Targeting protein tyrosine phosphatase σ after myocardial infarction restores cardiac sympathetic innervation and prevents arrhythmias

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Millions of people suffer a myocardial infarction (MI) every year, and those who survive have increased risk of arrhythmias and sudden cardiac death. Recent clinical studies have identified sympathetic denervation as a predictor of increased arrhythmia susceptibility. Chondroitin sulfate proteoglycans present in the cardiac scar after MI prevent sympathetic reinnervation by binding the neuronal protein tyrosine phosphatase receptor σ (PTPσ). Here we show that the absence of PTPσ, or pharmacologic modulation of PTPσ by the novel intracellular sigma peptide (ISP) beginning 3 days after injury, restores sympathetic innervation to the scar and markedly reduces arrhythmia susceptibility. Using optical mapping we observe increased dispersion of action potential duration, supersensitivity to β-adrenergic receptor stimulation and Ca2+ mishandling following MI. Sympathetic reinnervation prevents these changes and renders hearts remarkably resistant to induced arrhythmias.
Survivors of myocardial infarction (MI) remain at high risk for cardiac arrhythmias and sudden cardiac death. The infarct, or scar, generates an anatomical substrate that promotes re-entrant arrhythmias, and numerous studies indicate that altered sympathetic neurotransmission in the heart also plays a key role in the onset of post-infarct cardiac arrhythmias. Norepinephrine (NE) released from sympathetic nerves activates cardiac β-adrenergic receptors (β-AR) to modulate myocyte repolarization by altering transmembrane currents and Ca$^{2+}$ homeostasis, and simply disrupting the normal organization of sympathetic innervation in an otherwise healthy heart is arrhythmogenic. Cardiac sympathetic function is altered in a region-specific manner following MI, and studies in animals and humans reveal denervation of the infarct and adjacent, viable (peri-infarct) myocardium. Three recent studies in patients with implanted cardioverter defibrillators (ICDs) suggest that the amount of sympathetic denervation after MI predicts the probability of serious ventricular arrhythmias. A detailed electrical mapping study in intact human hearts revealed that sympathetic denervation of the normal myocardium adjacent to the infarct resulted in β-AR agonist supersensitivity and increased dispersion of repolarization that was arrhythmogenic. Studies and others led to the model that inappropriate heterogeneity of sympathetic transmission across the left ventricle, and subsequent electrical remodelling of cardiac myocytes, is a major contributor to post-infarct arrhythmias in humans.

The observation that the denervated myocardium adjacent to the infarct contributes to the generation of post-infarct arrhythmias was especially interesting to us because chondroitin sulfate proteoglycans (CSPGs) in the cardiac scar prevent reinnervation of the infarct and the adjacent myocardium by sympathetic axons. Although axons sprout and regenerate towards the scar, they are stopped near the outer edge of the infarct by CSPGs. In the absence of the CSPG receptor, protein tyrosine phosphatase receptor σ (PTPσ), sympathetic axons fully reinnervate undamaged peri-infarct tissue and hyperinnervate the infarct. Given the clinical significance of sympathetic denervation after MI, we were interested to determine whether restoring sympathetic innervation to the infarct and surrounding myocardium altered post-MI arrhythmia susceptibility.

We targeted PTPσ using both genetic and pharmacologic approaches in order to promote reinnervation of the infarct, and used electrocardiogram (ECG) telemetry to examine arrhythmia susceptibility. Transmembrane potential ($V_m$) and intracellular Ca$^{2+}$ dynamics were assessed using ex vivo optical mapping in order to investigate the mechanisms underlying changes in arrhythmia susceptibility. MI caused dispersion of action potential duration (APD), supersensitivity to β-AR stimulation and Ca$^{2+}$ mishandling. Restoring sympathetic innervation to the infarct and the surrounding tissue decreased arrhythmia susceptibility and normalized cardiac electrophysiology and Ca$^{2+}$ dynamics, despite the presence of a scar.

Results
Targeting PTPσ restores innervation after MI and prevents arrhythmias. We previously observed that CSPGs generated in the cardiac scar after ischaemia-reperfusion (I–R) prevented reinnervation of the infarct (Fig. 1a) despite high levels of nerve growth factor in the scar. The infarct becomes hyperinnervated in animals lacking the CSPG receptor PTPσ (Fig. 1b), confirming the crucial role for PTPσ in sympathetic denervation after MI. Since cardiac denervation is linked to risk for arrhythmia and cardiac arrest in human studies, we asked whether restoring sympathetic innervation to the infarct and surrounding myocardium affected arrhythmia susceptibility. Control mice heterozygous for PTPσ ($\text{ptprs}^{+/−}$; HET) and mice lacking PTPσ ($\text{ptprs}^{−/−}$; KO) were implanted with ECG telemetry transmitters and then subjected to sham or MI surgery. Ten days after surgery, the mice were injected with 10 μg of the beta agonist isoproterenol (ISO), which has also been used to induce arrhythmias in the clinical setting. ISO stimulated comparable increases in heart rate in all mice (Fig. 1c), but stimulated fewer premature ventricular complexes (PVCs) in sham mice of either genotype (Fig. 1d). In contrast, the arrhythmia response in post-MI animals differed on the basis of

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**Figure 1** | Absence of PTPσ restores innervation to the infarcted myocardium and prevents arrhythmias. Heart sections from HET (a) and KO (b) mice were stained for TH (green) to identify sympathetic nerve fibres and fibrinogen (red) to identify the infarct. Scale bar, 100 μm. (c) Heart rate was similar in both genotypes before (Base) and after (ISO) 10-μg ISO injection (mean ± s.e.m., $n = 8$ per genotype). (d) ISO induced comparable levels of PVCs in conscious Sham HET and KO mice; however, ISO induced significantly more PVCs in infarcted HET mice compared with KO mice and to Sham HET mice (mean ± s.e.m., $n = 4$ per group, ***$P<0.001$ versus HET sham and KO MI; two-way ANOVA with Bonferroni post test). (e) Fibrinogen-stained section after MI showing a transmural infarct after a 35-min occlusion. Scale bar, 500 μm. (f) Quantification of infarct size in HET and KO hearts following 35 min of occlusion (mean ± s.e.m., $n = 4$ per group; t-test, N.S. $P>0.05$).
the innervation status of the infarct. ISO stimulated a significant number of PVCs in HET mice with denervated infarcts (Fig. 1d); however, KO mice with innervated infarcts were resistant to ISO-induced arrhythmias, having the same number of PVCs as sham animals (Fig. 1d). Infarcts were generally transmural (Fig. 1e), and infarct size was the same in both genotypes (Fig. 1f) indicating that the difference in arrhythmia susceptibility could not be explained by scar size.

To confirm that reinnervation of the infarct and surrounding tissue decreased arrhythmia susceptibility, we sought to restore innervation in mice expressing normal levels of PTPs. In order to promote regeneration of sympathetic axons through the CSPGs-rich cardiac scar, we utilized ISP, developed by Lang and Silver26 to target the intracellular dimerization domain of PTPs. ISP restored growth of the central nervous system axons through CSPGs in vitro, and systemic injections of ISP enhanced axon sprouting through CSPGs after spinal cord injury in vivo26. We asked whether targeting PTPs with ISP could restore sympathetic axon regeneration into the cardiac scar. Wild-type (ptprs+/+) mice were implanted with ECG telemetry transmitters, and a week later subjected to I–R surgery. Beginning 3 days after MI, when the infarct was fully denervated24, mice were injected daily (intraperitoneal (IP)) with vehicle (5% dimethyl sulfoxide (DMSO)/Saline), ISP (10 μmol; 44 μg) or IMP (10 μmol; 42 μg) as a negative control. IMP targets the receptor PTPµ, which is not present in sympathetic neurons. Fourteen days after the MI surgery, the mice were treated with ISO to mimic circulating catecholamines and provoke arrhythmias, and hearts were collected for analysis of sympathetic innervation. Animals treated with either vehicle or IMP had denervated infarcts (Fig. 2a,b,d), whereas animals treated with ISP had normal levels of sympathetic innervation throughout the left ventricle, including the infarct (Fig. 2c,d). Thus, daily ISP injections beginning 3 days after the injury, when denervation was well established, allowed robust regeneration of sympathetic axons into the CSPG-laden cardiac scar.

Several studies indicate that newly regenerating sympathetic axons in the damaged heart have low levels of NE27,28, likely because of local depletion of Tyrosine Hydroxylase (TH) by inflammatory cytokines29. The cardiac scar in the mouse heart is mature by 10–12 days after reperfusion, and acute inflammation has resolved30. Therefore, we quantified NE levels in the infarct and peri-infarct myocardium 14 days after surgery to determine whether the axons that had reinnervated the infarct and surrounding myocardium had normal NE levels. NE content was low in the denervated infarct of vehicle-treated animals, but was normal in the innervated infarct of ISP-treated animals (Fig. 2e). NE content in the undamaged portion of the left ventricle was similar in both treatment groups (Fig. 2e), consistent with identical innervation densities (Fig. 2d). Likewise, NE content in the undamaged right ventricle was similar in both groups (Veh versus ISP; 5.50 ± 0.70 versus 6.38 ± 0.81 pmol mg⁻¹; P = 0.43). These data indicate that restoring innervation to the infarct with ISP normalizes NE levels across the left ventricle by 2 weeks after MI.

Since regional sympathetic denervation is thought to be an important source of post-MI arrhythmia susceptibility in humans, we quantified ISO-induced arrhythmias 14 days after MI in mice treated with vehicle, IMP or ISP. ISO triggered significantly fewer PVCs in ISP-treated mice, which had innervated infarcts and normal NE content, than in the vehicle or IMP-treated groups, which had denervated infarcts and low NE content (Fig. 2f). These data suggest that restoring sympathetic transmission throughout the innervated left ventricle, even days after denervation is established, is sufficient to decrease arrhythmia susceptibility.

Reinnervation limits dispersion of APD and β-AR supersensitivity. Activation of cardiac β receptors decreases myocyte APD by changing ion channel activity and Ca²⁺ handling to allow for adaptation to fast heart rates8-10. In order to assess directly the impact of infarct reinnervation on cardiac electrophysiology, we used optical mapping to simultaneously image intracellular Ca²⁺ and V_m in Langendorff-perfused isolated hearts. PTP KO mice were used to examine the effect of reinnervation on cardiac electrophysiology, with ptprs+/- (HET) mice serving as denervated controls.

In order to compare baseline electrical properties, a pacing electrode was positioned on the base of the LV epicardium (Fig. 3a), and APD at 90% repolarization (APD90) was assessed at a pacing interval of 150 ms (400 b.p.m. heart rate). Figure 3b (top) shows a representative example of action potential propagation across a heart from each group: HET Sham, KO Sham, HET MI and KO MI. Figure 3b (bottom) shows a representative map of...
APD₉₀ throughout each heart. Quantification of the mean APD₉₀ revealed that it was similar across all four groups (Fig. 3c). However, it is clear that the denervated HET MI heart exhibits greater extremes of APD₉₀ than the other hearts. The interquartile range (IQR) is a measure of APD₉₀ variability, or dispersion, across an individual heart. IQR was significantly higher in denervated HET MI hearts compared with all other groups (Fig. 3d), consistent with increased dispersion of APD following MI in human studies. Analysis of regional APD revealed a significant difference between the denervated infarct and normal myocardium (Supplementary Fig. 1). Surprisingly, sympathetic reinnervation of the infarct in KO hearts prevented an increase in APD₉₀ dispersion following MI (Fig. 3d), even though the infarct size in the KO MI group was identical to that in the HET MI group (Fig. 3e).

Cycle length-dependent changes in APD (known as APD restitution) have also been implicated in arrhythmia generation; therefore, we generated APD restitution curves using the pacing interval of 150 ms (S1) followed by a single extrastimulus (S2), with consecutively shorter S2 intervals. As expected, conduction velocity (Supplementary Fig. 2) and APD₉₀ decreased with shorter S2 intervals in all hearts (Fig. 3f), while APD₉₀ increased (Fig. 3g), consistent with previous studies of these properties in the mouse heart. Interestingly, the HET MI hearts exhibited limited APD₉₀ shortening compared with the other groups (Fig. 3f), and early repolarization (APD₃₀) was blunted in those hearts at shorter S2 intervals (Fig. 3g). The increased APD₃₀ observed in HET MI hearts is consistent with the reduction in transient outward K⁺ current (Iₒ) seen after MI or sympathetic denervation. However, KO MI hearts with sympathetic reinnervation exhibited APD restitution properties that were indistinguishable from sham hearts.

Sudden cardiac death is most common in the morning, when circulating catecholamines (NE and epinephrine) are rising rapidly. Given the protective role of beta blockers in humans, and the observation that PVCs induced by the beta agonist ISO are a good marker of arrhythmia propensity in humans, we examined the role of β-AR activation in arrhythmia generation in our infarcted mouse hearts. We treated hearts with tyramine to induce the release of endogenous NE from sympathetic axons within the heart, and with ISO to mimic circulating catecholamines. Release of endogenous NE with tyramine
resulted in similar APD shortening across all groups (% change: HET Sham − 0.5 ± 0.6, HET MI − 0.8 ± 0.6, KO Sham − 0.2 ± 0.2, KO MI − 2.1 ± 0.6; n = 3–5, mean ± s.e.m.). In contrast, treatment with ISO, which mimics the effect of circulating catecholamines in vivo, stimulated a significantly greater shortening of APD90 in denervated HET MI hearts compared with all other groups (Fig. 4a,b). This denervation-induced β-AR supersensitivity is consistent with recent human data2. Consistent with the baseline data, APD90 dispersion (IQR) remained elevated in the HET MI hearts following ISO treatment (Fig. 4c).

Sympathetic reinnervation prevents Ca2+ mishandling and PVCs. In order to identify additional mechanisms underlying changes in cardiac electrophysiology, we examined intracellular Ca2+ handling at various pacing frequencies and after ISO treatment. Disrupted Ca2+ homeostasis can result in beat-to-beat fluctuations between large and small intracellular calcium transients (CaT) called alternans, which is a sensitive noninvasive marker for the risk of sudden cardiac death in humans3–5. Intracellular Ca2+ handling appeared normal in sham hearts of both genotypes when paced with a 100-ms pulse interval (600 b.p.m. heart rate; Fig. 5). However, HET hearts with denervated infarcts (HET MI) exhibited significant intracellular Ca2+ alternans at the same pacing frequency (Fig. 5). Interestingly, KO hearts with innervated infarcts (KO MI) did not exhibit intracellular Ca2+ alternans (Fig. 5), suggesting that restoring sympathetic innervation to the infarct and surrounding tissue protects intracellular Ca2+ homeostasis. Denervated HET MI hearts also exhibited significant APD alternans (Fig. 5c), while innervated KO MI hearts did not, despite identical infarct size.

The notion that sympathetic reinnervation of the peri-infarct border and scar protects intracellular Ca2+ handling was confirmed by analysing intracellular Ca2+ dynamics after ISO administration. Under baseline conditions, normal excitation–contraction coupling occurred, in which membrane depolarization (V_m; black) preceded the CaT upstroke (red) in hearts from both genotypes and surgical groups (Fig. 6a, inset). In sham hearts treated with ISO, depolarization continues to precede the CaT (Supplementary Fig. 3). However, in the infarcted hearts, the response to ISO differed depending on the innervation status. Infarcted hearts with sympathetic innervation restored throughout the entire left ventricle (KO MI) continued to exhibit normal Ca2+ handling after ISO treatment (Fig. 6a,b), while hearts with sympathetic denervation (HET MI) exhibited pathological increases in diastolic intracellular Ca2+ (CaT; ) that preceded membrane depolarization (Fig. 6a,b). This Ca2+ elevation was prevented by addition of the β-AR blocker propranolol (Fig. 6a,b).

![Figure 4](image4.png)

**Figure 4** | Sympathetic innervation of the infarct prevents β-AR supersensitivity. (a) APD90 maps before (Baseline) and after 1-μM ISO (100-ms pacing interval or 600 b.p.m. heart rate for each condition), and representative traces of optical action potentials from sites marked with *. Scale bar, 50 ms. (b) Percent change in APD90 following ISO treatment (mean ± s.e.m.; n = 4–5 hearts per group; **P < 0.01 by two-way ANOVA with Bonferroni post test). (c) IQR of APD90 following ISO treatment (mean ± s.e.m.; n = 4–5 hearts; **P < 0.01 by two-way ANOVA with Bonferroni post test).

![Figure 5](image5.png)

**Figure 5** | Sympathetic innervation of the infarct prevents post-MI Ca2+ mishandling. (a) Representative optical Ca2+ transients and alternans maps from the sites on sham and post-MI hearts marked (*) paced with a 100-ms pacing interval (600 b.p.m. heart rate). Scale bar, 100 ms. Denervated hearts (HET MI) exhibit Ca2+ alternans, while sham hearts and infarcted hearts with sympathetic innervation of the scar (KO MI) do not. (b) Intracellular Ca2+ alternans magnitude was quantified by spectral analysis (mean ± s.e.m.; n = 4–5 hearts; **P < 0.01 versus other groups by two-way ANOVA with Bonferroni post test). (c) APD alternans magnitude was quantified by spectral analysis (mean ± s.e.m.; **P < 0.05 versus sham using Kruskal–Wallis test for non-Gaussian distribution).
We previously showed that strong localized β-AR stimulation can trigger Ca\(^{2+}\) release independent of membrane depolarization that is sufficient to cause PVCs\(^{38}\). Therefore, we asked whether ISO-stimulated Ca\(^{2+}\) mishandling led to the production of PVCs after MI. Quantification of in vitro arrhythmia severity in HET and KO MI hearts confirmed the striking difference in arrhythmia susceptibility observed during the in vivo ECG telemetry studies. Arrhythmia severity was scored on the basis of the most severe arrhythmia observed during a mapping procedure: PVCs, bigeminy or salvos, and 3:1 atrioventricular (A–V) block (Fig. 6c), even though infarct size was comparable to HET MI hearts during ISO treatment. Individual hearts are scored on the basis of the most severe arrhythmia observed in each heart over a 20-min continuous ECG recording (0 = no PVCs, 1 = single PVCs, 2 = bigeminy or salvos, and 3 = ventricular tachycardia\(^{39}\)). Sham animals of both genotypes exhibited PVCs only single PVCs, and ISO stimulated a similar number in both genotypes (HET versus KO; Base: 1.00 ± 0.71 versus 1.33 ± 0.88 and Iso: 1.75 ± 0.48 versus 1.33 ± 0.33). ISO triggered significantly more arrhythmias in HET MI hearts with denervated infarct/peri-infarct tissue compared with all other groups (Fig. 6c), and it stimulated arrhythmias of greater severity in those hearts, including ventricular tachycardia (Fig. 6d). In contrast, KO MI hearts with innervated infarct/peri-infarct tissue exhibited only individual PVCs and the number of PVCs was similar to that seen in the sham groups (Fig. 6c), even though infarct size was comparable to HET MI hearts. As expected, administration of propranolol prevented ISO-induced arrhythmias in HET MI hearts, confirming the role of β-AR (Fig. 6c). Importantly, Ca\(^{2+}\) mapping revealed that the Ca\(^{2+}\) elevation induced by ISO was associated with PVCs arising near the infarct that is sufficient to trigger PVCs. Restoration of sympathetic innervation to the infarct and surrounding tissue normalizes Ca\(^{2+}\) handling and makes hearts resistant to ISO-induced arrhythmias.
Discussion

The observation that local sympathetic denervation in the post-MI heart leads to high arrhythmia risk seems to contradict the many clinical studies showing that blocking sympathetic transmission in the heart, either with beta blockers or with surgical ganglionectomy, prolongs life after MI. However, a consensus has developed that inappropriate heterogeneity of sympathetic transmission in the heart, and subsequent electrical remodelling of cardiac myocytes, is arrhythmogenic. This heterogeneity can be caused by the loss of sympathetic transmission in a portion of the heart, or it can be caused by regional nerve sprouting and hyperinnervation. In this context, surgical denervation and administration of beta blockers can be seen as methods for ‘evening out’ sympathetic transmission across the heart by decreasing it everywhere. Our study fully supports the model that heterogeneity of sympathetic transmission after MI is pathological; however, our approach to correct that problem was to restore sympathetic transmission throughout the left ventricle by targeting PTPα. It is especially notable that we were able to restore innervation using a pharmacologic intervention that was initiated several days after injury when the infarct and peri-infarct myocardium was already denervated. Daily injections with ISP were sufficient to restore functional sympathetic innervation, and reinervation rendered hearts surprisingly resistant to arrhythmias. Indeed, infarcted hearts with restored sympathetic innervation were electrically indistinguishable from sham hearts, despite the presence of a scar.

This work has implications for patients who survive MI, and remain at risk for severe cardiac arrhythmias and sudden cardiac death. Those patients who have a left ventricular (LV) ejection fraction ≤35% often receive prophylactic implantation of an ICD; however, low ejection fraction has not proven to be a good predictor of those truly in need of ICDs, and that normalizing the heterogeneity can be caused by the loss of sympathetic transmission and that normalizing the heterogeneity of sympathetic transmission is protective for the heart. MI is inducible by regional nerve sprouting and hyperinnervation. In this study, we were able to restore innervation using a pharmacologic intervention that was initiated several days after injury when the infarct and peri-infarct myocardium was already denervated. Daily injections with ISP were sufficient to restore functional sympathetic innervation, and reinervation rendered hearts surprisingly resistant to arrhythmias. Indeed, infarcted hearts with restored sympathetic innervation were electrically indistinguishable from sham hearts, despite the presence of a scar.

Methods

Animals. PTPα (ptprs) transgenic mice (B6C3F1) were supplied by Michel Tremblay (McGill University) and were bred as heterozygotes. Age- and gender-matched mice that were 12-18 weeks old were used for all experiments. Ptpa+/− (HET) littermates were used as controls for ptpa−/− (KO) studies. Some ptpa+/− mice were given daily IP injections of vehicle (5% DMSO/Saline), IMP (Intracellular Mu Peptide; 10 μmol) or ISP (10 μmol) beginning 3 days after I-R. All mice were kept on a 12:12 h light-dark cycle with ad libitum access to food and water. All procedures were approved by the OHSU and the University of California-Davis Institutional Animal Care and Use Committee and comply with the Guide for the Care and Use of Laboratory Animals published by the National Academies Press (8th edition).

Surgery. (1) Myocardial I-R. Anaesthesia was induced with 4% isoflurane and maintained with 2% isoflurane. The left anterior descending coronary artery was reversibly ligated for 30 min (telemetry studies) or 45 min (ex vivo mapping) and then reperfused by release of the ligature. Occlusion was confirmed by sustained S-T wave elevation and regional cyanosis. Reperfusion was confirmed by the return of colour to the ventricle distal to the ligation and reperfusion arrhythmia. Core body temperature was monitored by a rectal probe and maintained at 37 °C, and a two-lead ECG was monitored. (2) Sham surgery. Sham animals underwent the procedure described above, except for the left anterior descending coronary artery ligature.

In vivo telemetry. ECGs were obtained from conscious adult mice using ETA-F10 (Data Sciences International) telemetry implants, and were analysed with the Datatrend ART software (Data Sciences International). Mice were anaesthetized with 4% and maintained on 2% inhaled isoflurane. A transmitter was implanted subcutaneously in a lead II configuration, with the negative lead placed in the right ventricle and the positive lead to the left of the xiphoid process. Devices were implanted at least 5 days before I-R or sham surgery. ECG recordings were obtained (IP, sham, transgenic, and wild-type) or 14 (ISP studies) days after sham or MI surgery. ECGs were analysed beginning 60 min before IP injection of a relatively low dose of the β-AR agonist ISO (10 μg or 0.5 mg kg−1) as a baseline, and then for 60 min after injection to identify ISO-induced arrhythmias. PVCs were defined as a single premature QRS complex.

Immunohistochemistry. Hearts were fixed for 1 h in 1% paraformaldehyde, rinsed in PBS, cryoprotected in 30% sucrose overnight and frozen in mounting medium for sectioning. Transverse 10-μm sections were cut on a cryostat and thaw-mounted on charged slides. To reduce fixative-induced autofluorescence, sections were rinsed in 10 mg ml−1 sodium borohydride for 3 × 10 min and then rinsed in PBS for 3 × 10 min. The sections were then blocked in 3% BSA/0.3% Triton X-100 in PBS for 1 h. The slides were then incubated with rabbit anti-TH (1:1,000, Millipore/Chemicon) and sheep anti-fibrinogen (1:500, AbD Serotec) antibodies overnight at 4 °C, rinsed for 3 × 10 min in PBS and incubated for 1.5 h with the AlexaFluor 488-conjugated rabbit IgG-specific antibody (1:1,000, Invitrogen) and AlexaFluor 568-conjugated sheep IgG-specific antibody (1:1,000, Invitrogen). Sections were rinsed for 3 × 10 min in PBS, incubated for 30 min in 10 mM CuSO4 in 50 mM ammonium acetate to reduce background signal further, rinsed for 3 × 10 min in PBS, coverslipped in a 1:1 PBS/glycerol solution and visualized using fluorescence microscopy.

Imaging and threshold analysis. TH staining was quantified to assess sympathetic innervation density. Images were taken of the infarct, proximal and distal peri-infarct zone of each section, and three sections obtained from a similar level of the base-to-apex axis were quantified in each heart using threshold discrimination analysis. Black and white photos were opened in ImageJ and the brightness/contrast tool was used to adjust each image so that the minimum was set at the left side of the histogram. The threshold tool was then used to identify nerve fibres. The automated function set a beginning threshold level; however, this did not reliably include all sympathetic fibres while excluding all non-neuronal tissues. The
 threshold was then manually adjusted to ensure that only specific TH staining was expressed as the percent of area that was above the threshold (TH+ fibres). Each image was quantified by two independent observers.

### Infarct size
Infarcts were identified with fibrinogen staining and infarct size was quantified using eight 10 μm sections, approximately 200 μm apart obtained from matched levels from each heart. Images were taken of the entire section, and the LV and infarct areas were measured by outlining the respective regions using the freehand selection tool in ImageJ. Infarct size was then calculated as a percentage of the LV area [(infarct area/LV area) × 100]. Sections were obtained from the upper, middle and lower regions of the infarct in each heart.

### NE content
NE levels in heart tissue were measured using HPLC with electrochemical detection. Frozen, pulverized tissue was weighed, and then homogenized in 0.2 M perchloric acid and 0.5 M dihydroxybenzylamine (DHBA). The tissue was refrigerated for 1 h and centrifuged at 14,000 r.p.m. for 4 min. Supernatant was adsorbed on alumina, followed by 15 min of tumbling. The alumina was washed twice with 1.0 ml of H2O followed by centrifugation in between. The NE was desorbed from the alumina with 0.5 ml of 0.1 M perchloric acid. Fifty-microlitre aliquots were fractionated by reversed-phase HPLC (C18; 5 μm particle size, Rainin) using a mobile phase containing 75 mM sodium phosphate (pH 3.0), 360 mg/l of sodium octane sulfonate, 100 μl/l triethylamine and 3.0% acetonitrile. A coulometric detector (Couлемh, ESA) was used to detect and quantify NE and DHBA. NE standards (0.5 μm) were processed in parallel with tissue samples and interspersed throughout the HPLC run. Retention time for NE was 50 min and for DHBA was 85 min.

### Langendorff perfusion and dual optical mapping of Vm and Ca2+
Mice were anaesthetized with pentobarbital sodium (150 mg·kg⁻¹·IP) containing 120 IU of heparin. Following a midline incision, hearts were rapidly excised and Langendorff-perfused at 37°C with oxygenated (95% O₂ and 5% CO₂) modified Tyrode’s solution of the following composition (in mM⁻¹): NaCl 128.2, CaCl₂ 1.3, KCl 4.7, MgCl₂ 1.05, NaH₂PO₄ 1.19, NaHCO₃ 20 and glucose 11.1 (pH 7.4). Flow rate (1.3–3.5 ml·min⁻¹) was adjusted to maintain a perfusion pressure of 60–80 mm Hg. One leaflet of the mitral valve was carefully damaged with sharp forceps inserted into the pulmonary vein to prevent solution convection in the LV cavity after suppression of ventricular contraction. Two Ag/AgCl disc electrodes were positioned in the bath to record an ECG analogous to a lead I configuration. ECG was continuously recorded throughout the duration of the experiment. A bipolar electrode was positioned on the base of the LV epicardium for pacing, which was performed at a basic cycle length of 150 ms using a 2 ms pulse width at twice the diastolic threshold.

### Mapping experimental protocol
Following dye loading, baseline electrophysiological parameters were recorded during normal sinus rhythm as well as LV epicardial pacing at intervals of 150 and 100 ms. Hearts were subjected to tyramine infusion (20 μM) followed by a washout period. Hearts were then subjected to acute β-AR stimulation with ISO (1 μM). Optical recordings were taken every 5 min following treatment, and ECG was continuously recorded. Following 15 min of ISO treatment, the β-AR blocker propranolol (10 μM) was added to the perifusate. Optimal recordings were repeated every 5 min for 15 min.

### Optical mapping data analysis
Optical mapping data analysis was performed using two commercially available analysis programmes (BY-Analyze, Brainvision, Tokyo, Japan and Optiq, Cairn, UK). Vm and Ca²⁺ data sets were spatially aligned and processed with a Gaussian spatial filter (radius 3 pixels). For both action potentials and Ca²⁺ activation time was determined as the time at 50% of the maximal amplitude. For action potentials, repolarization time at 30 and 90% return to baseline were used to calculate APD (APD₃₀ and APD₉₀). Relative APD₉₀ and APD₉₀ₐ used in restitution curves were calculated as a ratio of S2 APD/S1 APD. Conduction velocity was calculated along a vector from the base to apex using a polynomial fitting algorithm previously described in ref. 54. Separate measurements of longitudinal and transverse conduction velocity were not determined because of the basal pacing location and disruption of fibre orientation in the infarct region. Diastolic Ca²⁺ elevation was measured as the percentage of diastolic Ca²⁺ increase relative to the following CaT amplitude at baseline and 15 min of ISO treatment. The average diastolic Ca²⁺ elevation was calculated for each heart by averaging all Ca²⁺ signals from the entire anterior surface of the heart within the optical mapping field of view. PVC incidence was determined from the continuous ECG recording as the number of PVCs that occurred during a 20-min period of baseline activity (before initiation of treatment) and during the 20 min of treatment. Arrhythmia severity was scored on the basis of the most severe arrhythmia observed in each heart over a 20-min continuous ECG recording (0 = no PVCs, 1 = single PVCs, 2 = bigeminy or salvos and 3 = ventricular tachycardia).

The spectral method, which has been used clinically for detecting micro-volt T-wave alternans, was used to detect the presence of CaT alternans and APD alternans. The spectral method was chosen because of its high sensitivity and relative immunity to noise. This approach allowed us to determine whether an area within the mapping field of view was experiencing significant APD or CaT alternans (greater than the background noise levels) as well as the spatial extent of significant alternans. A spectral magnitude of ≥ 2 was used as the minimum threshold for significant APD or CaT alternans, corresponding to a beat-to-beat change in APD₉₀ ≥ 5 ms or beat-to-beat change in CaT amplitude ≥ 5% respectively.

### Statistics
Student's-t tests were used to compare two groups. Data sets that included more than two groups were analysed by one-way analysis of variance (ANOVA) using the Tukey post hoc test to compare all conditions. For experiments comparing different surgical groups and genotypes a two-way ANOVA was carried out using the Bonferroni post hoc test. All statistical analyses were carried out using Prism 5.0.

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