

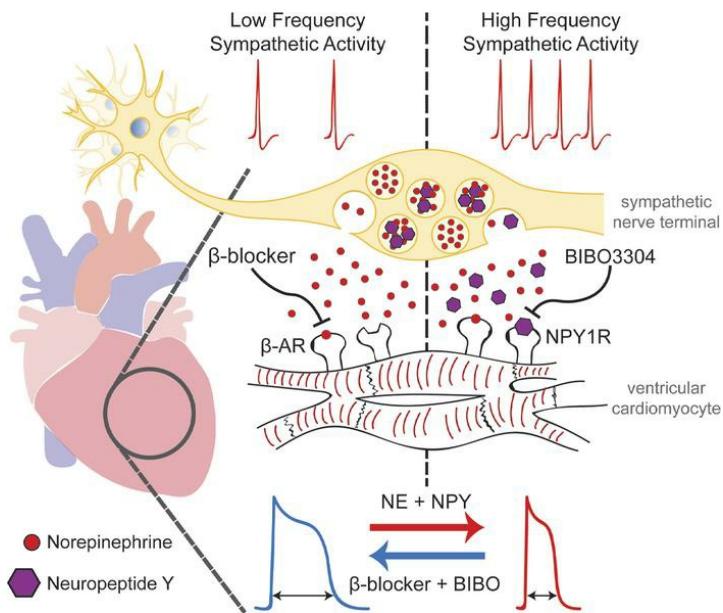
Cardiac sympathetic activation circumvents high-dose beta-blocker therapy in part through release of neuropeptide Y

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Graphical abstract



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Cardiac sympathetic activation circumvents high-dose beta blocker therapy in part through release of neuropeptide Y

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The sympathetic nervous system plays an important role in the occurrence of ventricular tachycardia (VT). Many patients, however, experience VT despite maximal doses of beta blocker therapy, possibly due to the effects of sympathetic cotransmitters such as neuropeptide Y (NPY). The purpose of this study was to determine, in a porcine model, whether propranolol at doses higher than clinically recommended could block ventricular electrophysiological effects of sympathoexcitation via stellate ganglia stimulation, and if any residual effects are mediated by NPY. Greater release of cardiac NPY was observed at higher sympathetic stimulation frequencies (10 and 20 vs. 4 Hz). Despite treatment with even higher doses of propranolol (1.0 mg/kg), electrophysiological effects of sympathetic stimulation remained, with residual shortening of activation recovery interval (ARI), a surrogate of action potential duration (APD). Adjuvant treatment with the NPY Y₁ receptor antagonist BIBO 3304, however, reduced these electrophysiological effects while augmenting inotropy. These data demonstrate that high-dose beta blocker therapy is insufficient to block electrophysiological effects of sympathoexcitation, and a portion of these electrical effects *in vivo* are mediated by NPY. Y₁ receptor blockade may represent a promising adjuvant therapy to beta-adrenergic receptor blockade.

Introduction

The sympathetic nervous system plays an important role in the occurrence of ventricular tachycardia (VT) and ventricular fibrillation (VF) (1–3). Cardiac sympathetic activation causes triggered activity (4, 5) and increases in heterogeneity and dispersion of ventricular repolarization (6–8), leading to VT, VF, and sudden cardiac death (9). Beta blocker therapy, by targeting beta-adrenergic receptors for norepinephrine (NE), remains the cornerstone of sympathetic neuromodulation for treatment of VT and VF (10). Recently, propranolol has been suggested to be more efficacious for control of recurrent ventricular arrhythmias than metoprolol, especially in the setting of VT/VF (electrical) storm (11).

However, despite beta blocker therapy at maximally tolerated doses, patients can continue to experience recurrent VT and VF episodes (11). It is possible that during states of significantly elevated sympathetic tone, beta blocker therapy is insufficient to completely suppress the electrophysiological effects of sympathetic activation. This may in part be due to release of sympathetic neuropeptides, such as neuropeptide Y (NPY), which are reported to be released during states of excessive sympathetic activation (12–14), and as yet are not therapeutically targeted. It has been reported that elevated coronary sinus (CS) plasma NPY levels in patients presenting with heart failure portends a poor outcome (15), and in patients with acute myocardial infarction (MI) is associated with higher ventricular arrhythmia scores (14), greater infarct size, and reduced ejection fraction, despite reperfusion therapy (16). In a rat Langendorff model, blockade of the myocardial NPY Y₁ receptor (NPY1R) by BIBO 3304 increased VF thresholds (17). These data suggest that NPY has proarrhythmic potential, which could be mediated through its Y₁ receptor on cardiomyocytes (18). However, direct ventricular electrophysiological effects of NPY *in vivo* remain to be evaluated.

Authorship note: JDH, SS, and NY contributed equally to this work.

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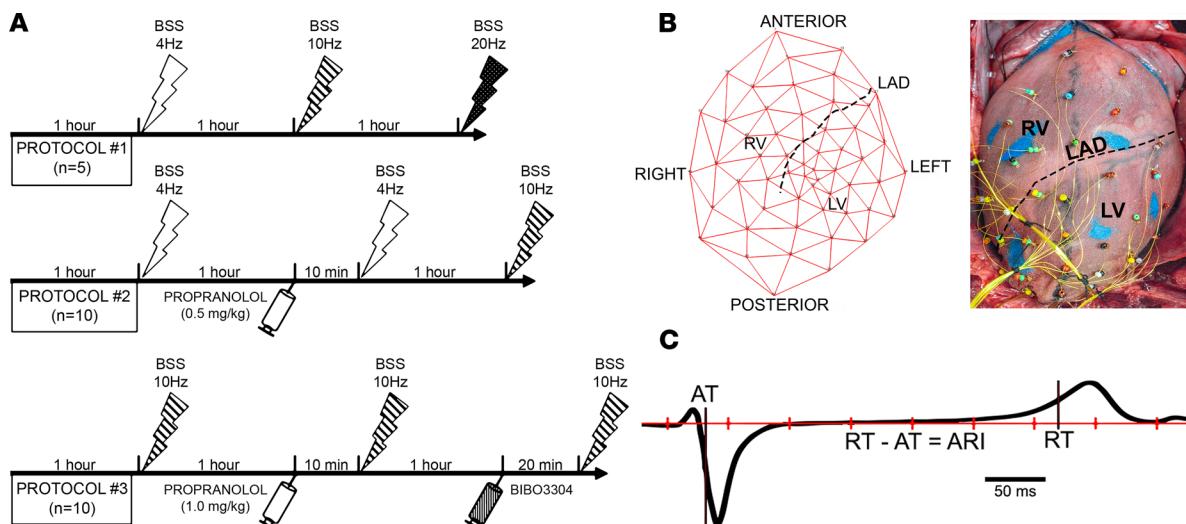


Figure 1. Study design and methods. (A) Time line of protocols used to evaluate effects of BSS under different pharmacological conditions. (B) Schematic of the 56-electrode sock used to acquire local electrograms from ventricular epicardium. Electrograms are mapped onto a 2-dimensional plane to assess regional differences. LAD, left anterior descending artery. (C) ARI, a surrogate for APD, is measured as the time from the most negative dV/dt of the activation wave front to the most positive dV/dt of the repolarization wave front.

The purpose of this study was to evaluate the effects of sympathetic activation via bilateral stellate ganglion stimulation (BSS) at different frequencies on cardiac electrophysiological indices, hemodynamic parameters, and NE and NPY levels, in a porcine model *in vivo*. In addition, we hypothesized that high doses of propranolol, at several times greater than clinically indicated doses, may not be sufficient to completely block the effects of sympathoexcitation. Finally, we assessed whether any remaining electrophysiological effects may be driven from the release of cardiac NPY and attenuated by infusion of the Y₁ receptor blocker BIBO 3304.

Results

In order to study the effects of sympathetic stimulation on hemodynamic and electrophysiological parameters and neurotransmitter/neuropeptide profiles with and without propranolol and the Y₁ inhibitor BIBO 3304, 3 protocols involving different groups of animals (protocols 1–3) were used (Figure 1A).

Effects of frequency of sympathetic stimulation on hemodynamic parameters, neurotransmitter/neuropeptide profiles, and electrophysiological parameters (protocol 1). In protocol 1, the effects of frequency on hemodynamic and electrical parameters as well as NE and NPY release were tested at 3 different frequencies (4, 10, and 20 Hz) but at the same fixed current (defined as 1.2 times the threshold current that led to a 10% increase in heart rate [HR] or systolic blood pressure at 4 Hz) *in vivo* to determine NE and NPY release profiles in Yorkshire pigs ($n = 5$; Figure 1). All tested frequencies of stimulation significantly increased HR, left ventricular (LV) systolic pressure (LVSP), and dP/dt_{max} from baseline ($P < 0.05$) (Figure 2, A–C). BSS at 10 Hz increased HR more than at 4 Hz (61.5 ± 7.0 bpm vs. 22.7 ± 5.5 bpm; $P = 0.02$). Further increases in HR at 20 Hz versus 10 Hz were not observed. There were no significant differences between frequencies of stimulation with regard to increases in LVSP or dP/dt_{max} .

The effects of frequency of BSS on electrical, hemodynamic, and plasma NE and NPY levels are shown in Figure 2, D and E. All frequencies of stimulation increased CS NE levels by 100- to 150-fold. BSS at 10 Hz and 20 Hz led to significantly greater release of CS NE compared with 4 Hz. There were no statistically significant differences in NE release profiles at 10 Hz vs. 20 Hz.

BSS at 4 Hz caused a significant but modest change in CS NPY levels (from 6.7 ± 2.6 pg/mL to 14.1 ± 1.3 pg/mL; $P = 0.046$) but not FA NPY levels (Figure 2E). However, BSS at 10 Hz evoked a 5-fold greater release of CS NPY than 4 Hz (39.3 ± 12.2 pg/mL with 10 Hz vs. 7.2 ± 2.7 pg/mL with 4 Hz; $P = 0.04$). BSS at 20 Hz further increased CS NPY levels compared with 10 Hz (from 7.0 ± 4.7 pg/mL to 91.4 ± 16.7 pg/mL; $P < 0.01$). CS and FA release profiles for NE and NPY are shown in Tables 1 and 2, respectively.

Table 1. Plasma NE concentrations in the CS and femoral artery at baseline and during BSS for protocols 1–3

			BL (ng/mL)	BSS (ng/mL)	Δ (ng/mL)
Protocol 1 (n = 5)	4 Hz BSS	CS	0.04 ± 0.0	4.7 ± 1.2^A	4.6 ± 1.2
		FA	0.2 ± 0.03	1.9 ± 0.5^A	1.7 ± 0.4
	10 Hz BSS	CS	0.07 ± 0.04	10.2 ± 2.3^A	10.2 ± 2.3
		FA	0.4 ± 0.3	4.7 ± 1.1^B	4.3 ± 0.9
	20 Hz BSS	CS	0.06 ± 0.03	9.4 ± 1.3^B	9.3 ± 1.3
		FA	0.2 ± 0.04	5.0 ± 0.6^B	4.8 ± 0.6
Protocol 2 (n = 10)	4 Hz BSS + 0.5 mg/kg propranolol	CS	0.2 ± 0.06	2.2 ± 0.7^A	2.1 ± 0.7
		FA	1.4 ± 0.5	8.9 ± 2.3^B	7.5 ± 2.0
	10 Hz BSS + 0.5 mg/kg propranolol	CS	0.2 ± 0.06	2.70 ± 0.4^C	2.5 ± 0.4
		FA	2.4 ± 0.6	18.7 ± 4.4^B	16.2 ± 4.2
Protocol 3 (n = 10)	10 Hz BSS	CS	0.1 ± 0.04	9.1 ± 1.6^C	9.0 ± 1.6
		FA	0.07 ± 0.03	4.3 ± 1.5^A	4.2 ± 1.5
	10 Hz BSS + 1.0 mg/kg propranolol	CS	0.1 ± 0.04	1.14 ± 0.38^A	1.0 ± 0.4
		FA	0.1 ± 0.05	0.8 ± 0.2^B	0.7 ± 0.2
	10 Hz BSS + 1.0 mg/kg Propranolol + BIBO 3304	CS	0.3 ± 0.08	1.50 ± 0.6	1.2 ± 0.6
		FA	0.2 ± 0.05	0.83 ± 0.2^C	0.7 ± 0.1

^AP ≤ 0.05, ^BP < 0.01, ^CP < 0.001 vs. baseline. Bold values represent statistically significant measurements compared with baseline.

Ventricular activation recovery intervals (ARIs), corrected for HR (ARIc), shortened during BSS compared with baseline with all frequencies of stimulation (Figure 3). Stimulation at 4 Hz shortened global ARIc by 75 ± 17 ms (from 397 ± 8 ms to 322 ± 17 ms; P = 0.01), and BSS at 10 Hz induced further shortening (139 ± 8 ms; from 392 ± 9 ms to 253 ± 10 ms; P < 0.001). BSS at 20 Hz also caused a 166 ± 8 ms shortening in global ventricular ARIc (from 415 ± 6 ms to 249 ± 10 ms; P < 0.001), but this was not significantly different from the changes observed at 10 Hz. No significant regional differences in ARIs at different frequencies of stimulation were noted (Supplemental Figure 1; supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.135519DS1>).

Effects of sympathetic stimulation after 0.5 mg/kg propranolol (protocol 2). Given the lack of electrophysiological and ARI differences between 10 Hz and 20 Hz of stimulation frequency, the effects of propranolol 0.5 mg/kg were evaluated during BSS at 4 Hz and 10 Hz (n = 10) (Figure 1). Despite this high dose of

Table 2. Plasma NPY concentrations in the CS and femoral artery at baseline and during BSS

			BL (pg/mL)	BSS (pg/mL)	Δ (pg/mL)
Protocol 1 (n = 5)	4 Hz BSS	CS	6.7 ± 2.6	14.1 ± 1.3^A	7.2 ± 2.7
		FA	8.0 ± 5.5	11.0 ± 6.2	3.1 ± 1.8
	10 Hz BSS	CS	4.6 ± 2.9	43.9 ± 12.3^A	39.3 ± 12.2
		FA	12.9 ± 8.2	15.6 ± 4.2	9.6 ± 3.0
	20 Hz BSS	CS	7.0 ± 4.7	91.4 ± 16.7^B	84.5 ± 14.5
		FA	11.7 ± 2.7	27.4 ± 8.5	15.7 ± 6.3
Protocol 2 (n = 10)	4 Hz BSS + 0.5 mg/kg propranolol	CS	9.0 ± 2.3	11.9 ± 2.0^B	2.3 ± 0.7
		FA	8.0 ± 1.8	9.6 ± 1.9	1.9 ± 0.9
	10 Hz BSS + 0.5 mg/kg propranolol	CS	7.3 ± 1.9	17.1 ± 3.6^B	9.9 ± 2.6
		FA	7.9 ± 2.2	11.25 ± 2.41	3.9 ± 1.5
Protocol 3 (n = 10)	10 Hz BSS	CS	7.0 ± 1.7	16.4 ± 2.1^B	9.5 ± 2.4
		FA	12.6 ± 4.2	14.5 ± 2.9	6.3 ± 2.1
	10 Hz BSS + 1.0 mg/kg propranolol	CS	9.6 ± 1.9	14.9 ± 1.9^B	5.3 ± 1.3
		FA	17.1 ± 6.1	15.9 ± 4.9	2.5 ± 1.2
	10 Hz BSS + 1.0 mg/kg propranolol + BIBO 3304	CS	9.3 ± 1.5	16.3 ± 1.5^C	7.0 ± 1.3
		FA	16.1 ± 6.0	17.8 ± 4.4	4.4 ± 1.3

BL, baseline. ^AP ≤ 0.05, ^BP < 0.01, ^CP < 0.001 vs. baseline. Bold values represent statistically significant measurements compared with baseline.

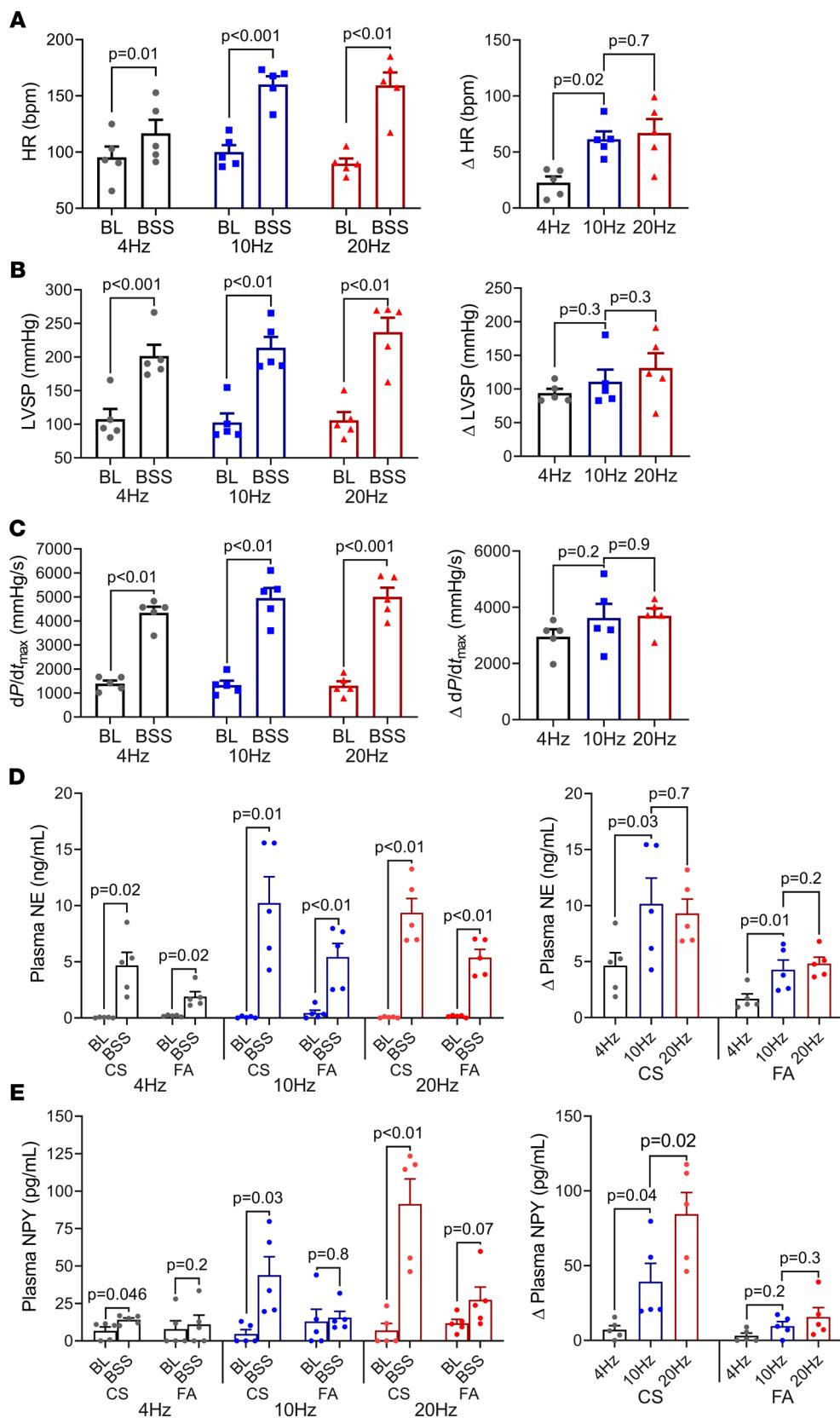


Figure 2. Effects of different frequencies of BSS on cardiac hemodynamic parameters, NE, and NPY. All frequencies of stimulation significantly increased (A) HR, (B) LVSP, (C) and dP/dt_{max} . HR, unlike LVSP or dP/dt_{max} , increased significantly more at 10 than 4 Hz. There were no significant differences in hemodynamic parameters between 10 and 20 Hz. (D) Plasma NE in the CS increased with all frequencies of stimulation, but release was significantly greater in the CS than FA. Release of NE was greater at the higher frequencies. There was no difference in the changes in CS NE levels between 10 and 20 Hz of stimulation. (E) CS NPY levels at 10 Hz BSS were greater than at 4 Hz, with further increases observed at 20 Hz. $n = 5$ animals for all comparisons; baseline (BL) vs. stimulation comparisons were performed using 2-sided paired Student's t test and comparisons of changes between different frequencies were performed using 1-way ANOVA with post hoc analysis. $P \leq 0.05$ was considered statistically significant.

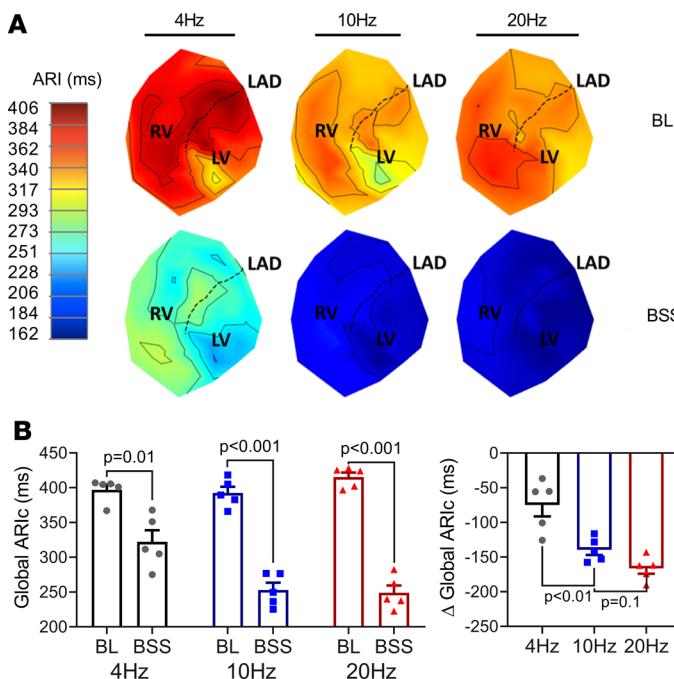


Figure 3. Effects of frequency of stimulation on ventricular electrophysiology. **(A)** Representative polar maps depicting the effects of BSS at 4, 10, and 20 Hz on ventricular ARIs. **(B)** All stimulation frequencies caused significant shortening of corrected ARIs. BSS at 10 Hz and 20 Hz shortened global ventricular ARIs more than at 4 Hz, even after correcting for HR. There was no difference in ARIC shortening between BSS at 10 vs. 20 Hz. Δ , change from BL. $n = 10$ animals for all comparisons; baseline vs. stimulation comparisons were performed using 2-sided paired Student's *t* test, and comparisons of changes between different frequencies were performed using 1-way ANOVA with post hoc analysis.

propranolol, BSS at 4 Hz and 10 Hz significantly increased HR, LVSP, and dP/dt_{max} (Supplemental Figure 2), with greater increases in LVSP and dP/dt_{max} at 10 Hz than 4 Hz.

BSS at 4 Hz and 10 Hz shortened global ventricular ARIC compared with baseline, despite beta blocker therapy. However, BSS at 10 Hz caused greater global ARIC effects than at 4 Hz (a decrease of 29 ± 4 ms at 10 Hz vs. 9 ± 3 ms at 4 Hz; $P < 0.001$) after propranolol treatment, despite similar levels of NE in the CS, further suggesting a potential role for other cotransmitters in mediating these effects. Raw (uncorrected) ARI values for BSS at 4 Hz and 10 Hz are reported in Supplemental Figure 3.

Effects of sympathetic stimulation after 1.0 mg/kg propranolol (protocol 3). Given the results of the above experiments, which demonstrated significant electrophysiological effects remaining with 0.5 mg/kg propranolol and significant release of NPY at 10 Hz, in protocol 3 we used stimulations at 10 Hz to evaluate effects of BSS after treatment with 1.0 mg/kg propranolol (to assure even greater blockade of β -adrenergic receptors) (Figure 1).

Propranolol (1.0 mg/kg) significantly mitigated BSS-induced CS NE release profiles (from 9.0 ± 1.6 to 1.0 ± 0.4 ng/mL), while there was only a modest reduction in BSS-induced release of CS NPY (Tables 1 and 2).

Although the effects of BSS on hemodynamic parameters and ventricular ARIs were significantly reduced after infusion of 1.0 mg/kg propranolol, BSS still increased HR, LVSP, and dP/dt_{max} ($P < 0.001$ for all parameters; Figure 4, A–C). Furthermore, after treatment with 1.0 mg/kg propranolol, BSS continued to significantly shorten global ventricular ARIC by 19 ± 5 ms (from 359 ± 12 ms to 341 ± 10 ms; $P < 0.01$; Figure 4, D and E). Raw (uncorrected) ARI values are reported in Supplemental Figure 3.

Expression of NPY1R on the ventricular myocardium. The presence of NPY1R protein in the ventricular myocardium was confirmed by Western blotting (Figure 5B; see complete unedited gel in the supplemental material). A major immunoreactive band was detected at approximately 51 kDa in the apex, mid-lateral wall, and base of the LV ($n = 3$). The higher molecular weight may represent an intermediate *N*- or *O*-glycosylated form, with bands up to 55 kDa reported in human tissue (19).

The localization of NPY1R was further validated by immunohistochemistry (Figure 5A). High expression of NPY1R was noted in the vascular smooth muscle of cardiac blood vessels with adjacent

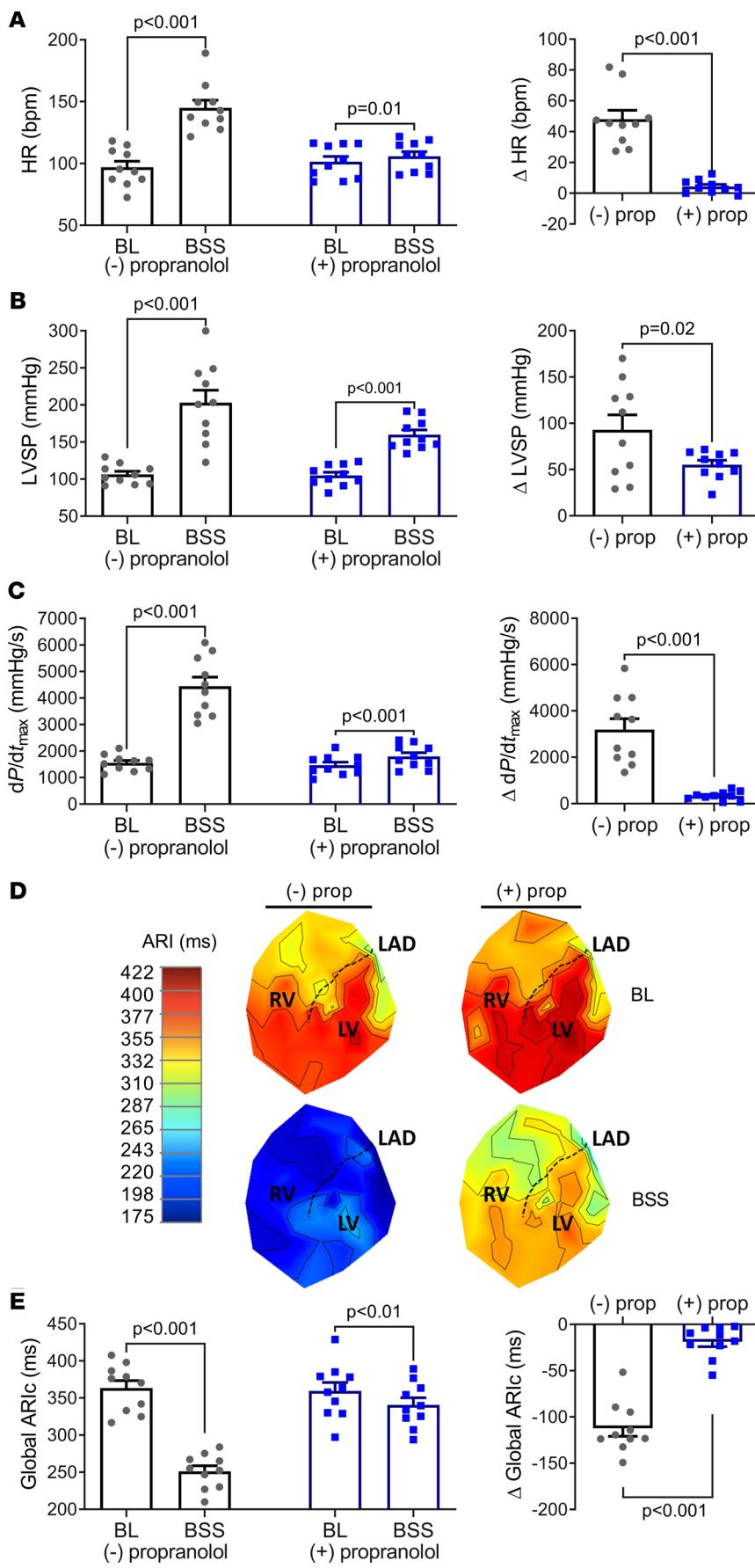


Figure 4. Effects of 1.0 mg/kg propranolol on BSS-induced changes in hemodynamic and electrophysiological parameters. Although the effects of BSS, especially on HR, were mitigated by 1.0 mg/kg propranolol, 10 Hz BSS continued to significantly increase (A) HR, (B) LVSP, and (C) dP/dt_{max} . (D) Representative polar maps comparing raw ventricular ARIs with and without propranolol. (E) Although 1.0 mg/kg propranolol did mitigate BSS-induced changes in global ARIs, it did not completely block the effects of BSS. (-) prop, untreated animals; (+) prop, 1.0 mg/kg propranolol. n = 10 animals for all comparisons; analyses were performed using 2-sided paired Student's t test.

NPY-immunoreactive nerve fibers. While highly expressing NPY, cardiac ganglia expressed only low levels of NPY1R. Adjacent myocardium showed moderate expression of NPY1R with NPY-immunoreactive nerve fibers running between myocytes. Neither NPY- nor NPY1R-immunoreactive puncta were observed in negative controls of any area.

Effects of adjuvant NPY1R antagonism on BSS-induced hemodynamic and electrophysiological changes (protocol 3). To assess the role of NPY via Y₁ receptors in mediating the observed residual effects of sympathoexcitation in the setting of high-dose propranolol, BIBO 3304, a selective NPY1R antagonist, was administered in the same animals that received 1.0 mg/kg propranolol.

Infusion of BIBO 3304 after propranolol had no significant additional effect on HR or dP/dt_{max} (Figure 5, C and E). However, it caused a modest reduction in LVSP ($P = 0.04$) and, importantly, prolonged global ventricular ARIc (from 381 ± 16 ms to 389 ± 15 ms; $P = 0.02$; Figure 5, F and G).

Following adjuvant therapy with BIBO 3304, BSS at 10 Hz continued to increase LVSP (from 98.1 ± 4.7 mmHg to 147.0 ± 10.9 mmHg; $P < 0.001$) and dP/dt_{max} (from 1234.6 ± 84.4 mmHg/s to 1780.1 ± 124.1 mmHg/s; $P < 0.001$) (Figure 6, B and C). Unlike with intravenous propranolol at 1.0 mg/kg, there was no longer a significant HR effect with BSS after treatment with BIBO 3304 and propranolol (103.7 ± 4.9 bpm to 104.7 ± 5.5 bpm; $P = 0.6$; Figure 6A). Thus, BSS-induced increases in HR in the setting of propranolol alone were inhibited after administration of BIBO 3304 (4.4 ± 1.4 bpm vs. 1.0 ± 1.9 bpm, respectively; $P = 0.02$). There was no significant difference in BSS-induced increases in LVSP, however, between propranolol alone and propranolol with BIBO 3304, suggesting that BSS continues to have significant effects on

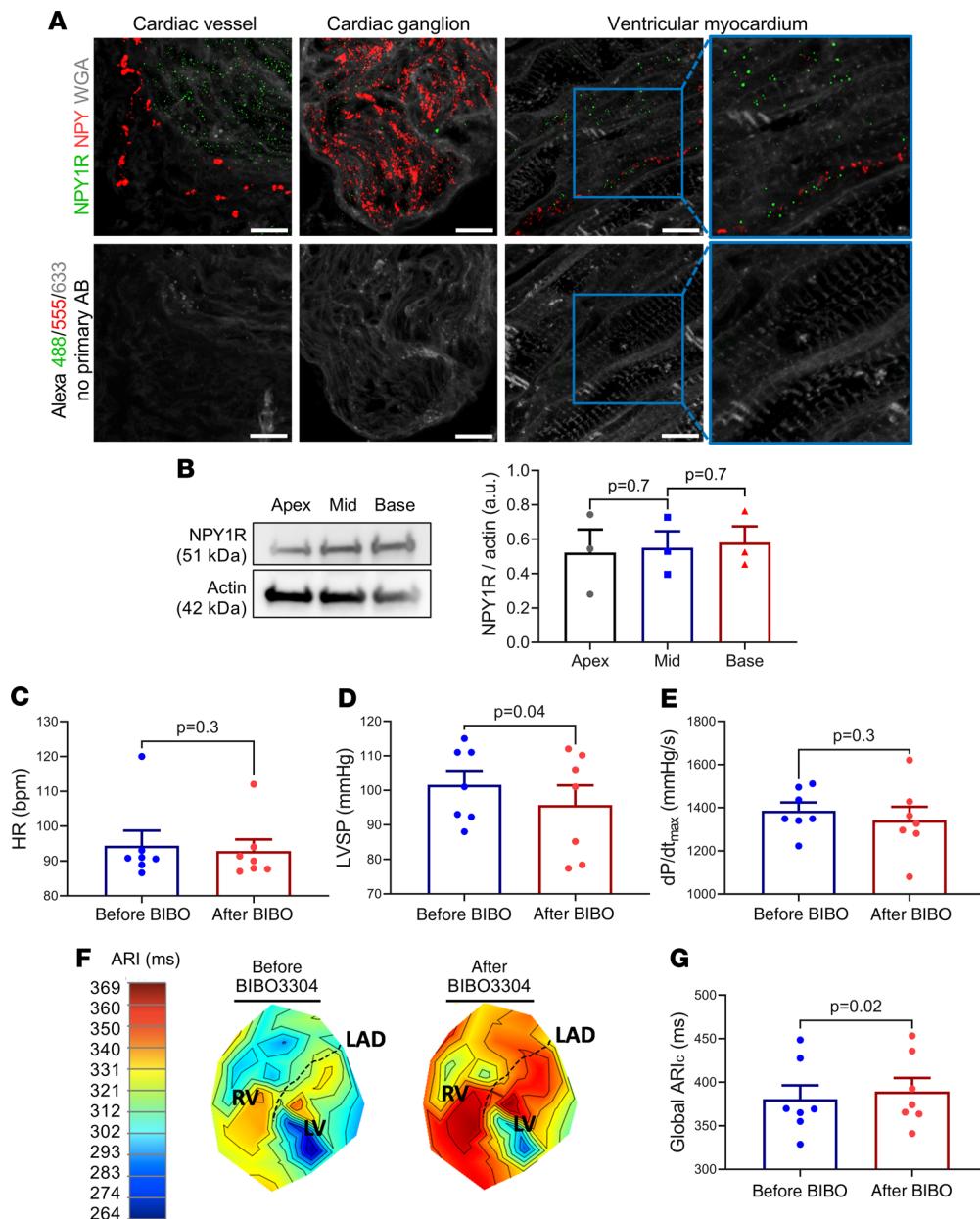


Figure 5. Expression of NPY1R in the porcine ventricular myocardium and the effects of infusion of BIBO 3304 on hemodynamic parameters and ARIs. (A) NPY1R expression was confirmed in the porcine heart. High expression of NPY1R (green) is evident in the vascular smooth muscle, with moderate expression in intracardiac ganglia and myocardium. NPY-immunoreactive nerve fibers (red) directly appose the NPY1R-immunoreactive vessels and myocardium. (B) The presence of NPY1R is confirmed by Western blot analysis of ventricular whole cell lysate and shows no regional differences in expression. BIBO 3304 had no significant effect on resting (C) HR or (D) dP/dt_{max} , while a modest reduction in (E) LVSP (101.6 ± 4.1 to 94.7 ± 5.7 mmHg) was observed. (F) Representative polar maps comparing the effects of BIBO 3304 on raw ARIs. (G) Global corrected ventricular ARIs were significantly prolonged following the administration of BIBO 3304. Scale bars: 10 μ m. **A** and **B**: $n = 3$ animals for all comparisons; comparisons were performed using 1-way ANOVA with post hoc analysis. **C–F**: $n = 7$ animals for all comparisons; comparisons were performed using 2-sided paired Student's *t* test. "Before BIBO" refers to data recorded just before infusion of BIBO 3304 but after infusion of 1.0 mg/kg propranolol for protocol 3, while "After BIBO" represents data taken after 20 minutes of BIBO 3304 infusion.

systolic blood pressure. Of note, inotropy, as measured by dP/dt_{max} , improved after BIBO 3304 infusion during BSS (an increase of 333.6 ± 59.8 mmHg/s before vs. 545.5 ± 78.3 mmHg/s after BIBO 3304 with BSS; $P = 0.047$; Figure 6C).

Despite high-dose propranolol, BSS significantly decreased global ventricular ARIC (from 359 ± 12 ms to 341 ± 10 ms; $P < 0.01$; Figure 6, D and E). After additional administration of BIBO 3304, however, effects of

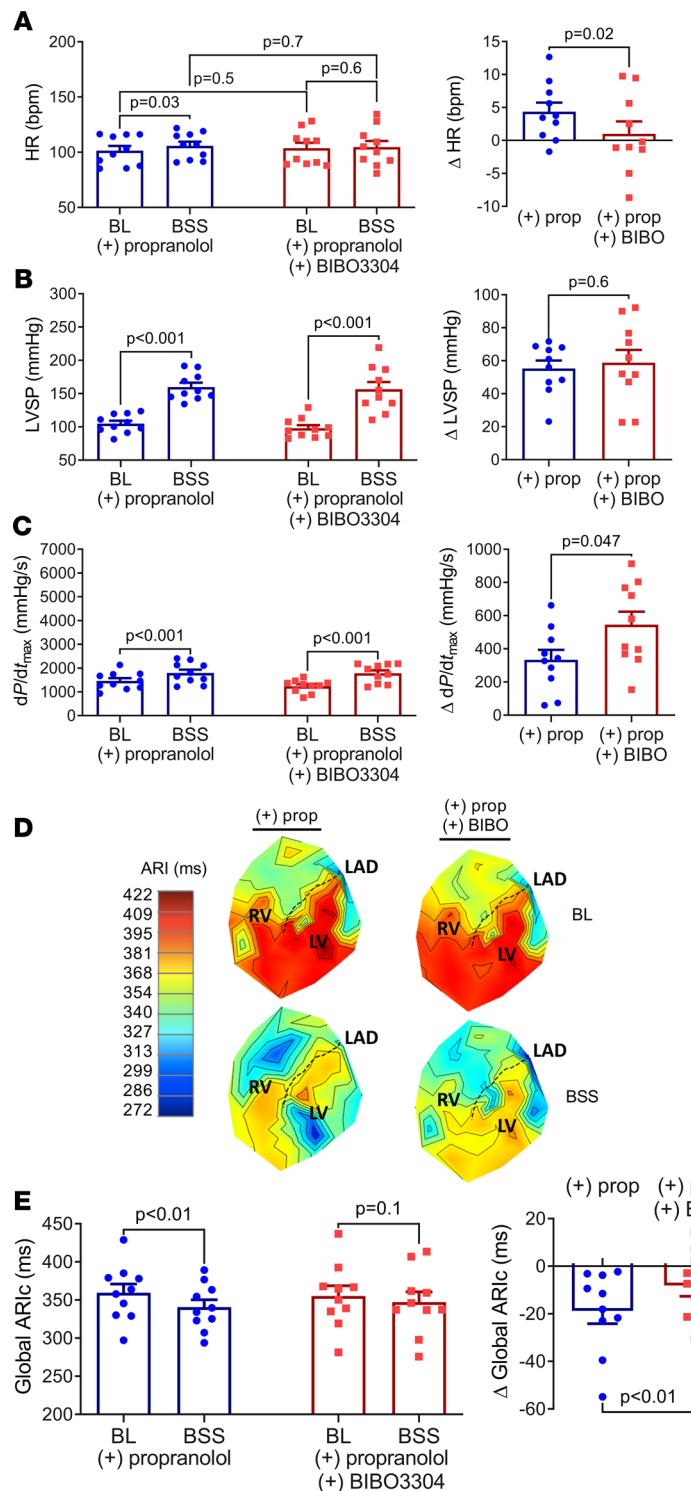


Figure 6. Effects of BSS on hemodynamic and electrophysiological parameters after 1.0 mg/kg propranolol i.v. and BIBO 3304 as compared with propranolol alone. BIBO 3304 completely blocked the effects of BSS on (A) HR, while residual effects on (B) LVSP and (C) dP/dt_{max} continued to be observed. BIBO 3304 augmented the changes in dP/dt_{max} . (D) Representative polar maps comparing effects of BSS on raw ventricular ARIs in the setting of high-dose propranolol, with and without BIBO 3304. (E) Global corrected ventricular ARIs significantly shortened despite administration of high-dose propranolol, but not after administration of BIBO 3304. Additional treatment with BIBO 3304 significantly reduced BBS-induced shortening of ARIc. (+) prop, 1.0 mg/kg propranolol, (-) BIBO, no NPY1R blockade, (+) BIBO, 0.2 mg/kg + 0.4 mg/kg/h BIBO 3304. $n = 10$ animals for all comparisons, analyses were performed using the 2-sided paired Student's t test.

BSS on ventricular ARIs were reduced (from 355 ± 13 ms to 347 ± 14 ms; $P = 0.1$). A mean difference in ARIs shortening of approximately 11 ms was observed after combined propranolol and BIBO 3304 compared with propranolol alone (19 ± 5 ms with propranolol alone to 8 ± 4 ms with propranolol + BIBO 3304; $P < 0.01$).

Discussion

Major findings. In the present study, we tested the hypothesis that elevated sympathetic tone circumvents the effects of high-dose propranolol and that its electrophysiological effects may, in part, be mediated via NPY. Using a porcine model, we first confirmed the frequency-dependent release of NPY in vivo in this species. We initially tested propranolol at 0.5 mg/kg i.v. to assess whether this dose would block electrophysiological effects of sympathoexcitation, as it represents 5 times the maximal clinically recommended dose for ventricular arrhythmias (10). Surprisingly, while 0.5 mg/kg propranolol reduced effects of BSS, significant effects on ARIs (APD) persisted, especially at 10 Hz of stimulation, suggesting that high doses of propranolol are insufficient to block the effects of sympathoexcitation and providing insight as to why patients may experience recurrent arrhythmias despite high doses of beta blocker therapy. Subsequently, in order to further isolate effects of NPY versus NE, we used 1.0 mg/kg propranolol to evaluate the electrophysiological effects of NPY. Detailed in vivo evaluation showed shortening of ventricular ARIs despite high-dose beta blocker therapy, effects that were mitigated by Y₁ receptor blockade. Our findings are in line with previous ex vivo data in rats and studies suggesting elevated arrhythmic risk in MI patients with higher NPY levels (14). Other key findings of this study include the following: (i) While cardiac NE release was significant with BSS at 4 Hz, this release profile plateaued at 10 Hz in vivo. Only modest amounts of NPY were released at 4 Hz of stimulation, with pronounced release of NPY at 10 Hz and 20 Hz of BSS. (ii) Beta blocker therapy decreased release of NE but did not affect NPY release. (iii) Although Y₁ receptor blockade caused a modest decrease in systolic blood pressure, suggesting afterload reduction, it did not affect dP/dt_{max} at baseline or during sympathetic activation, suggesting lack of deleterious inotropic effects. (iv) Y₁ receptor blockade demonstrated a significant and clinically meaningful reduction in ventricular ARIs, similar in magnitude to other neuromodulatory therapies, such as cardiac sympathetic denervation (CSD) (20), that are known to be antiarrhythmic. However, it did not inhibit BSS-induced increases in LVSP.

Beta blocker therapy and ventricular arrhythmias. Beta blocker therapy is the standard of care for patients who present with life-threatening VT. Recently, propranolol was shown to have better outcomes in treatment of patients presenting with VT storm than metoprolol. Despite treatment with beta blocker medications, however, many patients experience recurrent VT and VF (11, 21, 22). In this study, 0.5 mg/kg intravenous propranolol, which is 5–10 times the doses recommended by the American College of Cardiology/American Heart Association for treatment of VT (10), did not completely inhibit the effects of sympathoexcitation. Our data provide insight into why beta blocker therapy may be insufficient to prevent electrophysiological effects of sympathoexcitation. As a neuromodulatory therapy, CSD has shown benefit in the treatment of refractory VT in patients already treated with beta blocker therapy and in reducing VT inducibility in infarcted porcine hearts (20, 23). It is also possible that one of the mechanisms behind the beneficial effects of CSD is a reduction in NPY. CSD, by reducing afferent neurotransmission, can decrease efferent sympathetic outflow. In addition, by disrupting efferent sympathetic fibers, CSD can reduce both NE and NPY levels.

Beta blocker therapy can also act by presynaptic mechanisms to reduce NE release from sympathetic nerve terminals (24), as also observed in this study. We did not, however, observe a substantial reduction in NPY release after propranolol infusion. NE is stored in 2 different types of vesicles: small clear vesicles that primarily carry catecholamines; and large dense vesicles that also carry NPY (25–27). It is possible that beta blockers have differential presynaptic effects on the release of these vesicles. Unlike beta blocker therapy, which reduces the release of NE, it appears that NPY1R antagonism lacks presynaptic effects and does not affect the release of NPY, as shown in Table 2.

NPY, cardiovascular disease, and ventricular arrhythmias. NPY levels are increased in the setting of heart failure (28–30). Patients with MI who have higher CS NPY levels are at a greater risk of ventricular dysfunction, even despite reperfusion therapy (31), and those with higher plasma NPY were recently shown to have increased incidence of VT (14). Of note, in a rat Langendorff model, NPY significantly increased incidence of ischemia-driven ventricular arrhythmias (14). However, evaluation of the in vivo effects of NPY on ventricular APDs, especially in large animal models, is lacking. This is largely due to the overwhelming effect of NPY in causing vasoconstriction when given intravenously, which has prevented a detailed assessment of its electrophysiological effects. In this study, blockade of NPY1R with BIBO 3304

further mitigated the ventricular effects of sympathetic nerve stimulation on ARIs, a surrogate of APDs. Our study showed that on average, BIBO 3304 can mitigate ARI shortening by approximately 10 ms, even after correcting for HR, beyond beta blocker therapy. Although this is a modest reduction, previous studies of neuromodulation, such as CSD and vagal nerve stimulation, have shown that even 5- to 10-ms increases in ventricular ARIs or refractoriness are sufficient to significantly reduce VT/VF inducibility (20, 32, 33). Along this line, recently published data have shown that in an innervated rat Langendorff model, BIBO 3304 is able to increase VF threshold during sympathetic stimulation above and beyond beta blocker therapy with metoprolol, suggesting that Y₁ receptor blockade is antiarrhythmic ex vivo (17). Also, recent human data suggest that patients with ST-segment-elevation MI who have higher NPY levels also have higher incidence of ventricular arrhythmias, suggesting that NPY may be proarrhythmic in humans (17).

The effects of NPY on ventricular myocyte APD have been explored in cell culture using patch-clamping techniques. NPY has been shown to reduce APD in ventricular myocytes of guinea pigs (34). In addition, optical imaging of ex vivo rat hearts has shown that NPY significantly increases calcium transient amplitude, accompanied by a significant shortening of calcium transient duration (17). It has also been suggested that NPY1R activation enhances myocyte calcium release due to NE, acting in a synergistic fashion (35). While this mechanism can partly explain NPY-mediated APD shortening, the residual effects of BIBO 3304 beyond high-dose propranolol may also suggest that NPY1R has an independent mechanism for shortening APD in cardiomyocytes. We also noted a mitigation of effects of BIBO 3304 on HR, despite similar increases in LVSP, similar NE and NPY release profiles, and greater increases in inotropy with BSS. These data are in line with a previous study suggesting that NPY can cause Y₁ receptor-mediated stress-evoked tachycardia (36). The observed similar or greater effects on other hemodynamic parameters in this study suggest that repeat stimulation “fatigue,” which may occur with multiple stimulations of stellate ganglia if a sufficient waiting period between stimulations is not allowed, was not a factor in our studies (with a minimum of a 60-minute waiting period between stimulations).

NPY is a potent vasoconstrictor (37), but its effects on inotropy are unclear. A study in isolated rat cardiomyocytes suggested that NPY1R agonism may improve inotropy (38), while an in vivo study suggested that NPY inhibits the inotropic effects of sympathetic stimulation (39). It is also possible that by causing significant coronary vasoconstriction (16), NPY can interfere with myocardial metabolism and thereby cardiac function. Interestingly, NPY1R blockade with BIB O3304 did not reduce dP/dt_{max} and augmented the inotropic effects of BSS. Taken together, these data suggest that adjuvant myocardial blockade of NPY1R may be hemodynamically well tolerated. Despite combination of BIBO 3304 and propranolol, a modest residual effect on ARIs remained during BSS. This may be due to other sympathetic cotransmitters, such as galanin (13), whose levels are also elevated with cardiovascular disease (15) but whose electrophysiological effects remain unclear.

Of note, we tested the effects of BIBO 3304 at 10 Hz stimulation, given that we did not see electrophysiological differences between 10 Hz and 20 Hz. It is important to note that the levels of NPY observed in the CS of pigs in this study at 10 Hz (43.92 ± 12.25 pg/mL) were comparable to those observed in the CS of patients with acute MI (29.3 pg/mL, range 23.6–51.4 pg/mL), who had poorer outcomes at 6 months than patients who had lower levels of NPY (16).

Clinical implications. Recurrent ventricular arrhythmias occur in patients with cardiomyopathy, despite therapy with beta blocker medications, antiarrhythmic drugs, and catheter ablation, posing a significant therapeutic challenge (21, 22). Cardiac sympathetic activation is known to both trigger and maintain ventricular arrhythmias (1, 9). This study demonstrates that high-dose beta blocker therapy, at an order of magnitude higher than clinically recommended doses, still cannot overcome the electrophysiological effects of sympathoexcitation in normal porcine hearts, and that NPY has independent ventricular electrophysiological effects that are mediated through the Y₁ receptor. No adverse effects on inotropy were observed with Y₁ receptor blockade. As treatment of ventricular arrhythmias primarily relies on beta blocker therapy to reduce effects of sympathetic activation, our results in vivo in a large animal model provide some insight as to why patients may continue to experience recurrent arrhythmias despite this therapy. Studies in diseased hearts, however, are needed to further confirm our findings and evaluate the effects of Y₁ receptor blockade on ventricular arrhythmia inducibility.

Limitations. This study evaluated effects of BSS and Y₁ receptor blockade acutely; chronic effects of BIBO 3304 remain to be investigated. General anesthesia with isoflurane is known to blunt autonomic responses. To limit this effect, once surgical procedures had been completed, anesthesia was switched to α -chloralose. In this study, atropine was administered to prevent reflex bradycardia in response to rises in blood pressure that can occur as result of BSS. This may have led to an underestimation of effects of NPY acting via other receptors, such as Y₂ receptors, which are known to

decrease release of acetylcholine from parasympathetic fibers (13, 40). It is possible that a portion of the changes in ARIs during BSS were driven by HR. We did not correct for HR changes with pacing due to known effects of cardiac pacing on further exacerbating sympathoexcitation, cardiac NE levels, and cardiac autonomic neural activity (41, 42). Instead, we corrected ARIs at different HRs using the Fridericia formula for QT, as no formula exists for ARI correction and QT formula is without limitation. Of note, ventricular ARIs were prolonged with BIBO 3304 infusion, even after correcting for HR, and results of BSS on ARI before and after BIBO 3304 treatment were noted to be at the same HR. Finally, ventricular stimulation was not performed in these animals. Ventricular stimulation rarely causes VT in normal pig hearts (<10%), and aggressive programmed stimulation can produce nonspecific VF. Therefore, to observe the effects of BIBO 3304 on VT inducibility above and beyond beta blocker therapy, very large numbers of animals would be required to obtain sufficient power to see an effect. Studies on the effects of BIBO 3304 in the setting of chronic MI, where VT is more readily induced, could be used to evaluate effects of BIBO 3304 on the incidence of ventricular arrhythmias.

Conclusions. This study demonstrates that high doses of beta blockers cannot completely prevent the electrophysiological effects of sympathetic activation. In vivo NPY can act directly on the ventricles and modulate cardiac APDs via a Y₁ receptor-mediated mechanism. Cardiac contractility was preserved with NPY1R antagonism. Adjvant NPY1R blockade may present a promising therapeutic target in patients with refractory ventricular tachyarrhythmias.

Methods

Experimental protocol. Yorkshire pigs (S&S Farms; *Sus scrofa*; 3.6 ± 0.1 months old; *N* = 28) were used in the study. Pigs were housed for a minimum of a week to allow for acclimatization at the UCLA animal housing facility and subjected to a standard 12-hour light/12-hour dark cycle. Four groups of animals were used for the following studies (Figure 1):

Group 1 (49.1 ± 1.0 kg; *n* = 5): These animals underwent sequential BSS to evaluate the effect of a range of stimulation frequencies on hemodynamic, electrophysiological, and neurotransmitter/neuropeptide profiles in the porcine model, given the lack of previous data in this species. Stimulations were performed at 4 Hz, 10 Hz, and 20 Hz, with a 60-minute wait period in between stimulations.

Group 2 (52.2 ± 3.5 kg; *n* = 10): These animals underwent BSS at 4 Hz and 10 Hz (given the results of group 1) with and without 0.5 mg/kg propranolol i.v. and a minimum of a 60-minute wait period between stimulations.

Group 3 (44.8 ± 1.4 kg; *n* = 10): These animals underwent BSS at 10 Hz and repeat 10-Hz stimulations with 1.0 mg/kg propranolol i.v. and a combination of 1.0 mg/kg propranolol i.v. and BIBO 3304 infusion. A minimum of 60 minutes was allowed in between stimulations.

Group 4 (53.7 ± 3.8 kg, *n* = 3): These animals were only used for tissue collection to confirm the presence NPY1R in the porcine heart and did not undergo any stimulation or drug infusion protocols.

Experimental preparation. All animals were sedated with tiletamine-zolazepam (4–8 mg/kg, i.m.) and intubated. General anesthesia was maintained with isoflurane (1%–2%) and analgesia managed by intermittent boluses of fentanyl (total 20 mcg/kg, i.v.) during surgical preparation. Following the completion of surgical procedures, anesthesia was maintained by α-chloralose (50 mg/kg initial bolus, subsequently 20–30 mg/kg/h continuous infusion, i.v.). Hourly arterial blood gases were monitored, and appropriate ventilator adjustments were made to maintain pH at 7.35–7.45. Rectal temperature was assessed and adjusted to maintain body temperature at 35°C–38°C. Bilateral femoral veins and arteries were accessed and used for continuous saline and drug infusion and blood sampling, respectively. Right external jugular vein was used for insertion of a catheter into the CS for blood sampling. Left carotid artery was used for catheter insertion into the LV for the measurement of LV pressure and blood pressure, respectively. Twelve-lead surface ECGs were obtained via a Prucka Cardiolab System, and precordial leads were placed on the dorsal aspect of the animal given sternotomy. Animals underwent median sternotomy to expose the heart as well as bilateral stellate ganglia. Animals were euthanized by induction of VF under deep anesthesia. General anesthesia was maintained with isoflurane during surgical preparation and transitioned to α-chloralose following completion of surgical procedures.

Stellate ganglion stimulation. After median sternotomy, the right and left stellate ganglia were carefully isolated behind the parietal pleura, and bipolar platinum needle electrodes were placed in the ganglia for BSS as previously described (8, 20). The electrodes were connected to a stimulator (Grass Stimulator S88) and PSIU6

stimulation isolation units (Grass Technologies) for stimulation. The stimulation current that led to a 10% increase in HR and/or LVSP was determined unilaterally at 4 Hz, 4-ms pulse width (square wave) for each animal and defined as the threshold current. BSS was then performed at 2 times threshold according to one of 3 experimental protocols (Figure 1A). A minimum of 60 minutes was allowed for electrophysiological and hemodynamic parameters and neurotransmitter/neuropeptide profiles to return to baseline between stimulations.

Drug infusions. To evaluate whether beta blocker therapy could prevent BSS-induced changes in hemodynamic and electrophysiological parameters, effects of 2 high doses of i.v. propranolol were evaluated: 0.5 mg/kg (average dose of 26.1 ± 1.7 mg per animal; $n = 10$) and 1.0 mg/kg (average dose of 44.8 ± 1.4 mg per animal; $n = 10$). Both doses of propranolol were given as a bolus infusion over a 5- to 10-minute period. Both of these doses are more than 5- and 10-fold greater than the doses recommended by the American College of Cardiology/American Heart Association of 1–3 mg (0.01 mg/kg to 0.04 mg/kg) i.v. and up to 5 mg (0.07 mg/kg) i.v. for treatment of ventricular arrhythmias in 70-kg adult patients (10). Repeat BSS was performed 10 minutes after infusion of propranolol to allow for stabilization of hemodynamic parameters. Propranolol (1.0 mg/kg) was evaluated in a different set of animals after data from animals that received 0.5 mg/kg of propranolol showed significant residual sympathetic effects during BSS. Atropine sulfate (0.04 mg/kg) was used to prevent reflex bradycardia during BSS-induced rises in blood pressure. The NPY Y₁ receptor antagonist BIBO 3304 (Tocris) was dissolved in DMSO and diluted in saline to a final concentration of 660 mM (0.5 mg/mL) in 0.5% DMSO/saline. BIBO 3304 was administered at a dosage of 0.2 mg/kg, followed by a 0.4 mg/kg/h infusion (average dose 9.0 ± 0.3 –mg bolus followed by 17.9 ± 0.6 –mg/h infusion; $n = 10$) for 20 minutes before BSS and for the duration of the stimulation. BIBO 3304 was administered to the same animals that had received 1.0 mg/kg propranolol (Figure 1). All drugs were administered intravenously.

Hemodynamic assessment. A 5-Fr pressure-conductance catheter (SPR-350, Millar Instruments) was placed in the LV for continuous measurements of LV pressure throughout the experiment. Raw signals were digitized and recorded by CED Power1401 and subsequently analyzed using Spike2.

Cardiac electrophysiological recordings and analysis. A 56-electrode sock was placed over the ventricles to continuously record unipolar epicardial electrograms connected to a Prucka CardioLab System and band pass filtered at 0.05–500 Hz. Global ARI, a surrogate of APD, was analyzed from these 56 unipolar electrograms using a customized software, iScaldyn (University of Utah) as previously described (43, 44). Activation time (AT) was measured as the interval from onset to minimal dV/dt of the depolarization wave front; and repolarization time (RT) from onset to maximal dV/dt of the repolarization wave front. The difference between RT and AT was calculated as ARI, which has been shown to reflect the local APD at the electrode site ($ARI = RT - AT$) (44, 45). Polar maps and regional analyses reflect raw ARIs. Global ARIs were adjusted for the differences in HR using the Fridericia formula, to correct for the effect of HR on APD, and corrected ARIs are reported in all figures (46, 47). Unadjusted ARIs were used for regional analysis, as changes in various regions were compared at the same HR, as shown in Supplemental Figure 1.

Measurement of sympathetic neurotransmitter/neuropeptide concentrations. CS and FA blood were collected at baseline and during BSS to measure plasma NE and NPY concentrations. Blood was collected into K₂ EDTA blood collection tubes (BD Vacutainer), followed by immediate centrifugation at 1500 g for 15 minutes. The plasma was separated, snap-frozen in liquid nitrogen, and stored at –80°C until assay. NE and NPY were measured by ELISA (BA E-6200, sensitivity 0.093 ng/mL, Rocky Mountain Diagnostics; EZHNPY-25K, sensitivity 2.0 pg/mL, MilliporeSigma, respectively) according to manufacturer's instructions.

Evaluation of NPY Y₁ receptor expression. LV tissue was collected after euthanasia from naive normal animals ($n = 3$) and snap-frozen in liquid nitrogen for Western blot analysis or immersion fixed in 4% paraformaldehyde for 24 hours for immunohistochemistry. These animals did not undergo any type of sympathetic stimulation.

Snap-frozen tissues were Dounce homogenized and lysed in 8 M SDS-urea, and total protein concentrations were quantified by bicinchoninic acid assay. Protein (20 µg) was loaded per lane on 4%–20% polyacrylamide gels (Bio-Rad, 4561093), and proteins were transferred by Trans-Blot Turbo (Bio-Rad, 1704150) onto 0.2-µm PVDF membranes. Membranes were blocked with 5% milk in Tris-buffered-saline with 0.2% Tween, and incubated overnight with rabbit anti-NPY1R (1:1000; Abcam, ab91262) or rabbit anti-actin (1:2500; MilliporeSigma, A2066), followed by horseradish peroxidase anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., 711-035-152) for 1 hour at room temperature. Proteins were detected by chemiluminescence with Clarity Western ECL Substrate (Bio-Rad, 1706061) and imaged on ChemiDoc MP (Bio-Rad, 17001402). Densitometry was performed using ImageJ (NIH) to compare regional expression of NPY1R.

Fixed tissue was embedded in paraffin, sectioned (5 µm), and rehydrated in 2 xylene washes, followed by 3 ethanol washes and water. Epitopes were unmasked by heat-induced epitope retrieval in EDTA buffer, pH 8.0 (Abcam, ab64216) at 90°C. Slides were then blocked for 1 hour in 3% BSA-TBS/0.2% Triton X-100 with 5% donkey serum and incubated overnight at 4°C with rabbit anti-NPY1R (1:200; Alomone Labs, ANR-021) and mouse anti-NPY (1:500; Abcam, ab112473), followed by 2-hour incubation at room temperature with Alexa Fluor 488–donkey anti-rabbit IgG (1:200; Invitrogen, A-21206) and Alexa Fluor 555–donkey anti-mouse IgG (1:200; Invitrogen, A-31570). Slides were then incubated with wheat germ agglutinin conjugated to Alexa Fluor 633 (Thermo Fisher Scientific, W21404) for 30 minutes at room temperature and mounted with Antifade Mounting Medium with DAPI (Vector Laboratories, H-1200). Negative controls were performed on serial sections processed in tandem by omission of primary antibody. Slides were imaged on a Zeiss LSM 880 with Airyscan at $\times 630$ magnification and processed with Zen 2 (Zeiss).

Statistics. Data are reported as mean \pm SEM. Global ventricular ARIs were calculated as the mean ARI across all 56 electrodes and corrected for HR using the Fridericia formula. After confirmation of normality, 2-tailed paired Student's *t* test was used to compare parameters between baseline and BSS during each condition and responses to BSS between different conditions within each animal. Comparisons of changes in parameters between different frequencies (protocol 1) were performed using 1-way repeated-measures ANOVA with the FDR corrected for by the Benjamini-Hochberg procedure. $P \leq 0.05$ was considered statistically significant. All statistical analyses were performed with GraphPad Prism software v8.

Study approval. Care of the animals conformed to the *Guide for the Care and Use of Laboratory Animals* (National Academies Press, 2011). The study protocol was approved by the UCLA Institutional Animal Care and Use Committee.

Author contributions

JDH, SS, and MV contributed to the conception and design of the experiments. JDH, SS, NY, and MAS performed experiments, and JDH, SS, NY, and MV analyzed the data. DH contributed to the initial design of the study and helped perform preliminary experiments. JDH and MV drafted the manuscript, and all authors revised and approved the final version of the manuscript.

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