Adrenergic Hypersensitivity Drives Ventricular Arrhythmias Following Loss of Plexin-Mediated Cardiac Innervation

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Conflict of interest statement: UCLA has patents developed by K.S. relating to cardiac neural diagnostics and therapeutics. K.S. is co-founder of NeuCures, Inc.

Running title: A Model of Adrenergically Driven Ventricular Arrhythmia

Word count: 5,321

ABSTRACT

Background: Ventricular arrhythmias (VAs) are a leading cause of death and arise from a combination of cardiac muscle injury and dysfunction of the intramyocardial sympathetic nerves that control cardiac electrophysiology. The adrenergic mechanisms by which intramyocardial nerves contribute to arrhythmogenesis are poorly understood. Semaphorin-plexin signaling pathways are responsible for developmental guidance of sympathetic nerves onto the heart and have previously been associated with VAs in humans.

Objective: To investigate adrenergic control of arrhythmogenesis, we probed the cardiac electrophysiology of a *Plexin-A3/-A4* double knockout mouse with loss of cardiac adrenergic nerves.

Methods: We studied cardiac structure and function using tissue clearing, immunohistochemistry, and echocardiography. We utilized ECG *in vivo* and *ex vivo* in a Langendorff preparation to evaluate electrophysiologic responses to pharmacologic beta (β)-adrenergic stimulation and blockade, and we measured β-adrenergic receptor (βAR) density in cardiac membranes using radioligand binding. Finally, we performed a phenome-wide association study utilizing data from the UK Biobank to search for associations between *PLXNA4* and human arrhythmias.

Results: Mice with loss of plexin-dependent cardiac innervation had structurally normal hearts but displayed spontaneous VAs driven by adrenergic hypersensitivity, as well as increased cardiac βAR density. Several human *PLXNA4* variants were associated with arrhythmia phenotypes.

Conclusion: These data establish a model of VAs driven purely by enhanced adrenergic receptor signaling, in the absence of structural heart disease, which can be used to investigate adrenergic mechanisms of arrhythmogenesis and to identify novel antiarrhythmic targets.

Keywords: neurocardiology, ventricular arrhythmias, denervation supersensitivity, sympathetic nervous system, adrenergic signaling

INTRODUCTION

Ventricular arrhythmias (VAs) result in sudden cardiac death, which accounts for one-fifth of mortality worldwide[1,2]. VAs arise from a combination of abnormal myocardial electrophysiology and dysfunction of the intramyocardial sympathetic/adrenergic nerves[3,4]. The adrenergic nerves within the heart direct the cardiac response to stressors and control every aspect of myocardial function, including arrhythmia susceptibility[5,6]. Modern anti-arrhythmic therapies rely on targeting cardiomyocyte electrophysiology as well as inhibiting adrenergic influence on the heart, but at present they have demonstrated limited efficacy in preventing VAs[7–9]. Both the excess (hyperinnervation) and the lack (hypoinnervation) of adrenergic axons within the ventricular myocardium have been associated with VAs[10–15], but the mechanistic link leading from abnormal innervation to arrhythmogenic electrophysiology has not been elucidated.

Adrenergic axons infiltrate the heart during embryonic and early postnatal development, stimulated by nerve growth factors and spatially guided by trophic molecules released from coronary vessels[16–18]. This innervation process becomes quiescent in adulthood but is reactivated in acquired heart diseases such as myocardial infarction (MI) and heart failure[19–21]. One important mechanism directing cardiac innervation is semaphorin-3A signaling, which has previously been associated with cardiac arrhythmias in both humans and animal models[11,22,23]. Semaphorin-3a (*Sema3a*) knockout (KO) mice have abnormal cardiac innervation and arrhythmias[12], but their arrhythmogenic mechanisms are challenging to study due to lethality in the early postnatal period[12,24]. The receptors Plexin-A3 and Plexin-A4 mediate Sema3A signaling in developing sympathetic neurons, with Plexin-A4 transducing most of the Sema3A effect[25]. Mice deficient in Plexin-A4 have reduced cardiac innervation but are viable and fertile[25,26], and their cardiac physiology has not been previously characterized.

In this study, we investigate the electrophysiologic consequences of abnormal cardiac adrenergic innervation in *Plxna3/a4* double KO (dKO) mice. Since the two plexins function redundantly[25,27] in Sema3A signal transduction, we primarily studied the dKO to maximize the

phenotypic effect of losing Sema3A signaling in cardiac innervation. We assess adrenergic axon density and cardiac structure using tissue clearing and immunofluorescence (IF) imaging in intact hearts. Using ECG recording *in vivo* and in a Langendorff preparation, we evaluated post-synaptic adrenergic responses in *Plxna3/a4* dKO mouse hearts and identify a phenotype of hyperadrenergic arrhythmogenesis.

METHODS

Animals

Plxna3 and *plxna4* (MMRRC_030406-MU) alleles have been described previously[25]. All experiments complied with National Institutes of Health guidelines and were reviewed and approved by the UCLA Animal Research Committee, and both the MUSC and Duke Institutional Animal Care and Use Committees.

Immunofluorescence and tissue clearing

Mice were given intraperitoneal (i.p.) heparin (100U) and euthanized with isoflurane and cervical dislocation. Once all reflexes subsided, a midsternal incision was made and animals were transcardially perfused with 50 mL ice-cold 0.01 M PBS containing 100U heparin followed by 50 mL freshly prepared, ice-cold 4% paraformaldehyde (PFA; EMS, RT 15714) in PBS. Hearts were post-fixed in 4% PFA overnight at 4 °C, washed, and stored in PBS with 0.01% sodium azide. Langendorff-perfused hearts were immersion fixed in 4% PFA overnight at 4 °C at the end of the functional experiments.

Hearts were stained and cleared using a modified iDISCO protocol[28] as previously described[14]. Antibodies for cleared hearts were anti-TH (EMD Millipore, AB1542, 1:200) and donkey anti-sheep Alexa Fluor 647 (Jackson ImmunoResearch, 713-605-147, 1:300) diluted in 0.01 M PBS with 0.2% Tween-20 and 10 mg/ml heparin (PTwH). Primary and secondary Ab were

replenished approximately halfway through the incubation period. Hearts were stored in benzyl ether (Millipore Sigma, 108014 ALDRICH; refractive index: 1.55) for up to 7 days prior to imaging.

For qualitative assessment of lateral left ventricular (LV) myocardial and atrioventricular (AV) nodal sections, 300 μm-thick vibratome sections were created from fixed hearts and immunolabeled with anti-HCN4 (1:400; Alomone Labs APC-052), anti-TH (1:400: Millipore AB1542) and anti-SYP (1:500; SCBT sc-17750) antibodies overnight at 37°C, and followed by Alexa Fluor-conjugated secondary antibodies (Life Science) for 3 hr at 37°C. Immunolabeled sections were counterstained with DAPI and cleared with ScaleU2[29].

Confocal imaging and image analysis

Whole hearts were mounted in benzyl ether with adhesive plastic spacers (Sunjin Labs, IS012 and IS013). Images were acquired on a confocal laser scanning microscope (Zeiss, LSM 880) at 5x magnification. Whole heart images were taken in tiles with XY-resolution of 1.661μm and Z-resolution of 8.29μm. Heart sections were flat-mounted to slides and imaged at 40x magnification.

To quantify TH-positive axons in whole hearts, tiles were stitched with Zeiss Zen software and a maximum intensity projection (MIP) was created of each heart. The TH-positive pixel area was measured by binary threshold selection using Fiji-ImageJ software. This was divided by the whole heart area, as measured by muscle autofluorescence, to calculate percentages.

Echocardiography

Mice were anesthetized with 3% isoflurane and maintained with 1% isoflurane in 95% oxygen. Anesthesia was adjusted to obtain a target heart rate of 470 ± 50 beats per minute (bpm). Transthoracic echocardiography was conducted with a Vevo 3100 high-frequency, high-resolution digital imaging system (VisualSonics) equipped with a MX550S MicroScan Transducer. Fur was removed a day prior to imaging studies. Mice were immobilized in a supine position at a 20-30 degree incline. Ultrasound gel was warmed to approximately body temperature and applied to the area overlying the heart. A parasternal short axis view, indicated by the presence of papillary

muscles, was used to obtain M-mode images for analysis of fractional shortening, ejection fraction (LVEF), and other cardiac structural parameters. All parameters were measured two to three times, and means were used for analysis. At the end of the procedures, all mice recovered from anesthesia without difficulties.

Electrocardiography and analysis

ECG data was recorded in awake, freely moving mice by implanting radio telemetry devices (ETA-F10, Data Science International). Mice were anesthetized with 4% isoflurane and maintained with 2% isoflurane. Telemetry units were implanted into the peritoneal cavity. The electrical leads of the telemetry units were tunneled through the abdominal wall with the free ends secured to the pectoral muscles using non-absorbable suture in a lead II orientation. Mice were recovered for 14-20 days following telemeter implantation surgery to ensure minimal lead motion artifact and to avoid the effects of postoperative pain and inflammation on baseline autonomic state. Mice were housed singly in cages over antenna receivers connected to a computer system with Ponemah software (Data Science International) for recording. Approximately 48 hours of continuous ECG recording were collected for each mouse, with brief interruptions for the intraperitoneal injection of isoproterenol (1 μg/g) and propranolol (4 μg/g).

Anesthetized ECGs were performed in mice during 2% isoflurane inhalation, using platinum needle electrodes (AD Instruments) placed subdermally in the lead II configuration. Recording was performed using a Powerlab 35 acquisition system (AD Instruments). Once stable baseline heart rate was confirmed for 5 minutes, isoproterenol 1 μg/g was administered by intraperitoneal injection and data was collected for an additional 20 minutes after isoproterenol administration. LabChart 8.0 software (AD instruments) was used for anesthetized ECG analyses.

Analysis of telemetry recordings, including premature ventricular contraction (PVC) and ventricular tachycardia (VT) quantification, was performed using ecgAUTO software (Emka Technologies). ECG interval measurements were taken from the average of a 5-min recording at

9am. Baseline heart rates (HR) were averages of 2 hours prior to isoproterenol administration, starting at 4pm. Post-drug arrhythmia quantification was performed for the 2 hours following isoproterenol or propranolol administration. The arrhythmia severity scores were defined as: 0 = no ventricular ectopy, 1 = isolated PVCs, 2 = bigeminy or couplets, 3 = VT (3 or more continuous ectopic beats).

Langendorff perfusion and pseudo-ECG

Mice were injected with intraperitoneal heparin (100 U) 10-15 minutes prior to sacrifice per protocol by anesthesia with 5% isoflurane followed by cervical dislocation. Hearts were removed immediately and perfused via the aortic root with Tyrode's solution (130mM NaCl, 1.25mM $CaCl_2$, 5mM KCl, 1.2mM NaH_2PO_4, 1.1mM $MgCl_2$, 22mM $NaHCO_3$, and 50mM dextrose, aerated by bubbling 95% oxygen and 5% carbon dioxide via a fritted gas dispersion tube). Hearts were immobilized and immersed in Tyrode's solution bath within a custom, 3-D printed chamber to reduce motion artifact. Perfusate and bath temperature were maintained at 36.6-37°C. Aortic root pressure was monitored with a Powerlab Bridge Amp (AD Instruments) and maintained constantly at 70-80 mmHg for the duration of the experiments, with meticulous flow rate adjustments (3-6 ml/min). Following confirmation of stable heart rate for 20 minutes, isoproterenol was administered via addition to the perfusate reservoir at 100 nM concentration, and an additional 20 minutes of data was recorded.

Pseudo-ECG was recorded with platinum needle electrodes (Grass Instruments) stabilized in Sylgard 184 silicone elastomer (Electron Microscopy Sciences) using a P511 amplifier (Grass Instruments) connected to a Powerlab 35 acquisition system (AD Instruments). ECG analysis was performed using LabChart software (AD Instruments).

Membrane extraction from cardiac ventricular tissue

Mice were sacrificed per protocol by anesthesia with 5% isoflurane followed by cervical dislocation. Hearts were dissected, ensuring complete removal of atria, pulmonary and aortic vasculature,

then flash frozen in liquid nitrogen and stored at -80°C until needed. Frozen ventricles were resuspended in ice-cold lysis buffer (Tris–HCl 25 mM, EDTA 5 mM, and 1X of the proteinase inhibitor mix aprotinin and leupeptin [LB]; pH 7.4), minced, and dounce homogenized (30 strokes). Homogenates were centrifuged at 500 g for 5 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended in binding buffer (Tris–HCl 75 mM, EDTA 2 mM, MgCl2 12.5 mM, and 1X LB mix; pH 7.4). The homogenates were centrifuged at 35,000 g for 30 minutes at 4°C to obtain the membrane fraction. The supernatant was discarded, and the pellet was resuspended in binding buffer with 10% glycerin, homogenized, and stored at −80°C until needed.

Radioligand Saturation Binding

Protein quantification of cardiac membrane fractions was performed using the bicinchoninic acid (BCA) protein assay following the manufacturer's instructions. Then, 18 to 20 µg of protein diluted in 50 µl of binding buffer was added per well, followed by 50 µl of binding buffer or propranolol (30 µM) to determine non-specific binding (NSB). Finally, increasing concentrations of Iodo-(-)-Cyanopindolol, [125I] (125I-CYP) from 35.7 to 300 pM were used to measure total binding (TB) at a constant volume of 200 µl/well. The mixture was incubated for 90 minutes at room temperature to reach equilibrium. The radioactivity was measured as counts per minute using a gamma counter (PerkinElmer, USA). The saturation binding curves are reported as specific binding (SB), where SB = TB − NSB. The receptor density (Bmax) was determined using one site-specific binding regression curve (Graphpad Prism 10). The Bmax values were converted from counts per minute to fmol/mg of protein.

Phenome-wide association study

To evaluate the association between plexins and arrhythmias in humans, we searched for associations between all phenotypes containing the word “arrhythmia” and the gene “*PLXNA4*” via the AstraZeneca PheWAS portal (https://azPheWAS.com/). This analysis was based on data from the UK Biobank, which included approximately 15,500 binary phenotypes and 1,500

continuous phenotypes derived from around 500,000 participants who had undergone exome sequencing[30]. The detailed methodology is documented in the original publication.

Statistics

All statistical analyses for comparison are indicated in figure legends or main text and were performed in Prism 10 (GraphPad). Statistical tests were all non-parametric and two-tailed, unless otherwise noted in the text.

RESULTS

Plxna3/a4 dKO mice exhibit cardiac adrenergic denervation but have structurally normal hearts

To evaluate cardiac adrenergic innervation in *Plxna3/a4* dKO mice, we performed whole-mount immunofluorescence (IF) staining for the adrenergic neuron marker tyrosine hydroxylase (TH) in intact hearts and cleared them using a modified iDISCO+ protocol[28]. Confocal imaging revealed a significant global reduction in cardiac adrenergic nerves in *Plxna3/a4* dKO hearts compared to wild-type (WT) controls (Figure 1A). Quantification of TH staining as percent of total heart area in maximum intensity projection (MIP) images showed that TH-positive axons were less than half as dense in *Plxna3/a4* dKO hearts (Cliff's $\delta = 1$) compared with WT hearts. Qualitatively, this denervation was observed throughout the whole heart, as demonstrated by decreased staining for TH and the synaptic marker synaptophysin (SYP) in tissue sections from the working myocardium of the lateral LV and from the AV nodal region as defined by anti-HCN4 staining (Figures 1C-D).

Despite the significant reduction in adrenergic innervation, *Plxna3/a4* dKO mice showed no abnormalities in cardiac structure or systolic function. Transthoracic echocardiography demonstrated normal LV ejection fraction (LVEF), posterior wall thickness (LVPW;d) and end-diastolic dimension (LVID;d) in *Plxna3/a4* dKO mice compared to WT controls (Figures 1E-H,

Mann-Whitney test, $p > 0.05$ for all parameters). There was no fibrosis seen in tissue sections from *Plxna3/a4* dKO hearts stained with Masson's trichrome (Supplementary Figure 1).

Plxna3/a4 dKO mice have spontaneous ventricular arrhythmias

To determine whether adrenergic denervation affects underlying electrophysiology in *Plxna3/a4* dKO mice, we performed 48-hour continuous ECG recordings in conscious, freely-moving mice using implanted telemeters. Both WT and *Plxna3/a4* dKO mice had normal PR, QRS and QT intervals, and their resting HRs were not significantly different (Supplementary Figure 2). While WT mice displayed normal sinus rhythm without any observed ventricular ectopy at baseline (Figure 2A), *Plxna3/a4* dKO mice frequently exhibited spontaneous premature ventricular contractions (PVCs) that were overall pleiomorphic in nature (Figure 2B-C). These PVCs occurred with both fixed (Figure 2B) and variable coupling intervals (Figure 2C). *Plxna3/a4* dKO mice also had episodic ventricular tachycardia (VT) (Figure 2D). Quantification of arrhythmia severity demonstrated significantly higher arrhythmia scores in *Plxna3/a4* dKO mice compared to WT controls at baseline (Figure 2E, one-tailed Mann-Whitney test, $p = 0.0303$), and the frequency of these arrhythmias was also increased (Figure 2F, one-tailed Mann-Whitney test, $p = 0.0011$).

Ventricular arrhythmias in *Plxna3/a4* dKO mice are exacerbated by beta-adrenergic stimulation and attenuated by beta-adrenergic blockade

The presence of both fixed- and variable-coupled PVCs in *Plxna3/a4* dKO mice suggested that the underlying electrophysiologic mechanisms of their ectopic impulse initiation likely includes abnormal automaticity and triggered activity – both of which are downstream of adrenergic signaling and thus should be exacerbated by catecholamines[31–34]. Indeed, we found that the arrhythmia severity in *Plxna3/a4* dKO mice was amplified following intraperitoneal administration of the beta (β)-adrenergic agonist isoproterenol (1 μg/g) (Figure 3A, Mann-Whitney test, $p = 0.0216$). The frequency of VAs, defined by ventricular ectopic beats per hour, was also significantly higher in *Plxna3/a4* dKO mice following isoproterenol administration (Figure 3B,

Mann-Whitney test, p = 0.0077). The peak HR following isoproterenol administration was not changed in *Plxna3/a4* dKO mice compared to WT (Supplementary Figure 2B). Administration of the β-blocker propranolol (4 μg/g, i.p.) significantly reduced VA frequency in *Plxna3/a4* dKO mice (Figure 3C, one-tailed Wilcoxon paired rank test, p=0.0312) in the 2 hours after injection, when compared with the 2 hours prior to propranolol administration.

Plxna3/a4* dKO mouse hearts display intrinsic adrenergic hypersensitivity *ex vivo

Despite their decreased quantity of cardiac adrenergic nerves, *Plxna3/a4* dKO mice display arrhythmic susceptibility that is dependent on sympathetic influence, leading us to hypothesize that their VAs arise from intrinsic adrenergic hypersensitivity of the myocardium, rather than from overactivity of the sympathetic nervous system. To test this hypothesis, we isolated hearts from *Plxna3/a4* dKO and WT mice and perfused them at constant pressure in an *ex vivo* Langendorff preparation, thus removing all autonomic nervous system influence on cardiac physiology. In Langendorff, WT and *Plxna3/a4* dKO hearts had similar HR at baseline and similar peak HR with isoproterenol infusion (100 nM), and none of the hearts exhibited VAs at baseline during 20-minute pseudo-ECG recordings (Supplementary Figure 3). However, compared to WT, hearts from *Plxna3/a4* dKO mice demonstrated a significantly accelerated chronotropic response to isoproterenol (Figure 4A), with a shorter time to reach peak sinus rate (Figure 4B, Mann-Whitney test, p=0.0397).

Isoproterenol also induced VAs in all *Plxna3/a4* dKO hearts in Langendorff, with greater arrhythmia severity (p=0.0476, Mann Whitney test) and frequency (p=0.0079, Mann Whitney test) of ventricular ectopy, when compared with WT hearts (Figure 4C-D). In 2 of 8 *Plxna3/a4* dKO hearts, sustained VT was seen at peak isoproterenol effect, lasting several minutes (Figure 4E). These *ex vivo* findings suggest that the arrhythmic susceptibility in *Plxna3/a4* dKO mice is at the post-synaptic level, i.e. due to intrinsic hyperadrenergic response of the myocardium.

Plxna4 KO mice exhibit adrenergically driven ventricular arrhythmias and increased post-synaptic β-adrenergic receptor density in ventricular membranes

Given the challenge of breeding *Plxna3/a4* double KO mice, we considered whether *Plxna4* single KO mice would also display denervation supersensitivity, since previous reports have shown that Plexin-A4 transduces the majority of Sema3a signaling in developing sympathetic nerves[25], and *Plxna4* KO mice are also known to have decreased cardiac adrenergic innervation[26]. We performed 10-minute baseline ECG recordings in *Plxna4* KO mice and WT controls during isoflurane inhalation, which showed no baseline arrhythmias (data not shown), followed by 20-minute recordings after administration of isoproterenol (1 μg/g, i.p.). *Plxna4* KO mice displayed significantly higher severity and frequency of VAs (Figure 5A-B, one-tailed Mann-Whitney tests, $p = 0.0143$) compared to WT controls.

To determine the mechanism underlying the hyperadrenergic phenotype in *Plxna4* KO hearts, we performed saturation binding assays using the radiolabeled beta-adrenergic receptor (βAR) antagonist [125I]-cyanopindolol. Representative binding curves revealed an approximately two-fold increase in total βAR density (Bmax) in ventricular membranes from *Plxna4* KO mice compared to WT controls (Figure 5, Mann-Whitney test, $p = 0.0121$). This finding supports our contention that the arrhythmic physiology in mice with loss of plexin-dependent cardiac innervation is due to adrenergic hypersensitivity of the myocardium secondary to an increase in density of post-synaptic βARs.

Human _PLXNA4_ variants are associated with cardiac arrhythmias

To investigate the clinical relevance of our findings, we performed a phenome-wide association study (PheWAS) between phenotypes containing the word “arrhythmia” and the gene “*PLXNA4*” via the AstraZeneca PheWAS portal (https://azPheWAS.com/). This analysis identified 13 rare, single-nucleotide variants with nominally significant association p-values of <0.01 with arrhythmias (Figure 6A). One of these variants was a synonymous coding-region variant

associated with two arrhythmia phenotype codes, with “re-entry ventricular arrhythmia” being the most significantly associated phenotype (Figure 6B), with a large effect size (odds ratio = 141). The p-values for individual variants did not reach the PheWAS significance threshold of $p < 4.6e-6$ (false discovery rate < 0.1)[35]. However, these associations found among different ancestries suggests that the *PLXNA4* gene may play a role in human arrhythmogenesis. The full variant list with detailed case-control comparisons and confidence intervals can be found in Supplementary Table 1.

DISCUSSION

In this study, we determined that loss of plexin-mediated cardiac adrenergic innervation results in VAs driven by post-synaptic adrenergic hypersensitivity. We demonstrated that this hyperadrenergic physiology is due to increased βAR density in cardiac membranes. The observation that *Plxna3/a4* dKO mice exhibit spontaneous VAs in the absence of cardiac structural abnormalities or intentional ion channel mutations is of particular significance. Compared with humans, mice are far less prone to arrhythmias due to numerous differences in their cardiac electrophysiology[36,37]. Even in humans, VAs are rarely sustained in the absence of myocardial injury or ion channelopathy[38]. Our finding that adrenergic stimulation alone can cause sustained VT in *Plxna3/a4* dKO mouse hearts suggests that its physiology represents a unique platform to specifically study adrenergically driven VAs.

Plxna3/a4 dKO and *Plxna4* single KO mice display significantly decreased density of adrenergic nerves in the myocardium yet have enhanced cardiac adrenergic response. This phenomenon, known as denervation supersensitivity, has previously been shown to be important in arrhythmogenic heart diseases such as MI and heart failure[13,39,40], where there is injury to myocardium and degeneration of adrenergic axons. Denervation supersensitivity has also long been observed during development and disease states in other synapses of both the central

nervous system and skeletal muscle[41], though its precise molecular mechanisms remain poorly understood.

Several limitations of our study should be acknowledged. First, while we demonstrate a clear link between hypoinnervation and cardiac arrhythmogenesis, the precise molecular mechanisms by which deficiency of Plexin-A3 and Plexin-A4 leads to abnormal cardiac electrophysiology requires further investigation, and we anticipate doing this by first using high-throughput screens of gene expression in *Plxna3/a4* dKO mouse hearts. Second, our study focused on βARs, but other components of the adrenergic signaling pathway, such as G proteins, adenylyl cyclase, and downstream effectors, may also contribute to the observed phenotype. Future studies should examine these additional components to provide a more comprehensive understanding of the molecular mechanisms underlying hyperadrenergic arrhythmias.

We identified several rare variants in human *PLXNA4* that were associated with arrhythmia phenotypes at nominally significant p-values of < 0.05. Based on our experimental evidence thus far, *PLXNA4* variants would be anticipated to play a disease-modifying rather than a disease-causing role in human arrhythmias. While disease-causing arrhythmia genes, such as KCNQ1, frequently have association p-value that exceeded genome-wide significance, it is not surprising that PheWAS of *PLXNA4* as a likely disease modifier may not yield a significant p-value, when corrected for multiple-hypothesis testing. The absence of significant associations may also be due to negative selection of lethal arrhythmogenic variants and the relatively modest impact of even common variants on human arrhythmia. Though we recognize that PheWAS may be prone to false positive associations, the presence of multiple associated variants across different ancestries and variant types warrants future investigations in plexin-mediated cardiac innervation in human arrhythmias.

CONCLUSIONS

In conclusion, our findings demonstrate that loss of plexin-mediated cardiac adrenergic innervation leads to ventricular arrhythmias through a mechanism involving post-synaptic βAR upregulation and enhanced adrenergic sensitivity, which may be relevant in human arrhythmogenesis. Adrenergic dysfunction is becoming an increasingly important area of investigation in arrhythmia biology, and neuromodulatory therapies have shown promise in the clinical management of VAs[42]. Thus, the *Plxna3/a4* dKO mouse is a novel tool for ongoing investigation into adrenergic mechanisms of arrhythmogenesis, which may identify new therapeutic targets for the prevention of sudden cardiac death.

DATA AVAILABILITY

All data used in this study are available upon request to the corresponding author.

AUTHOR CONTRIBUTIONS

C.Z., T.M., K.S., and H.A.R. designed the studies. C.Z. performed the animal surgeries, tissue collection, IF and tissue clearing, confocal imaging, telemetric ECG recordings, Langendorff experiments and data analyses. E.Y.L.G. performed cardiac tissue membrane extraction and saturation binding assays. R.P. and T.M. supplied mice and performed tissue section IF and imaging, as well as ECG recordings under anesthesia. J.J.W. assisted with the PheWAS. Y.C. performed echocardiography. C.Z. prepared the figures and wrote the manuscript with assistance from E.Y.L.G. All coauthors contributed to the final version of the manuscript.

ACKNOWLEDGEMENTS

We are grateful to Dr. Kristina Bostrom and Dr. Conrad Hodgkinson for their thoughtful review and suggestions for the manuscript. This work was supported by an American Heart Association Career Development Award (24CDA1268188) to C.Z. and NIH grants: Stimulating Peripheral Activity to Relieve Conditions (OT2OD023848) and P01HL164311 to K.S., and R01HL056687 to H.A.R.

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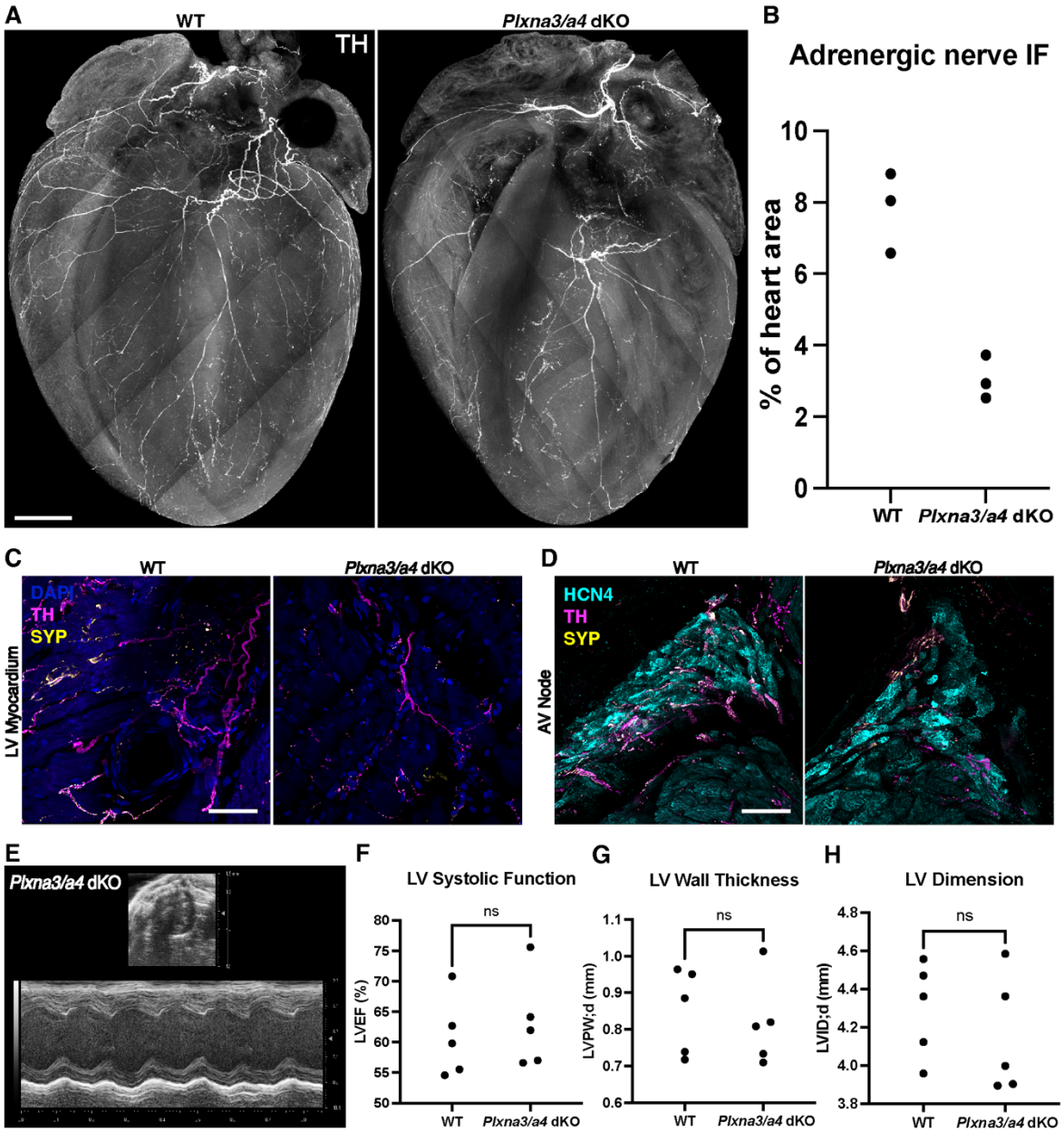

Figure 1. Decreased adrenergic innervation and normal cardiac structure in *Plxna3/a4* dKO hearts. A) Representative maximum intensity projection images of whole-mount IF in iDISCO-cleared hearts demonstrates globally reduced adrenergic nerve marker TH in *Plxna3/a4* dKO mouse ventricles (right image) compared with WT (left image). **B)** TH quantification, calculated as percent of whole heart area (defined by muscle autofluorescence), confirms *Plxna3/a4* dKO hearts have less than half the adrenergic nerve IF compared with WT hearts (Cliff's δ = 1, each dot represents one mouse). **C,D)** IF of tissue sections from Plxna3/a4 dKO hearts qualitatively confirms the adrenergic denervation phenotype in both working lateral LV myocardium and conduction system (AV nodal region) defined by HCN4. **E-H)** Transthoracic echo demonstrates *Plxna3/a4* dKO mice have normal heart structure and LV systolic function compared with WT mice (Mann-Whitney test, n = 5 mice per group). SYP = synaptophysin, used to stain for nerve endings. ns = not significant. Scale bar in **(A)** = 200μm, in **(C)** and **(D)** = 100μm.

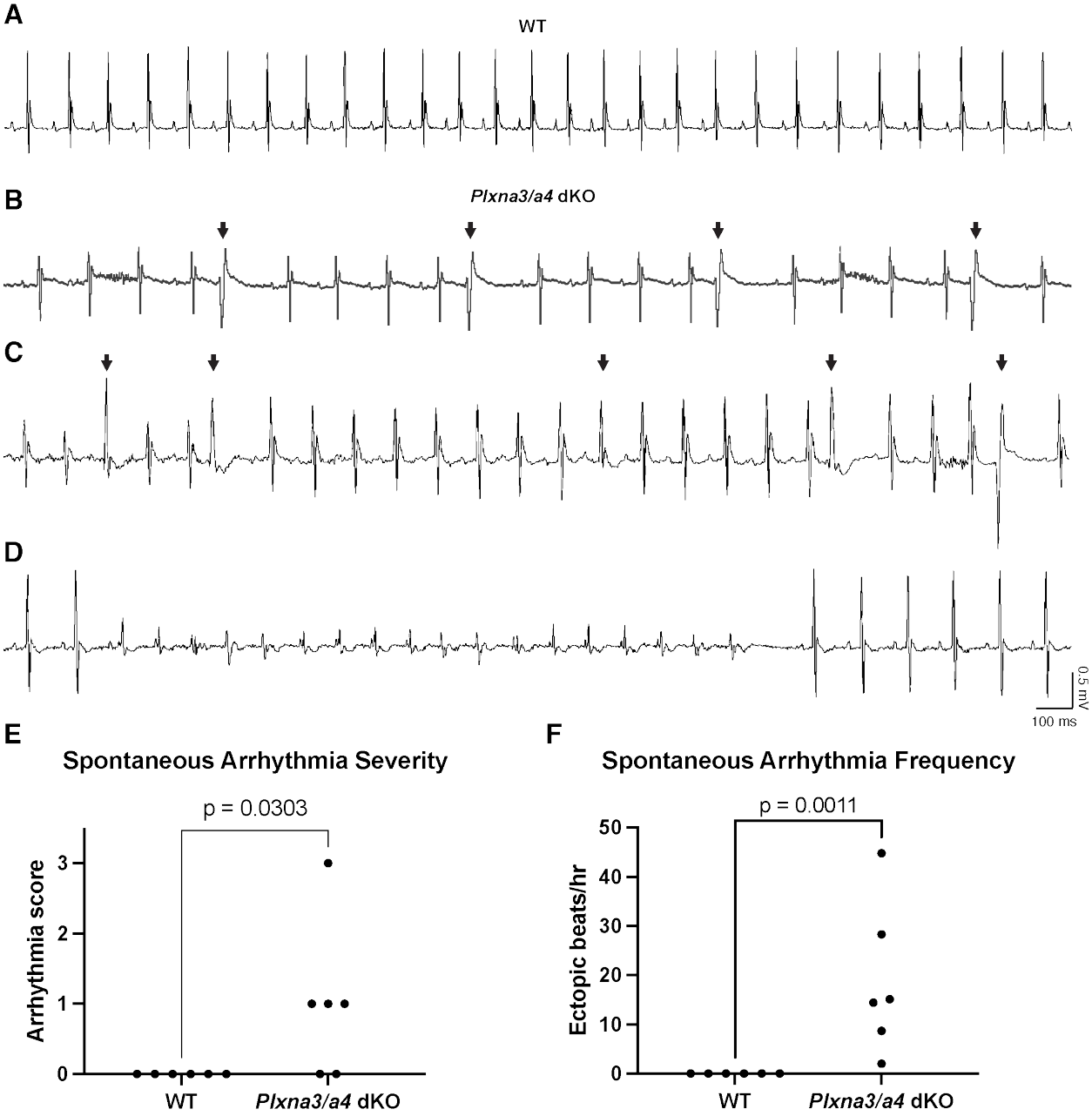

Figure 2. Spontaneous ventricular arrhythmias in *Plxna3/a4* dKO mice. A) Representative ECG recording from a conscious, freely moving WT mouse shows entirely normal sinus rhythm. **B)** Frequent, spontaneous PVCs of fixed coupling interval in *Plxna3/a4* dKO mice. **C)** Spontaneous PVCs of variable coupling intervals with fusion beat (third arrow-demarcated beat) and **D)** VT in conscious *Plxna3/a4* dKO mice. **E)** Ventricular arrhythmia severity and **F)** frequency in *Plxna3/a4* dKO mice versus WT. Arrhythmia severity scores: 0 = no PVCs, 1 = isolated PVCs, 2 = couplets or bigeminy, 3 = VT (3 or more consecutive ventricular ectopic beats). All p-values are from one-tailed Mann-Whitney tests and each dot in **(E-F)** represents one mouse.

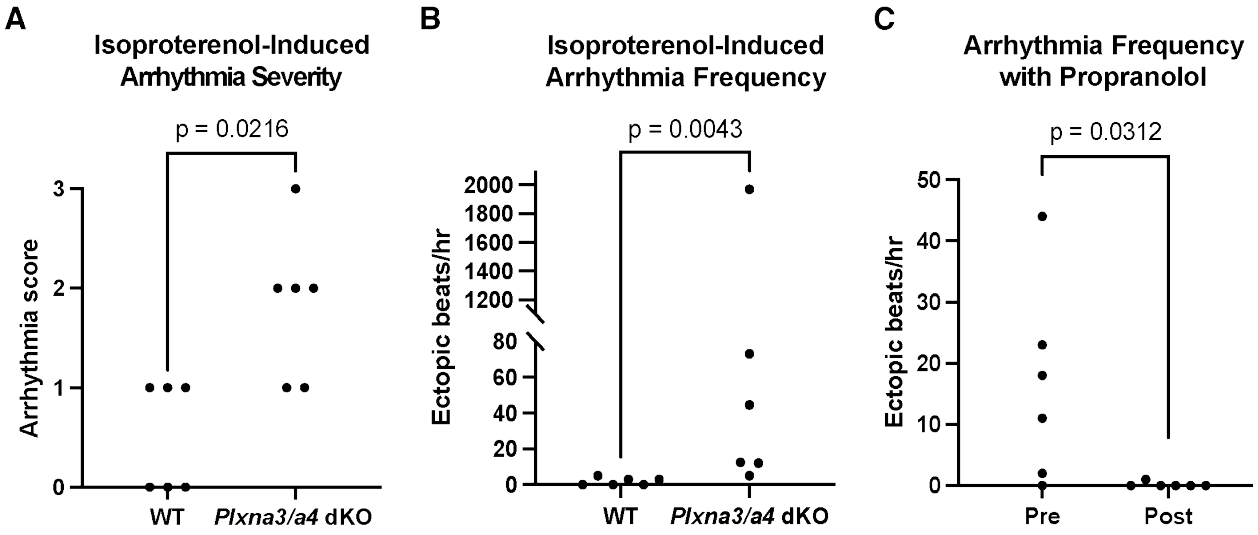

Figure 3. Ventricular arrhythmias are exacerbated by beta-adrenergic stimulation and inhibited by beta-adrenergic blockade in *Plxna3/a4* dKO mice. A) Arrhythmia severity scores following isoproterenol (1 µg/g i.p.) injection in *Plxna3/a4* dKO mice versus WT. **B)** Frequency of induced arrhythmias in the 2 hours following isoproterenol administration, calculated as ventricular ectopic beats per hour, in *Plxna3/a4* dKO mice versus WT. **C)** Arrhythmia frequency in *Plxna3/a4* dKO mice during 2-hour baseline recording (pre) compared to the 2 hours after (post) propranolol administration (4 µg/g i.p.) administration. P-values in **(A-B)** are from Mann Whitney tests and p-value in **(C)** is from one-tailed Wilcoxon paired rank test. Each dot represents one mouse. Arrhythmia severity scores: 0 = no PVCs, 1 = isolated PVCs, 2 = couplets or bigeminy, 3 = VT (3 or more consecutive ventricular ectopic beats).

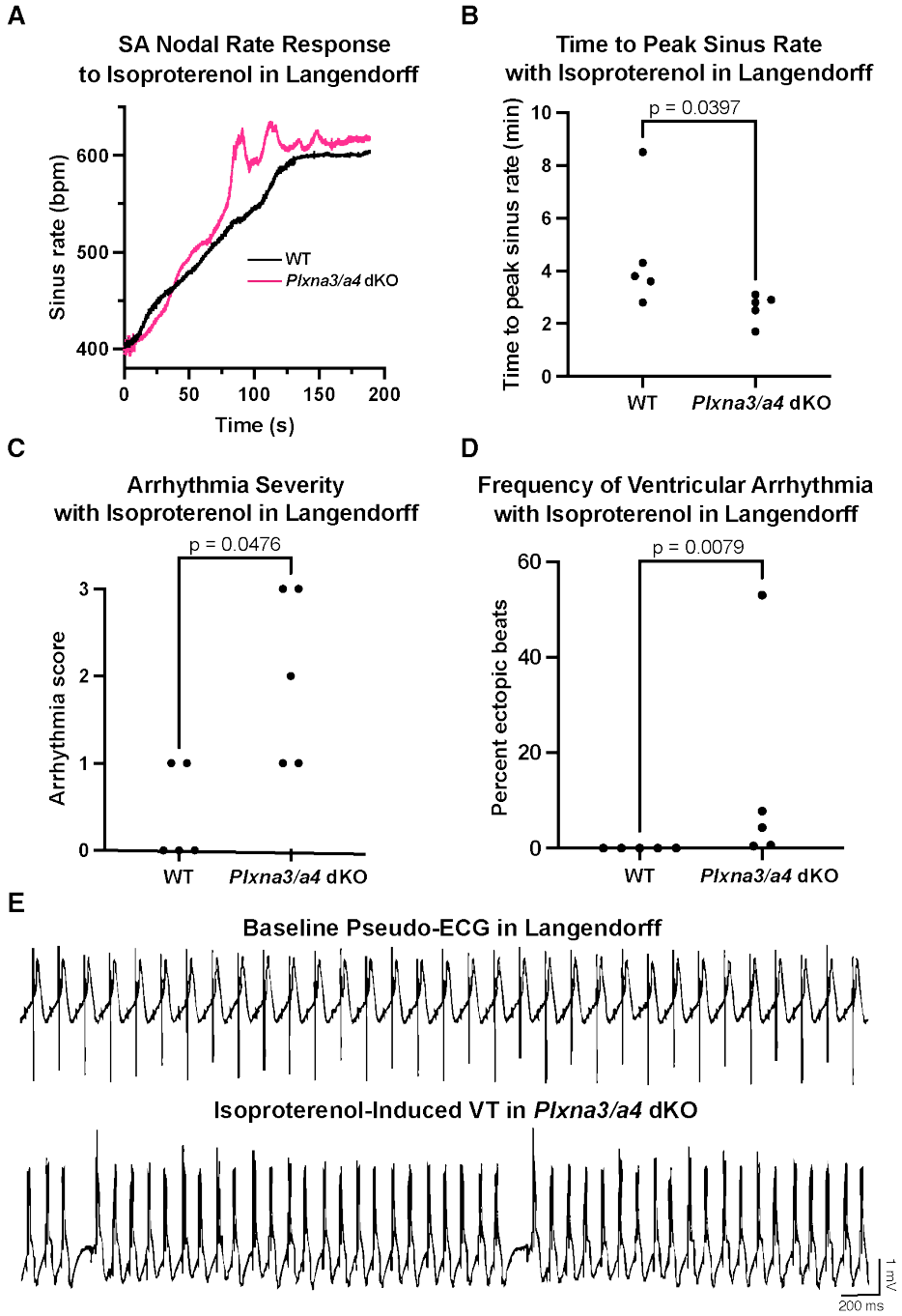

Figure 4. Hyperadrenergic electrophysiologic response in Langendorff-perfused *Plxna3/a4* dKO hearts. A) Representative sinoatrial nodal rate response curves with isoproterenol perfusion (100 nM). **B)** Time to reach peak sinus rate after isoproterenol perfusion in *Plxna3/a4* dKO hearts versus WT. **C)** Isoproterenol-induced arrhythmia severity and **D)** frequency of ventricular ectopic beats in Langendorff-perfused WT hearts versus *Plxna3/a4* dKO, calculated as percent of total beats. All p-values are from Mann-Whitney tests and each dot in **(B-D)** represents one mouse. Arrhythmia severity scores: 0 = no PVCs, 1 = isolated PVCs, 2 = couplets or bigeminy, 3 = VT (3 or more consecutive ventricular ectopic beats). **E)** Representative pseudo-ECG tracings showing baseline sinus rhythm in a *Plxna3/a4* dKO heart (top) and sustained VT induced with isoproterenol (bottom).

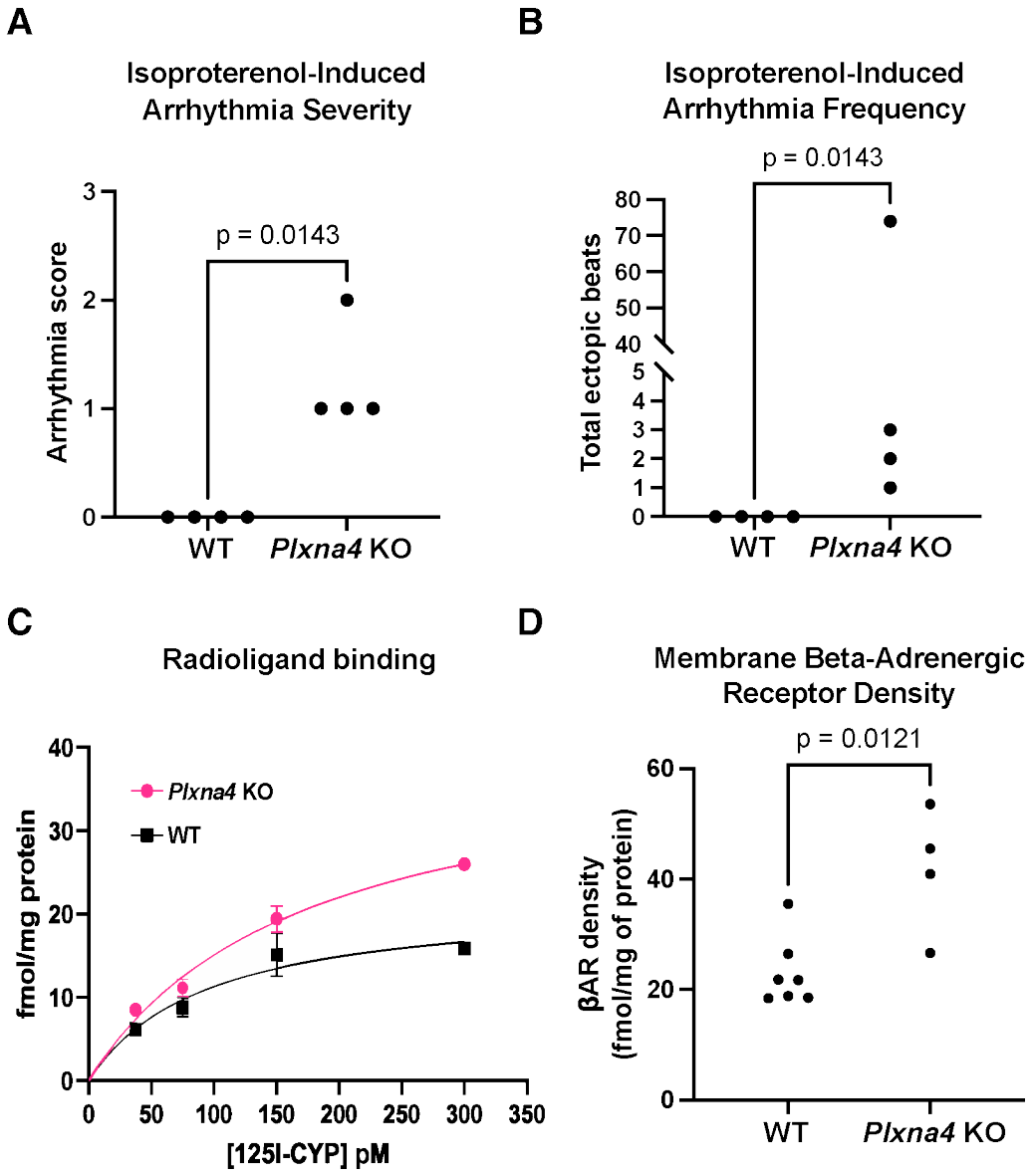

Figure 5. *Plxna4* KO mice have adrenergically driven ventricular arrhythmias and increased density of beta-adrenergic receptors in ventricular membranes. A) ECG recordings under anesthesia with isoflurane reveal that *Plxna4* KO mice have significantly increased isoproterenol-inducible ventricular arrhythmia severity and **B)** frequency as measured by total ventricular ectopic beats during a 20-minute ECG recording. **C)** Representative competition binding curves using increasing concentrations of the radiolabeled βAR antagonist cyanopindolol (125I-CYP) show **D)** significantly higher βAR density in hearts of *Plxna4* KO mice when compared to WT hearts. P-values in **(A-B)** are from one-tailed Mann-Whitney tests, and p-value in **(D)** is from a two-tailed Mann-Whitney test. Each dot represents one mouse in **(A, B, D)**.

A

Variant	Phenotype	Ancestry	p-value	Model	Consequence	Amino Acid Δ	Odds ratio
7-132185330-C-T	Other cardiac arrhythmias	European	8.60E-03	dominant	missense	p.Val1043Met	2.89
7-132198552-C-T	Other cardiac arrhythmias	European	7.10E-03	recessive	missense	p.Ala891Thr	5.54
7-132227591-G-A	Cardiac arrhythmia unspecified	European	9.30E-03	dominant	missense	p.Thr581Met	14.41
7-132484961-T-G	Cardiac arrhythmia unspecified	European	1.30E-03	dominant	missense	p.Glu475Ala	2.10
7-132489414-C-T	Cardiac arrhythmia unspecified	European	9.80E-03	dominant	missense	p.Asp417Asn	0.35
7-132133091-T-C	Cardiac arrhythmia unspecified	European	5.00E-03	recessive	synonymous	n/a	1.54
7-132146585-G-A	Other cardiac arrhythmias	European	8.30E-03	dominant	synonymous	n/a	3.00
7-132146585-G-A	Re-entry ventricular arrhythmia	European	1.13E-04	dominant	synonymous	n/a	141.37
7-132179832-T-C	Other cardiac arrhythmias	South Asian	9.00E-03	allelic	synonymous	n/a	0.54
7-132179832-T-C	Other specified cardiac arrhythmias	European	9.00E-03	dominant	synonymous	n/a	1.26
7-132187512-G-A	heart arrhythmia	European	9.40E-03	dominant	synonymous	n/a	3.57
7-132198588-G-T	Other cardiac arrhythmias	African	6.80E-03	dominant	synonymous	n/a	8.71
7-132484990-C-G	Cardiac arrhythmia unspecified	European	1.30E-03	dominant	synonymous	n/a	2.10
7-132164145-G-T	Other cardiac arrhythmias	African	5.20E-03	recessive	synonymous	n/a	n/a
7-132145293-A-G	Other cardiac arrhythmias	African	5.20E-03	recessive	splice region	n/a	n/a

B

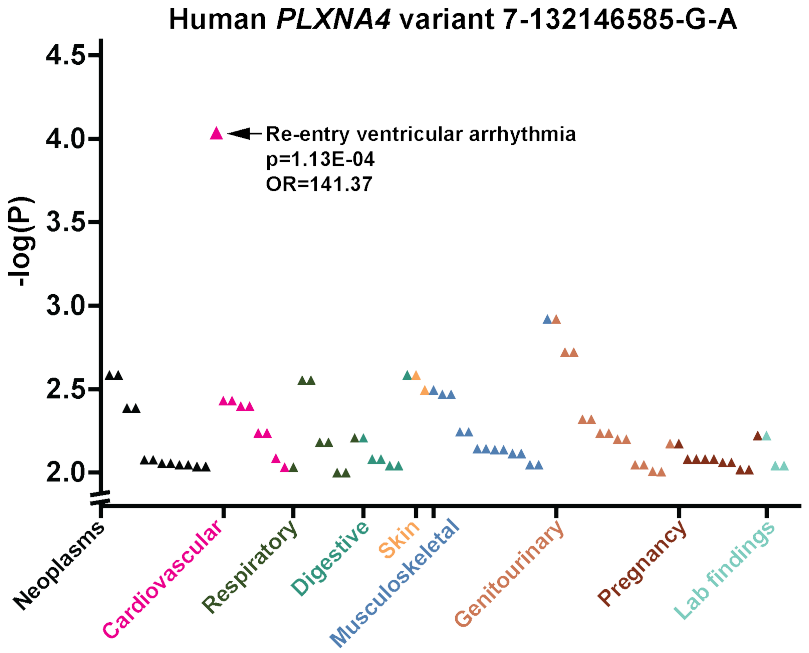

Figure 6. Association between rare *PLXNA4* variants and human arrhythmia phenotypes. Survey of the UK Biobank revealed **A)** 13 rare coding and splice single-nucleotide variants with nominally significant ($p<0.01$) associations with arrhythmia phenotypes, across several different ancestry groups and with several different inheritance patterns (models). One of these variants (highlighted in pink) was a synonymous variant associated with two arrhythmia phenotype codes. This variant was examined more closely in **B)** the Manhattan plot depicting the negative log-transformed P-values (y-axis) for its association with all phenotypes (x-axis), organized by disease categories. The most significantly associated phenotype for this variant was "Re-entry ventricular arrhythmia" (nominal p = 1.13e-4). OR = odds ratio.