type natriuretic peptide (BNP), possibly contributing to adverse cardiovascular events including ventricular arrhythmias (VAs) and LV remodelling. We investigated the cardiac effects of chronic BNP elevation in healthy mice and compared the results with a model of HF after myocardial infarction (PMI mice).

**Methods and results** Healthy mice were exposed to circulating BNP levels (BNP-Sham) similar to those measured in PMI mice. Telemetric surface electrocardiograms showed that in contrast with fibrotic PMI mice, electrical conduction was not affected in BNP-Sham mice. VAs were observed in both BNP-Sham and PMI but not in Sham mice. Analysis of heart rate variability indicated that chronic BNP infusion increased cardiac sympathetic tone. At the cellular level, BNP reduced  $\text{Ca}^{2+}$  transients and impaired  $\text{Ca}^{2+}$  reuptake in the sarcoplasmic reticulum, in line with blunted SR  $\text{Ca}^{2+}$  ATPase 2a and S100A1 expression. BNP increased  $\text{Ca}^{2+}$  spark frequency, reflecting  $\text{Ca}^{2+}$  leak through ryanodine receptors, elevated diastolic  $\text{Ca}^{2+}$ , and promoted spontaneous  $\text{Ca}^{2+}$  waves. Similar effects were observed in PMI mice. Most of these effects were reduced in BNP-Sham and PMI mice by the selective  $\beta$ 1-adrenergic blocker metoprolol.

**Conclusion** Elevated BNP levels, by inducing sympathetic overdrive and altering  $\text{Ca}^{2+}$  handling, promote adverse cardiac remodelling and VAs, which could account in part for the progression of HF after MI. The early use of  $\beta$ -blockers to prevent the deleterious effects of chronic BNP exposure may be beneficial in HF.

**Keywords** Natriuretic peptide • Heart failure • Arrhythmia • Calcium • Remodelling

## 1. Introduction

B-type natriuretic peptide (BNP) is synthesized in cardiac myocytes and released in excess into the blood circulation following left ventricular (LV) wall stretching.<sup>1</sup> During the onset of ventricular remodelling after myocardial infarction (PMI), BNP is chronically elevated and is a strong indicator of heart failure (HF) severity.<sup>1</sup> Despite favourable haemodynamic effects, high blood BNP is associated with the risk of ventricular arrhythmias (VAs) and sudden cardiac death (SCD).<sup>2,3</sup> Consequently, the effectiveness of therapies aimed at increasing BNP during HF is still questionable.<sup>4</sup> While short-term BNP infusion is of haemodynamic benefit, with natriuretic, diuretic,

and vasorelaxant effects, chronic BNP treatment increases the risk of mortality.<sup>3,5,6</sup> Sympathetic activation related to a reflex response triggered by these haemodynamic effects could be implicated in this process.<sup>7,8</sup> Although the signalling pathways mediating the effects of BNP are poorly understood, a therapeutic strategy that could prevent the deleterious effects of BNP without affecting its benefits remains of interest.

Altered  $\text{Ca}^{2+}$  cycling, characterized by increased sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$  leak, decreased SR  $\text{Ca}^{2+}$  uptake leading to diastolic  $\text{Ca}^{2+}$  elevation, and decreased systolic  $\text{Ca}^{2+}$  levels, is a common feature in chronic HF.<sup>9–11</sup> Such altered  $\text{Ca}^{2+}$ -handling and chronic sympathetic overdrive are established components of chronic HF

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triggering VA.<sup>12</sup> BNP decreases the expression of SR Ca<sup>2+</sup> ATPase 2a (SERCA2a)<sup>13,14</sup> and induces pro-adrenergic effects.<sup>15</sup> We thus hypothesized that BNP could influence two key players in the triggering of VA and, more globally, in cardiac remodelling during HF.

Our present study attempts to determine the effect of chronic BNP treatment on LV function both *in vivo* in healthy mice and at the cellular level. Here, we show, by heart rate variability (HRV) analysis and the use of the  $\beta$ -blocker metoprolol, that increased sympathetic tone associated with Ca<sup>2+</sup>-handling alterations contributes to BNP-mediated LV remodelling and VA.

## 2. Methods

### 2.1 Animals and BNP

All procedures conformed to European Parliament Directive 2010/63/EU and the 22 September 2010 Council on the protection of animals and were approved by the local institutional animal research committee (Languedoc Roussillon, No. CE-LR-0714).

For cardiomyocyte experiments, the heart was explanted after euthanasia by cervical dislocation. Seven-week-old male C57BL/6 mice (Janvier, Le Genest-Saint-Isle, France) were randomly assigned to the following groups: (i) Sham; (ii) Sham treated with BNP (BNP-Sham); (iii) mice subjected to HF by left coronary artery ligation (PMI); (iv) BNP-Sham mice treated with the  $\beta$ 1-adrenergic blocker metoprolol (BB-BNP-Sham); and (v) PMI mice treated with metoprolol (BB-PMI). For PMI, a left thoracotomy was performed under anaesthesia and cardiac monitoring (2% isoflurane/O<sub>2</sub>, Aerrane<sup>®</sup>, Baxter, France). The artery was ligated 1–2 mm beyond its point of emergence from the top of the left atrium, using an 8-0 suture. A subcutaneous injection of 0.01 mL buprenorphine solution (0.3 mg/mL) was administered for post-operative analgesia. Sham mice were subjected to the same surgical procedure but without coronary artery ligation. Two mice died during surgery, i.e. before their inclusion in the study, and were replaced. Metoprolol (Sigma-Aldrich, 100 mg/kg/day) was administered in the drinking water. The dose was determined based on the literature. In the range of 60–350 mg/kg/day, metoprolol has beneficial effects on cardiac and cellular function.<sup>16–20</sup> Here, the dose of 100 mg/kg/day reduced the heart rate and improved the HRV parameters in PMI mice without affecting the water intake. Mouse BNP(1–45) (Ref 14-5-30A, American Peptide, Sunnyvale, CA, USA) was administered to Sham mice at a rate of 0.03  $\mu$ g/kg/min for 14 days, using a micro-osmotic pump (Alzet 1002, Charles River, France), to achieve plasma BNP levels similar to the steady-state level observed in PMI mice. Circulating BNP levels were measured in duplicate using a commercial kit (Phoenix Pharmaceuticals, Belmont, CA, USA). The timeline of experiments is shown in the Supplementary material online, *Figure S1*.

### 2.2 Histology

Haematoxylin–eosin and Sirius red staining were performed as described.<sup>21</sup> Results indicate the area of Sirius red-stained tissue as a percentage of the total area of myocardial tissue.

### 2.3 *In vivo* analysis

Cardiac function was assessed by echocardiography (Vivid7Pro, GE Medical Systems, USA). LV mass, LV shortening fraction (SF), end-diastolic LV dimension (LVEDd), and end-systolic LV dimension (LVESd) were measured.<sup>10</sup> Electrocardiograms (ECGs) were recorded by telemetry (DSI, St Paul, MN, USA, and EMKA Technologies, France).<sup>10</sup> HRV, the PR, QRS, and QTc intervals, and arrhythmias were analysed using 12 h nocturnal ECGs (ECG-auto, EMKA Technologies).<sup>21</sup>

### 2.4 Ca<sup>2+</sup> handling

LV myocytes were enzymatically dissociated, loaded with a fluorescent Ca<sup>2+</sup> indicator Fluo-4 AM (5  $\mu$ mol/L, Molecular Probes, Paris, France), and field-stimulated at 1.0 Hz to assess intracellular Ca<sup>2+</sup> transients and cell shortening.<sup>10</sup> Ca<sup>2+</sup> sparks were recorded in quiescent cells (1.5 ms/line, LSM510 Zeiss confocal microscope;  $\times$ 63 water-immersion objective; NA: 1.2) at 25°C.<sup>10</sup> Cell volume was estimated using Z-stack (*x–y* projection, front view) image acquisition. Data were analysed using ImageJ and ‘SparkMaster’. Cellular arrhythmias and diastolic Ca<sup>2+</sup> levels were measured with ratiometric Indo-1 AM (10  $\mu$ M, Invitrogen, France; IonOptix System, Hilton, USA).<sup>10</sup> Ca<sup>2+</sup> fluorescence was measured during a 30 s pacing period (1.0 Hz), followed by a 30 s rest period. Diastolic Ca<sup>2+</sup> levels and the number of cells developing ectopic Ca<sup>2+</sup> transients during the rest period were quantified.

### 2.5 Ca<sup>2+</sup>-handling proteins

LV proteins were separated using 2–20% SDSi–PAGE, blotted onto PVDF membranes (Protean, Germany) and incubated overnight at 4°C with primary antibodies: RyR-2 (Covalab, France), Phospho Ser<sup>2809</sup>-RyR-2 (A010-30, Badrilla, UK), SERCA2a (A010-20, Badrilla), phospholamban (PLB; A010-14, Badrilla), PhosphoSer16-PLB (A010-12, Badrilla), NCX1 (R3F1, Swant, Switzerland), and S100A1 (SP5355P, Acris Antibodies GmbH, Germany). Protein levels were expressed relative to calsequestrin (PA1-913, ABR, USA). Immunodetection was performed using the ECL Plus system (Amersham, UK).

### 2.6 Statistical analysis

All data are reported as means  $\pm$  standard deviation. Statistical analyses were performed using GraphPad Prism and Origin Softwares. One-way ANOVA for multiple comparisons was used, followed by a parametric *t*-test with Bonferroni’s correction for all parameters. A *P*-value of 0.05 or less indicated a statistically significant difference.

## 3. Results

### 3.1 Plasma BNP levels in BNP-Sham and PMI mice

Sham mice had levels of circulating BNP lower than the detection limit (<0.34 ng/mL). In PMI mice, BNP increased to  $5.4 \pm 1.2$  ng/mL ( $n = 8$ ) 14 days after MI and remained stable over the next 2 weeks. At week 4, BNP-Sham mice presented BNP levels ( $6.8 \pm 1.4$  ng/mL,  $n = 8$ ) similar to those measured in PMI animals ( $5.1 \pm 0.9$  ng/mL,  $n = 7$ ).

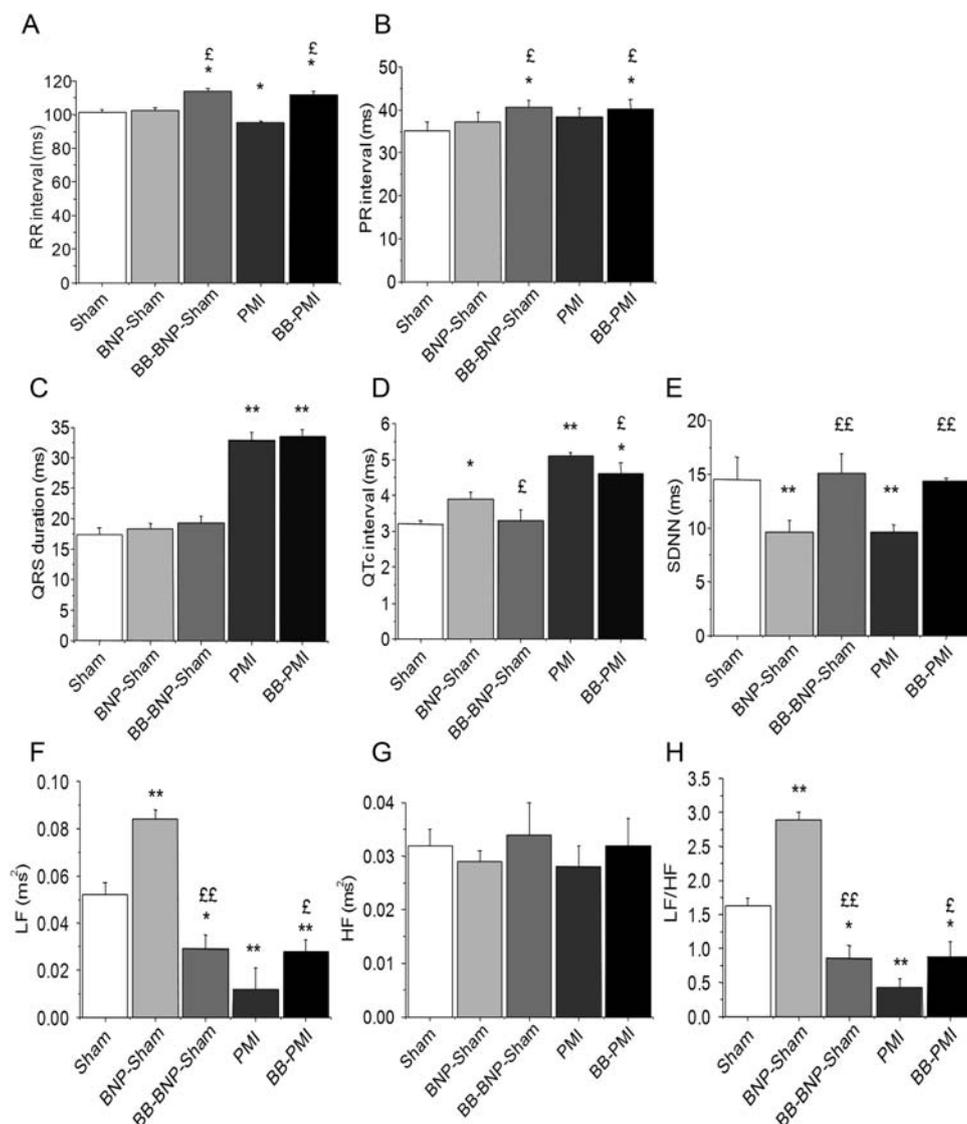
### 3.2 Morphofunctional and electrocardiographic effects of BNP

Chronic BNP treatment increased the heart weight–body weight ratio (HWR) of BNP-Sham and PMI mice (*Table 1*). Unlike modifications in PMI mice, echocardiography revealed an unchanged LVEDd and SF in BNP-Sham mice (*Table 1*). ECG showed an unchanged heart rate in BNP-Sham mice, whereas it was increased in PMI mice as indicated by the decreased RR interval (*Figure 1A*). The PR interval, corresponding to the conduction time from the sinus node through the atrioventricular (AV) node to the ventricle, was unaltered in both BNP-Sham and PMI mice (*Figure 1A*), whereas the QRS duration, representing the time to ventricular depolarization and early repolarization, was lengthened in PMI mice (*Figure 1C*). In addition, multiple spikes within the complex (fragmented QRS),<sup>22</sup> probably caused by myocardial scarring, were observed in PMI but not in BNP-Sham mice. The QTc interval was increased in both BNP-Sham and PMI

**Table I Echocardiography, histology, and cellular hypertrophy**

	LVEDd (mm)	SF (%)	HWR	Collagen (%)	Cell volume ( $10^{-5} \text{ mm}^3$ )
Sham	26.2 ± 0.4	61.7 ± 1.2	3.30 ± 0.23	0.1 ± 0.0	3.5 ± 0.1
BNP-Sham	27.1 ± 0.1	57.9 ± 1.2	3.72 ± 0.08*	0.2 ± 0.0	5.0 ± 0.3**
PMI	48.6 ± 0.2**	17.9 ± 1.4**	4.01 ± 0.32*	5.1 ± 1.8**	5.3 ± 0.5**

End-diastolic diameter of left ventricular cavity (LVEDd), shortening fraction (SF), and heart weight–body weight ratio (HWR) (10 mice/group). Collagen content, expressed as a percentage of the total area of myocardial tissue analysed (7 mice/group) and cell volume ( $n = 30$  cells, 3 mice/group) were estimated \* $P < 0.05$ , \*\* $P < 0.01$ , BNP-Sham/PMI vs. Sham animals.



**Figure 1** ECG analysis. Parameters estimated from 12 h nocturnal ECGs: heart rate (RR interval) (A), PR interval (B), QRS duration (C), and QTc interval (D). Heart rate variability analysed in the time-domain (SDNN) (E) and frequency-domain with low-frequency (LF; F) and high-frequency bands (HF; G) and LF/HF ratio (H). \* $P < 0.05$ , \*\* $P < 0.01$ , BNP-Sham/PMI vs. Sham animals; £ $P < 0.05$ , ££ $P < 0.01$  for metoprolol-treated vs. untreated animals,  $n = 12$  for each group.

mice (Figure 1D). ECG analyses showed that chronic BNP treatment did not alter electrical conduction. In accordance with this finding, BNP had no effect on fibrosis as quantified by collagen content, whereas a large increase was observed in PMI mice (Table 1).

### 3.3 Effect of BNP on cardiac sympathovagal regulation

We examined the regulation of the cardiac rhythm by the autonomic nervous system (ANS) by examining the HRV.<sup>10,21,23,24</sup> The HRV, assessed by the standard deviation of the mean of all normal sinus intervals (SDNN), was comparably decreased in BNP-Sham and PMI mice (Figure 1E), suggesting an elevation of sympathetic tone.<sup>23,24</sup> Frequency-domain analysis showed that oscillations in 'low'-frequency bands (LF), reflecting parasympathetic and sympathetic components, were increased in BNP-Sham mice (Figure 1F), whereas 'high'-frequency oscillations (HF), reflecting exclusively vagal activity, were unchanged (Figure 1G). The increase in the LF bands in BNP-Sham mice is consistent with increased sympathetic activity without a detectable modification of the mean heart rate.<sup>25</sup> The typical profile of HRV parameters measured in PMI mice, with a blunted LF band and LF/HF ratio (Figure 1F–H), confirmed the loss of rhythmicity of ANS activity on the heart and the severity of cardiac pathology in PMI animals.<sup>24,26,27</sup> This blunting could result from the saturating

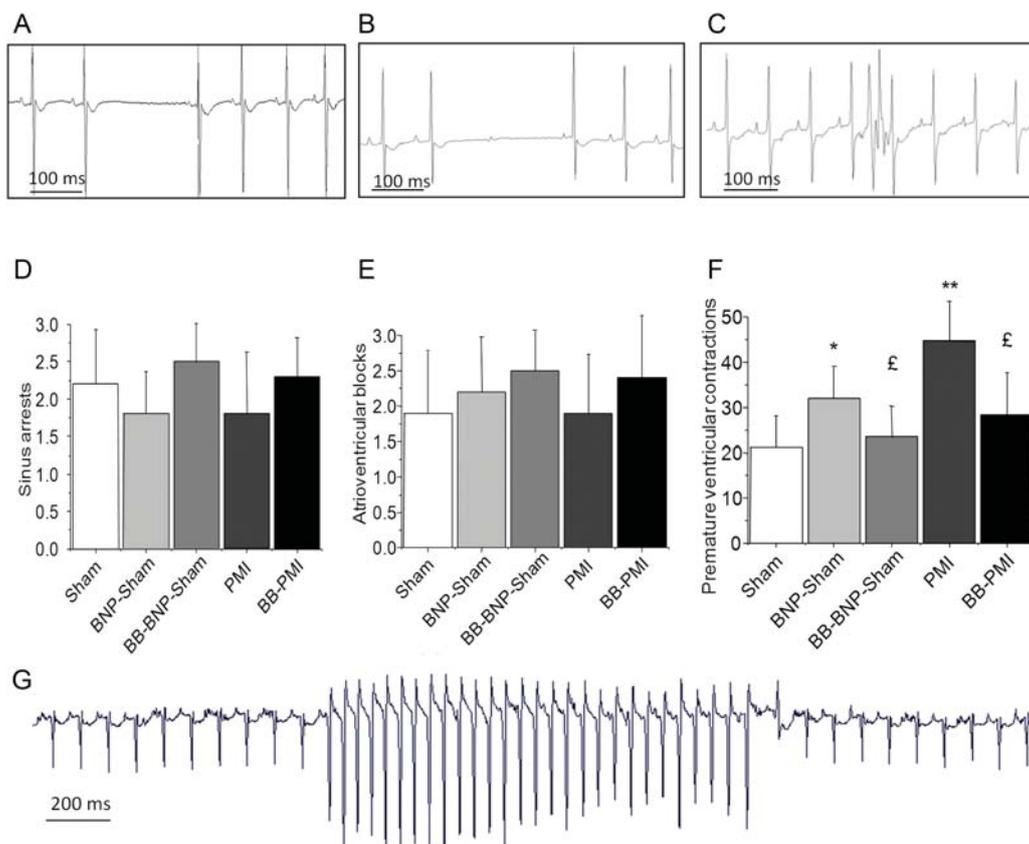
influence of persistent high sympathetic tone on the sinus node<sup>24</sup> (as attested by the decreased RR interval), or from an impairment of  $\beta$ -adrenergic receptor signalling following chronic sympathetic activation.<sup>26,27</sup>

### 3.4 BNP promotes rhythm disorders

Mice, like humans, display spontaneous arrhythmias (Figure 2A–C). Sham, BNP-Sham, and PMI mice exhibited comparable incidences of sinus arrests (Figure 2D) and AV blocks (Figure 2E). In contrast, more VAs were observed in BNP-Sham and PMI than in Sham mice (Figure 2F). Ventricular tachycardia (VT, defined as more than five consecutive ectopic beats; Figure 2G) was observed in BNP-Sham (2/8 mice) and PMI (3/12) but not Sham mice (0/12).

### 3.5 Preventive effect of the $\beta$ -blocker metoprolol

The influence of the BNP-induced neurohormonal imbalance on the ANS was further investigated using the  $\beta$ 1-adrenergic blocker metoprolol. Metoprolol reduced the heart rate, as shown by the increased RR (Figure 1A) and PR intervals (Figure 1B) in BB-BNP-Sham and BB-PMI mice. It had no effect on QRS lengthening either in BB-BNP-Sham, or in BB-PMI mice, as would be expected from the



**Figure 2** Arrhythmic events. Sinus arrests (A), atrioventricular blocks (B), and PVC (C) were counted and averaged (D–F). Typical spontaneous non-sustained VT recorded in a BNP-Sham mouse (G). \* $P < 0.05$ , BNP-Sham/PMI vs. Sham animals; \*\* $P < 0.01$ ;  $\epsilon P < 0.05$  for metoprolol-treated vs. untreated animals.

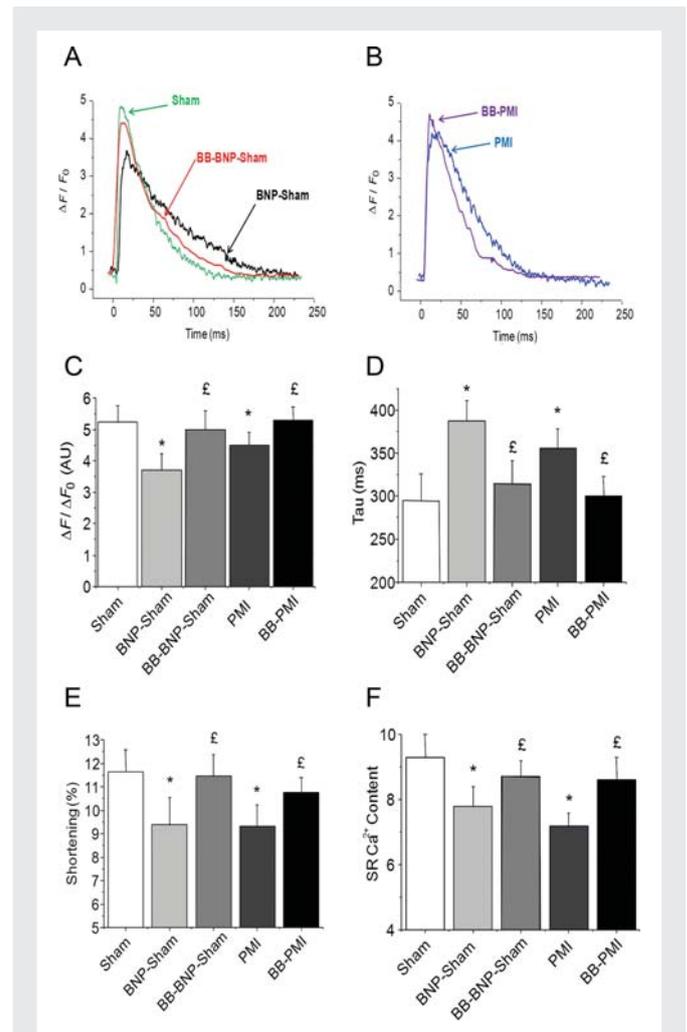
structural alterations (fibrosis) in PMI mice (Figure 1B). In addition, myocardial collagen deposits were unchanged in BB-BNP-Sham when compared with BNP-Sham mice ( $0.2 \pm 0.0$ ,  $n = 7$  vs.  $0.2 \pm 0.0\%$ ,  $n = 4$ ) and in BB-PMI when compared with PMI mice ( $4.4 \pm 1.9$ ,  $n = 7$  vs.  $5.2 \pm 1.8\%$ ,  $n = 4$ ), consistent with a previous study.<sup>28</sup> Metoprolol had a weak effect on morphofunctional parameters such as LVEDd ( $26.6 \pm 0.4$  and  $39 \pm 0.6$  mm) and SF ( $60.1 \pm 4.6$  and  $22 \pm 2.2\%$ , respectively, in BB-BNP-Sham and BB-PMI mice,  $n = 5$  each). However, metoprolol decreased the QTc in both BB-BNP-Sham and BB-PMI mice (Figure 1D).

Metoprolol also abolished the decrease in the SDNN in both BB-BNP-Sham and BB-PMI mice (Figure 1E). In the frequency domain, metoprolol decreased the LF in both BB-BNP-Sham and BB-PMI mice (Figure 1F), without modifying the HF (Figure 1G). Overall, metoprolol prevented the sympathovagal imbalance observed both after BNP treatment and in PMI mice (Figure 1H). Metoprolol also reduced premature ventricular contractions (PVC) both in BB-PMI and BB-BNP-Sham mice (Figure 2F). No VT was observed with metoprolol, but two of eight BB-BNP-Sham and one of eight BB-PMI mice presented a high incidence of sinus arrests and AV blocks (first degree), in line with the slowing of the AV conduction time by metoprolol.<sup>29</sup>

### 3.6 BNP promotes alterations in $\text{Ca}^{2+}$ handling

The detection of VAs in the absence of conduction disorders or fibrosis led us to investigate the cellular mechanisms underlying the pro-arrhythmogenic effect of BNP in single LV cardiomyocytes. First, we found that both BNP and MI increased cell size. BNP increased the cell volume (Table 1) and cell length when compared with Shams, consistent with cell hypertrophy, which was prevented by metoprolol (data not shown). We next measured intracellular  $\text{Ca}^{2+}$  transients using Fluo-4 AM (Figure 3A–F) and Indo-1 AM (Figure 4A–C). In BNP-Sham and PMI mice, the amplitude of  $\text{Ca}^{2+}$  transients was smaller (Figure 3A–C) and decay kinetics were slower (Figure 3D) than in Sham mice. Cell shortening was similarly decreased in BNP-Sham and PMI when compared with Sham mice (Figure 3E). Cardiomyocyte shortening depends on the amount of  $\text{Ca}^{2+}$  released from the SR by ryanodine receptors (RyRs) during systole. We assessed SR  $\text{Ca}^{2+}$  content by measuring the  $\text{Ca}^{2+}$  transient induced by rapid caffeine application, which instantly opens all RyRs. SR  $\text{Ca}^{2+}$  content was lower in Sham-BNP and PMI than in Sham mice (Figure 3F). Metoprolol prevented the effects of BNP and MI on  $\text{Ca}^{2+}$  transient amplitude,  $\text{Ca}^{2+}$  reuptake, and SR  $\text{Ca}^{2+}$ -store depletion, resulting in improved cell shortening in BB-BNP-Sham and BB-PMI mice (Figure 3E).

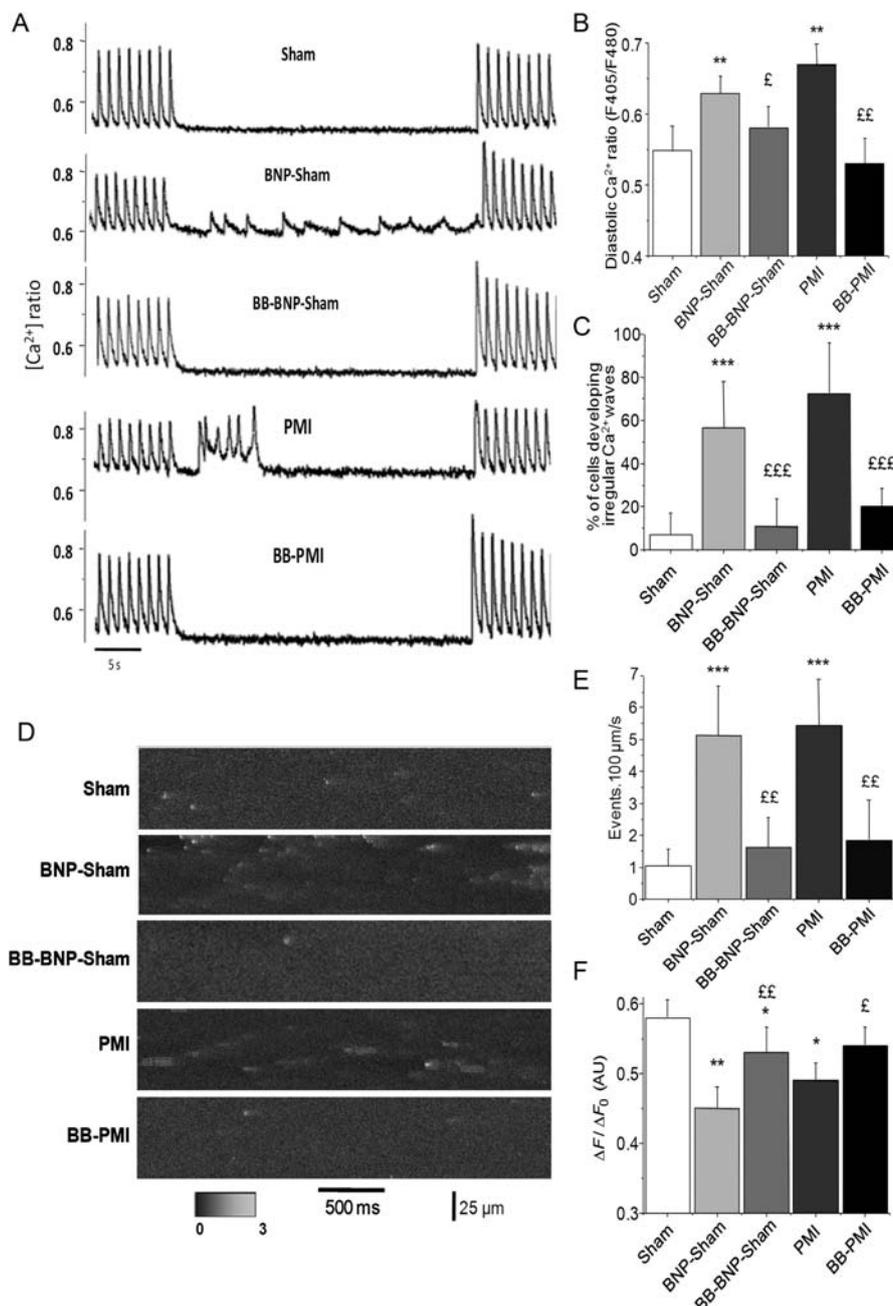
We next examined the arrhythmogenic propensity of cardiomyocytes. In BNP-Sham and PMI mice, diastolic  $\text{Ca}^{2+}$  was higher and spontaneous  $\text{Ca}^{2+}$  waves more frequent than in Shams (Figure 4A–C). Metoprolol prevented the diastolic  $\text{Ca}^{2+}$  increase similarly in BB-BNP-Sham and BB-PMI mice and decreased  $\text{Ca}^{2+}$  wave occurrence (Figure 4C). Since elevated diastolic  $\text{Ca}^{2+}$  and ectopic  $\text{Ca}^{2+}$  waves are suggestive of abnormal spontaneous RyR opening, we investigated RyR activity by directly visualizing  $\text{Ca}^{2+}$  sparks.<sup>9,10</sup> Representative linescan images are shown in Figure 4D. Both BNP-Sham and PMI cells exhibited more  $\text{Ca}^{2+}$  sparks, with lower amplitudes, than Sham cells, a change prevented by metoprolol (Figure 4E and F).



**Figure 3**  $\text{Ca}^{2+}$  transients and cell shortening.  $\text{Ca}^{2+}$  transients in single LV cardiomyocytes field-stimulated at 1.0 Hz visualized (Fluo-4 AM), expressed as  $\Delta F/F_0$  in Sham, BNP-Sham, and BB-BNP-Sham mice (A) and in PMI and BB-PMI mice (B); averaged  $\text{Ca}^{2+}$  transient amplitude (C); averaged decay time constant, Tau, of  $\text{Ca}^{2+}$  transients (D); cellular shortening (E); and averaged SR  $\text{Ca}^{2+}$  content, estimated from the caffeine-induced peak in  $\text{Ca}^{2+}$  transients (F). \* $P < 0.05$ , BNP-Sham/PMI vs. Sham animals; <sup>£</sup> $P < 0.05$  for metoprolol-treated vs. untreated animals. Sham,  $n = 60$  cells, 6 mice; BNP-Sham, BB-BNP-Sham, PMI, and BB-PMI,  $n = 40$  cells; 4 mice.

### 3.7 BNP decreases SERCA2a and S100A1 expression

Although SR  $\text{Ca}^{2+}$  leak reduces SR  $\text{Ca}^{2+}$  content and cell shortening, it does not explain the slowing of  $\text{Ca}^{2+}$  transient decay kinetics, known to result mainly from  $\text{Ca}^{2+}$  reuptake into the SR via SERCA2a, and  $\text{Ca}^{2+}$  extrusion by the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX1). SERCA2a activity is inhibited by PLB, and PLB phosphorylation (P-PLB) relieves this inhibition.<sup>12</sup> SERCA2a activity is also modulated by the small  $\text{Ca}^{2+}$ -binding protein S100A1.<sup>30</sup> Here, we found reduced SERCA2a and S100A1 protein expression in the LV of BNP-Sham mice (Figure 5A and C), with no modification of PLB or the P-PLB/PLB ratio (Figure 5D–F). RyR protein content was decreased (Figure 5G), whereas the Pser<sup>2809</sup>RyR/RyR ratio remained

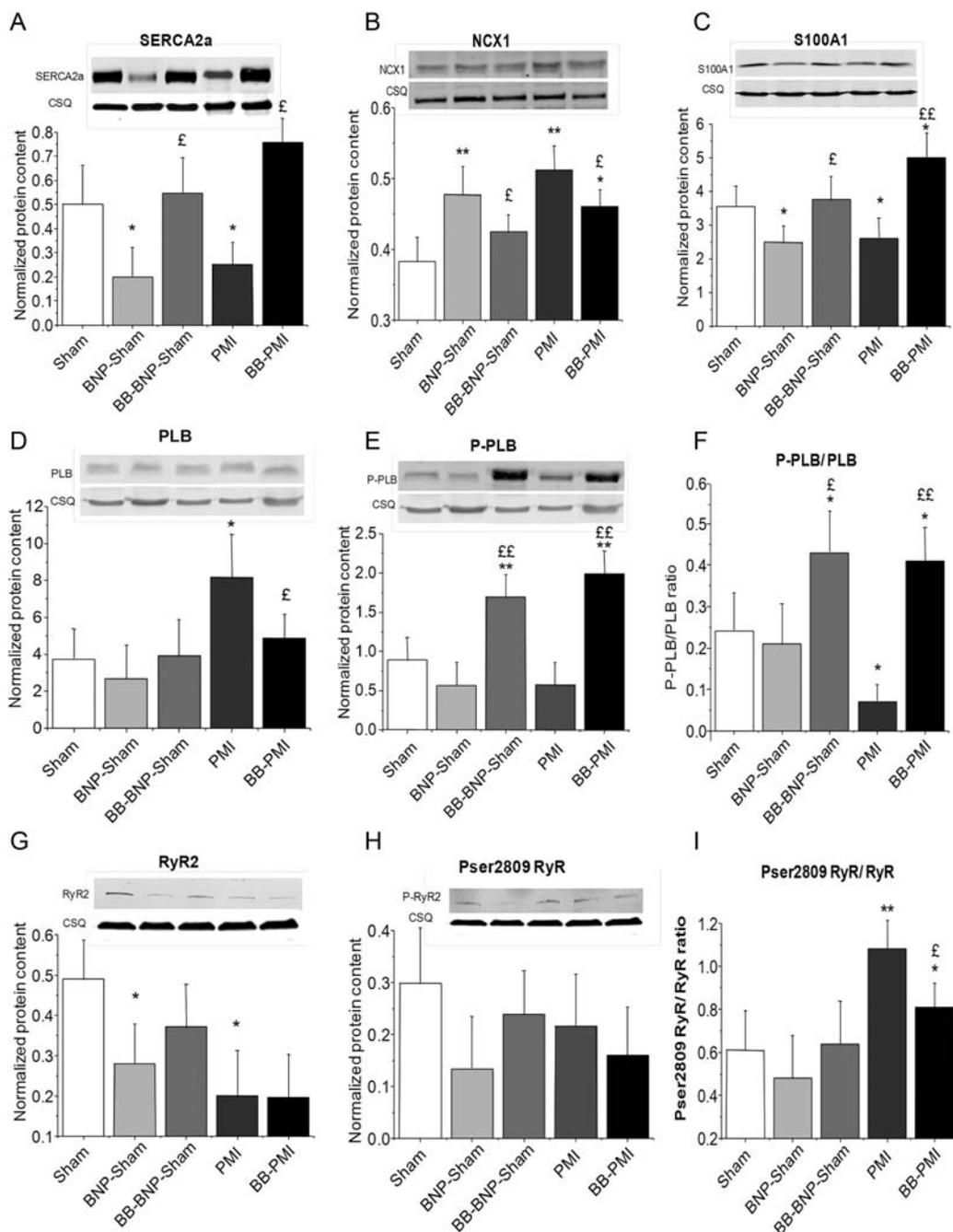


**Figure 4** Diastolic Ca<sup>2+</sup> levels, spontaneous Ca<sup>2+</sup> waves, and Ca<sup>2+</sup> sparks. Ca<sup>2+</sup> transients evoked by field stimulation (1.0 Hz) and spontaneous Ca<sup>2+</sup> waves in cardiomyocytes loaded with Indo-1 AM (A); averaged diastolic Ca<sup>2+</sup> levels (B); percentage of cells exhibiting spontaneous Ca<sup>2+</sup> waves (C); representative  $\Delta F/F$  linescan images of Ca<sup>2+</sup> sparks, recorded in intact Fluo-4 AM-loaded cardiomyocytes (D); averaged Ca<sup>2+</sup> spark frequency (E); averaged Ca<sup>2+</sup> spark amplitude (F). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , BNP-Sham/PMI vs. Sham animals; <sup>£</sup> $P < 0.05$ , <sup>££</sup> $P < 0.01$ , and <sup>£££</sup> $P < 0.001$  for metoprolol-treated vs. untreated animals. Sham,  $n = 60$  cells, 6 mice; BNP-Sham, BB-BNP-Sham, PMI, and BB-PMI,  $n = 40$  cells; 4 mice.

constant (Figure 5H and I). NCX1 protein content was increased (Figure 5B). In PMI mice, the results were similar overall, notably regarding SERCA2a, NCX1, and S100A1. However, the P-PLB/PLB ratio was decreased (Figure 5D–F), and the Pser<sup>2809</sup>RyR/RyR ratio was increased (Figure 5G–I). Metoprolol had substantial beneficial effects on both BB-BNP-Sham and BB-PMI mice by preventing the reduction in SERCA2a and S100A1 and the increase in NCX1, and by increasing the P-PLB/PLB ratio (Figure 5A–F). Metoprolol also decreased the Pser<sup>2809</sup>RyR/RyR ratio in BB-PMI mice (Figure 5G–I).

## 4. Discussion

In the present study, we show that in healthy mice, circulating BNP, at levels consistent with those measured during HF following MI, severely alters cellular and molecular functions in cardiomyocytes. Chronically elevated BNP sets the stage for alterations in cellular contraction and Ca<sup>2+</sup> handling and promotes the occurrence of spontaneous cellular Ca<sup>2+</sup> waves and VAs *in vivo*. Most of these changes are prevented by the  $\beta$ -blocker metoprolol, suggesting a



**Figure 5**  $\text{Ca}^{2+}$ -handling proteins. Normalized protein content and ratios obtained by western blotting for SERCA2a (A), NCX1 (B), S100A1 (C), PLB (D), P-PLB (E), P-PLB/PLB (F) RyR2 (G), P-RyR (H), and P-RyR/RyR (I). \* $P < 0.05$ , \*\* $P < 0.01$ , BNP-Sham/PMI vs. Sham animals;  $\epsilon P < 0.05$  and  $\epsilon\epsilon P < 0.01$  for metoprolol-treated vs. untreated animals;  $n = 8$  animals per group.

role for the sympathetic nervous system in the cardiac effects of circulating BNP.

#### 4.1 High blood BNP levels promote $\text{Ca}^{2+}$ waves and VA in healthy mice

A striking finding was that chronic exposure of the heart to high BNP levels promoted PVC, consistent with the correlation between high BNP levels and VA and SCD in HF patients.<sup>2</sup> VA occurred in the

absence of fibrosis, in line with the normal QRS duration, and was associated with mechanisms intrinsic to LV cardiomyocytes and consistent with the participation of spontaneous diastolic  $\text{Ca}^{2+}$  waves. The accumulation of aberrant RyR openings in diastole, observed functionally by the higher incidence of  $\text{Ca}^{2+}$  sparks, provided the substrate for VA/VT by generating  $\text{Ca}^{2+}$  waves.<sup>9,10</sup> Spontaneous  $\text{Ca}^{2+}$  waves are known to induce a transient inward depolarizing current ( $I_{ti}$ ) via NCX activation.<sup>31</sup> The increase in NCX1 protein was also likely to favour cellular arrhythmias.<sup>11</sup>

## 4.2 High blood BNP blunts SERCA2a and S100A1

Chronic exposure to BNP increased the frequency of  $\text{Ca}^{2+}$  sparks and reduced SR  $\text{Ca}^{2+}$  content, normally regulated by the activity of SERCA2a and its regulatory proteins PLB and S100A1.<sup>12,30</sup> In a key finding, BNP blunted the expression of both SERCA2a and S100A1, explaining both the impairment of SR  $\text{Ca}^{2+}$  uptake and RyR-mediated SR  $\text{Ca}^{2+}$  leak. S100A1 is a  $\text{Ca}^{2+}$ -dependent molecular inotrope that regulates cardiac SR  $\text{Ca}^{2+}$ -handling and myofibrillar  $\text{Ca}^{2+}$  responsiveness.<sup>32,33</sup> S100A1 colocalizes and interacts with both the SERCA2a/PLB complex and RyR, thereby playing a key role in the coordinated enhancement of RyR2-mediated  $\text{Ca}^{2+}$  release during systole and SR- $\text{Ca}^{2+}$  uptake during diastole.<sup>34,35</sup> S100A1 enhances SR  $\text{Ca}^{2+}$  load without changing the PLB/SERCA2a ratio or PLB phosphorylation.<sup>36</sup> S100A1 also inhibits spontaneous RyR activity and decreases SR  $\text{Ca}^{2+}$  leakage.<sup>34,35</sup>

The lack of change of Pser<sup>2809</sup>RyR/RyR and P-PLB/PLB ratios in BNP-Sham mice and the fact that neither PKA nor CaMKII is involved in the regulation of RyR and PLB by S100A1<sup>36</sup> together suggest that phosphorylation is not a key player in the chronic effects of BNP. Our data are also consistent with the functional consequences of an S100A1 deficit.<sup>36</sup> Abnormal RyR openings in diastole occurred independently of increased PKA-dependent RyR phosphorylation, known to favour RyR opening.<sup>37</sup> An increase in spark frequency in the absence of increased PKA-dependent RyR phosphorylation is already known.<sup>10</sup> Therefore, the elevation in diastolic  $\text{Ca}^{2+}$  due to impaired SR  $\text{Ca}^{2+}$  uptake may contribute to SR  $\text{Ca}^{2+}$  leak, notably in relation with the biphasic  $\text{Ca}^{2+}$ -dependent effect of S100A1 on RyR activity, which depends on cytosolic  $\text{Ca}^{2+}$  levels.<sup>30,36</sup> Other mechanisms, such as S-nitrosylation, cannot be excluded.<sup>10</sup>

## 4.3 The role of ANS and the $\beta$ 1-adrenergic pathway

A recent report shows the beneficial effects of local BNP on SERCA2a following intramyocardial gene delivery.<sup>38</sup> This apparent inconsistency with our data, together with other reports,<sup>13–15</sup> may in fact reflect different aspects of BNP action: localized vs. systemic effects, and/or acute vs. chronic effects. A dose-dependent effect of circulating BNP has been described.<sup>7,39</sup> Our study points to the critical importance of systemic effects of chronically elevated BNP on cardiac function. Indeed, BNP induces a neurohormonal imbalance and affects the myocardium through sympathetic activation. Decreased HRV is a strong adverse prognostic marker for heart distress and cardiac mortality<sup>40</sup> in patients with decompensated HF<sup>7</sup> or sympathetic overdrive.<sup>24</sup> A decrease in SDNN is an established marker of sympathetic activation in HF, where its reduction parallels disease severity.<sup>24</sup> The increase in the LF and the LF/HF ratio, a sympathovagal index, in healthy mice treated with BNP further confirms enhanced sympathetic tone.<sup>23,24</sup> This is consistent with findings showing that high-dose BNP increases sympathetic activity in decompensated HF<sup>7</sup> or in patients with essential hypertension.<sup>8</sup> Overactivation of the sympathetic system is in part responsible for the cellular alterations observed in our model. These effects could result from a reflex response<sup>7,8</sup> or an enhancement of the adrenergic pathway,<sup>15,41,42</sup> which is known to promote ventricular hypertrophy and  $\text{Ca}^{2+}$ -cycling alterations.<sup>43</sup>

The use of metoprolol, which counteracts sympathetic overdrive, provides strong evidence for an active role of the  $\beta$ -adrenergic

system in the adverse cardiac remodelling induced by chronic BNP. Indeed, metoprolol prevented several functional alterations, including  $\text{Ca}^{2+}$  mishandling and the triggering of cellular  $\text{Ca}^{2+}$  waves and VA in healthy BNP-treated animals. This is in line with results showing that high BNP sensitizes the  $\beta$ 1-adrenergic response via NPR-B,<sup>42</sup> the predominant natriuretic peptide receptor in the failing heart.<sup>41</sup> At the protein level, metoprolol prevented SERCA2a and S100A1 blunting, thus maintaining normal SR  $\text{Ca}^{2+}$  re-uptake, correcting RyR-mediated  $\text{Ca}^{2+}$  leakage, and retaining low diastolic  $\text{Ca}^{2+}$  levels.<sup>17,44</sup> In short, metoprolol contributed to preserving SR  $\text{Ca}^{2+}$  content and  $\text{Ca}^{2+}$  transient amplitude, and consequently, cardiomyocyte contraction.<sup>45</sup>

## 4.4 Role of endogenous BNP during the progression of HF?

There were certain phenotypic differences between BNP-Sham and PMI mice: unlike PMI, BNP had no noticeable effect on myocardial function, heart rate, electrical conduction (QRS), or fibrosis in Shams. At the cardiomyocyte level, however, BNP-Sham mice exhibited established features of HF (reproduced in PMI mice) regarding  $\text{Ca}^{2+}$  handling. In addition, both BNP treatment and MI blunted the expression of S100A1, which contributes to  $\text{Ca}^{2+}$ -handling alterations and depressed contraction.<sup>36,46</sup> S100A1 is decreased in HF,<sup>30</sup> promotes SR  $\text{Ca}^{2+}$  leak by increasing the probability of RyR2 opening,<sup>47</sup> and hampers  $\text{Ca}^{2+}$  reuptake due to reduced SERCA2a activity.<sup>30</sup> Both altered SERCA2a expression and BNP production are considered early indicators of HF and are inversely correlated in human cardiac hypertrophy and HF.<sup>14,48,49</sup> Our finding regarding blunted S100A1 and SERCA2a expression in BNP-Sham mice further strengthens the concept that BNP contributes to adverse cardiac remodelling early in the progression of HF.<sup>13,14</sup> This could explain the ineffectiveness of nesiritide in treating HF, despite its beneficial haemodynamic effects.<sup>15</sup> Both HRV analysis and the prevention of the deleterious effects of BNP by metoprolol suggest that BNP acts in part through adrenergic overdrive. We observed similar effects of metoprolol in BB-PMI mice, confirming previous studies describing an increase in  $\text{Ca}^{2+}$  reuptake through increased SERCA2a expression, increased phosphorylation of PLB,<sup>50</sup> and decreased RyR2 phosphorylation. Our study also highlights the functional consequences of the previously unsuspected but recently described cardiac pro-adrenergic property of BNP,<sup>15</sup> which could potentially lead to a cellular HF-like profile with  $\text{Ca}^{2+}$ -cycling defects or aggravate existing HF.

### 4.4.1 Clinical implications

Our work shows that elevated blood BNP is not only a biomarker for guided therapy in HF but contributes *per se* to adverse cardiac remodelling and the triggering of VA, making the rapid lowering of BNP levels in HF patients a highly desirable end, in harmony with early in-hospital treatment aimed at decreasing BNP levels to improve survival.<sup>51–53</sup> Our study brings into question the treatment of HF patients with synthetic natriuretic peptide-like drugs, whose efficacy is uncertain<sup>54,55</sup> and whose link to increased mortality has been suspected.<sup>5,6</sup> Indeed, the ventricular remodelling induced by chronically elevated natriuretic peptides may limit their haemodynamic benefits. Overall, our work supports the general concept that the very early normalization of SERCA2a<sup>56</sup> or/and S100A1 expression<sup>30</sup> is critical in limiting adverse cardiac remodelling in HF. Last, but not least,  $\beta$ -blocker therapy should be considered as soon as BNP levels rise, even in patients without cardiac symptoms.

## Supplementary material

Supplementary material is available at

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