Supplemental Material

METHODS

A total of sixteen mongrel dogs (20 to 40 Kg), were intubated, mechanically ventilated (Harvard Apparatus Co., Natick, MA) and underwent general anesthesia, protocol 1 with isoflurane and protocol 2 with sodium pentobarbital. A 6F sheath was inserted into the right femoral artery and connected to a pressure transducer (AD Instruments Inc., Colorado Springs, CO) for continuous arterial blood pressure (BP) recordings. ECGs were continuously recorded using the Animal Bio Amplifier (AD Instruments). Oxygen saturation (SaO_2) and end-tidal CO_2 were continuously recorded by a pulse oximeter in the dog's tongue, and an Expression MRI 865214 monitor (Invivo Co., Gainesville, FL). Core body temperature was maintained at 36.5±1.5° C by Bair Hugger 505 (3M, St. Paul, MN). Data analysis was performed off-line using LabChart 7 software (AD Instruments).

Protocol 1

In 7 dogs, simultaneous neural activity recordings from the extrinsic (right and left cervical vagal nerves and left stellate ganglion) and intrinsic (anterior right ganglionated plexi) cardiac autonomic nervous system (ANS) were obtained before and during apnea, and before and after local resiniferatoxin (RTX) administration (manuscript Figure 1B, C and D). A midline neck incision was performed exposing the carotid arteries and both cervical vagal nerves. The vagal nerves were isolated from the carotid sheath and bipolar microelectrodes (F. Haer Co., Bowdoin, Maine) were fastened around each nerve. After a median sternotomy, the heart was suspended in a pericardial cradle. Microelectrodes were attached to the anterior right ganglionated plexi (GP) -located within the epicardial fat pad between the right superior pulmonary vein and the right inferior pulmonary vein- and the left SG -identified between the vertebral junctions of the first and second ribs. Electrodes were connected to four mini DC

Coupled Headstages and to AM1700 amplifier (A-M systems Inc., Sequim, WA), set with bandpass filters (300 Hz-10 KHz, gain 100-1000X). Prior to nerve recordings, anesthesia was switched from isoflurane to alpha-chloralose (4.5 g; Sigma-Aldrich, St. Louis, MO) with a loading dose of 50 mg/kg, given over 10 minutes. Then, a maintenance rate of 60 mg/kg/hr was used. Signals were digitized and collected with a multichannel recorder at 1-20 kHz (AD Instruments). To induce apnea, the ventilator was switched off during end expiration, and the endotracheal tube disconnected and covered. We aimed to maintain apnea duration as long as needed to reach SaO_2 of at least 80%. Apnea was terminated if attempted respiratory movements were observed. In between each apnea episode the animal was allowed to recover to baseline SaO_2, CO_2, heart rate (HR) and BP.

RTX administration. RTX (0.5 mg; Sigma-Aldrich) was dissolved in 0.5 ml of ethanol and mixed with 9.5 ml of isotonic saline [1]. Two ml of RTX (50 µg/ml) was locally injected in the anterior right GP (ARGP) (manuscript Figure 1E) using a 20G needle, creating a wheal over the ARGP. Apnea episodes were performed before and after RTX administration. The mean duration of apnea episodes before RTX injection was 2.5 ±1 min and after injection was 2.75 ±2 min, p=NS.

Protocol 2

In 9 dogs, atrial effective refractory period (ERP) and atrial fibrillation (AF) inducibility with left atrial (LA) single extrastimulation were determined before and during apnea, and before and after closed-chest intrapericardial RXT administration (manuscript Figure 1F, G, H and I). Vascular sheaths were inserted in the right and left femoral veins, and right internal jugular vein. A percutaneous pericardial subxiphoid puncture was performed using a Tuohy needle, using a posterior approach (manuscript Figure 1G and H). Subsequently, multipolar electrophysiologic recording catheters were introduced into the coronary sinus and right atrium. The left atrium was accessed via a transeptal puncture. A mapping catheter was then inserted and a 3-dimensional

reconstruction of the left atrium and pulmonary vein geometry was made (NavX system, St. Jude Medical, Inc., St. Paul, MN).

An EP-Med stimulator (St. Jude Medical) was used to deliver 2× threshold current, 2-ms duration pulses using an S1S2 protocol with a basic cycle length (S1 of 400 ms) [2]. The S1-S2 duration was lowered in 5-10 ms decrements until S2 failed to capture the LA or pulmonary veins (PV). Left atrial ERP measurements were obtained at three different locations, the ostium of the right superior pulmonary vein (RSPV) (location 1), left superior pulmonary vein (LSPV) (location 2) and right inferior pulmonary vein (RIPV) (location 3). Single extrastimulation was performed in those three sites to assess AF inducibility.

AF was defined as an irregular fast atrial rate (> 300 bpm) that persisted for at least 10 s after the end of extra-stimulation. AF inducibility was quantified as the successful percentage of the total induction attempts. We purposefully avoided aggressive means of AF induction, such as burst pacing or during vagal stimulation, in order to test apnea-induced AF susceptibility as specifically as possible. If AF lasted >10 minutes, external electrical cardioversion was performed. Apnea was induced as described in protocol 1. Additionally, apnea episodes of up to 6 min were induced. The animal was allowed to recover to baseline SaO_2, CO_2, HR and BP levels in between each apnea episode.

RTX administration. RTX preparation was as in protocol 1. A deflectable sheath (Agilis NxT, St. Jude Medical) was advanced over the wire into the pericardium. Its tip was deflected to direct the wire towards the posterior left atrium in the oblique sinus of the pericardium and 10 ml of RTX (50µg/ml) was slowly infused into the pericardial sheath over 1 minute duration [1]. Cardiac resuscitation and external DC cardioversion was undertaken in dogs that developed ventricular fibrillation. Apnea episodes were performed before and after RTX administration.

Protocols 1 and 2 are summarized in manuscript Figure 1J.

Histopathology studies

After extraction, the heart was fixed in 10% neutral buffered formalin (NBF) for 24h. The ARGP and inferior right and left GP and superior left GP were then sampled and fixed for an additional 5 days in 10% NBF. Following fixation, tissue samples were either routinely processed in a vacuum infiltration processor over approximately 11 hours' time period or were processed using an extended 18 hours protocol (Tissue-Tek VIP 6, Sakura, Torrance, CA). The samples were embedded in paraffin, sectioned at 4 µm and stained routinely with hematoxylin and eosin. Sections designated for immunohistochemical staining for TRPV1 channels, expressed in the GP sensory neurons, were saved on chrome alum coated slides. TRPV1 immunoreactivity was detected using an automated immunostainer, the Ventana Discovery XT platform (Ventana Medical Systems, Inc., Tucson, AZ). Heat-induced antigen retrieval was done using the CC2 standard program and a pH6 Ribo CC, Citrate-based buffer (Roche, Basel, Switzerland). The primary rabbit polyclonal anti-VR1-TRPV1 antibody (LSBio, Seattle, WA) was used at a 1:200 dilution for one hour at room temperature. Immunoreactivity was detected using the Discovery ChromoMap DAB (diaminobenzidine) Kit (Roche) and OmniMap anti-Rb HRP (RUO) Discovery (Roche). The anti-rabbit horseradish peroxidase secondary antibody was applied for 12 minutes at room temperature. Slides were counterstained with hematoxylin II (Roche) for 8 minutes at 37°C. Hematoxylin was enhanced with bluing reagent (Roche) for 4 minutes at room temperature. Sections of canine dorsal root ganglia served as positive controls. Negative control slides were stained by substituting the primary antibody with IntelliPath FLX Universal negative control (Biocare Medical, Pacheco, CA). The CGRP immunohistochemistry analyses was conducted using formalin- fixed, paraffin-embedded tissue sections with an automated immunostaining platform. The assay was developed on the Ventana Discovery XT platform (Ventana). The primary rabbit polyclonal CGRP antibody (Abcam, Cambridge, MA) was used at a 1:500 dilution for one hour at room temperature. On the Discovery XT platform, heat-induced antigen retrieval was conducted using the CC1 standard program and a pH9, Tris-based buffer (Ventana). Primary antibody was detected using the

Discovery ChromoMap DAB (diaminobenzidine) Kit and Discovery OmniMap anti-Rb HRP (Ventana). The anti-rabbit horseradish peroxidase secondary antibody was applied for 12 minutes at room temperature. Slides were counterstained with hematoxylin for 8 minutes at 37ºC. Hematoxylin was enhanced with bluing reagent for 4 minutes at room temperature. Terminal deoxynucleotidyl transferase dUTP-mediated nick end labeling (TUNEL) assay was also performed using a commercially available In Situ apoptosis detection Kit (Abcam) to detect apoptosis.

Statistical analysis

According to the data distribution Wilcoxon signed rank sum test or paired Student *t* test using SigmaStat 3.1 (Systat Software Inc., San Jose, CA) were used to compare the median and interquartile range or mean and standard deviation values of the parameters before and during apnea, before and after RTX injection. Bonferroni correction was used to consider statistical significance in multiple comparisons. The significance level set for the *P* values after the Bonferroni correction is reported.

RESULTS

Protocol 1

Seven dogs completed this protocol. The mean duration of apnea episodes before RTX injection was 2.5 ±1 min and after injection was 2.75 ±2 min, p=NS. Maximum SaO_2 drop during apnea was 31±10% pre-RTX, and 30±11% post-RTX (p= 0.3).

Hemodynamic response to apnea

Seven dogs completed this protocol. The percentage of SaO_2 drop during apnea was 31 ±10%. There was variability in time to reach a SaO_2 of 80% and it often continued to drop after resumption of breathing. Hence apnea duration was variable. To compare across apnea episodes and across animals, apnea episode durations were divided in deciles, and HR and BP were normalized to their eupneic baselines. After termination of each apnea, HR, BP and SaO_2 recovered back to baseline values.

There was significant inter-individual variation in the hemodynamic response to apnea (Supplemental Figure 1). Overall, HR gradually decreased throughout apnea (supplemental Figure 2A). The highest decrease being at decile 7, dropping by 12% (17.9, 6.2), p<0.001. In one dog, however, this HR decrease was not sustained and HR increased towards the end of apnea (Supplemental Figure 1A).

In all animals, initially, apnea induced a mild, steady decline in BP. However, as apnea continued and SaO_2 declined, an abrupt elevation in BP- "BP rebound", the increase from nadir BP to BP at end of apnea- occurred. Systolic BP (SBP) and diastolic BP (DBP) rebounds before RTX injection were 25.39 ±6.07 % and 18.74 ±5.59 %, respectively. See Supplemental Figures 2B, C and D. Compared with eupneic baselines, the highest SBP increase during apnea was at decile 10, 12.5% (0.7,25.9), p=0.065.

Within these overall trends in HR and BP during apnea, fine periodic oscillations in HR and BP occurred as apnea progressed (see manuscript Figure 3A). These were not associated with

respiratory movements (as opposed to BP oscillations during ventilation), and exhibited a crescendo pattern that replicated that of phasic vagal firing (see manuscript Figure 3A).

Hemodynamic response to apnea after RTX administration

The mean duration of apnea episodes before RTX injection was 2.5 ±1 min and after injection was 2.75 ±2 min, p=NS. The percentage of SaO_2 drop during apnea was 31 ±10% pre-RTX, and 30 ±11% post-RTX (p= 0.3).

Overall, the gradual HR decrease seen throughout apnea did not significantly change after RTX injection (Supplemental Figure 1A). However, the animal that had an increase in HR towards the end of apnea presented with HR decrease after RTX (Supplemental Figure 1A). The most salient apneic hemodynamic change after RTX was a reduction in the BP rebound at the end of the apnea episodes: the rise in BP significantly decreased after RTX injection (systolic BP rebound: pre-RTX 25.39 ±6.07 %, post-RTX 7.35 ±8.58 %, p=0.005; diastolic BP rebound: pre-RTX 18.74 ±5.59 %, post-RTX 6.12 ±8.52 %, p=0.02) (Supplemental Figure 2D). The abrupt elevation in systolic BP that followed was blunted after RTX (Supplemental Figure 1B). Oscillations in HR and BP during apnea still occurred after RTX (Supplemental Figure 3) with identical phasic correlations between vagal firing and HR and BP oscillations.

Protocol 2

Nine dogs initiated protocol 2, however, during baseline apnea induction (pre-RTX), 1 dog showed profound bradycardia and hypotension requiring resuscitation. This case was hence excluded from the series. Another dog suffered ventricular fibrillation and cardiac arrest after epicardial RTX infusion but could not be resuscitated and was excluded from analysis as well. In the remaining seven dogs, the protocol was completed as described.

Electrophysiological response to apnea before RTX administration

Effective refractory period in each site. Atrial ERP (AERP) decreased during apnea from 118 ±35.1 ms to 83 ±25.8 ms (p=0.006) in RSPV, from 112 ±33.65 ms to 106 ±35.1 ms (p=0.08) in LSPV and from 106 ±26.1 ms to 90 ±19 ms (p=0.04) in RIPV.

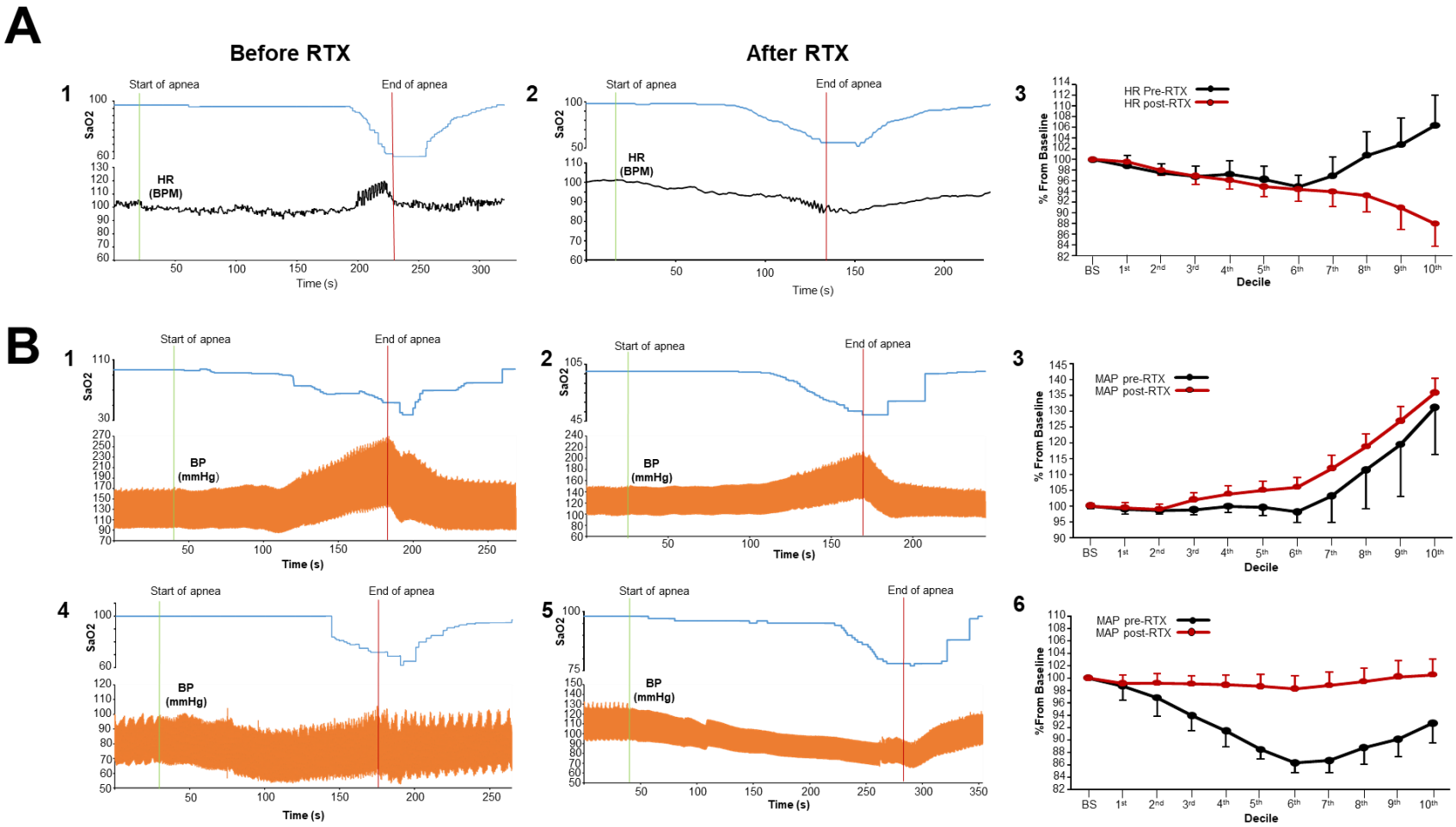
Electrophysiological response to apnea after RTX administration

Post-RTX apnea induced significant *increases* in AERP in different sites. It changed from 100.7 ±16.7 ms to 122.9 ±17.3 ms (p=0.1) in RSPV, from 117.5 ±22.8 ms to 132.5 ±21.6 ms (p=0.1) in LSPV and from 108.57 ±31.4 ms to 126.4 ±40.9 ms (p=0.006) in RIPV (manuscript Figure 7). The eupneic AERP was unchanged from baseline (110.2±31.3 vs 107.6±23.9, p=0.9), which suggests a lack of RTX-induced changes in eupneic myocardial refractoriness.

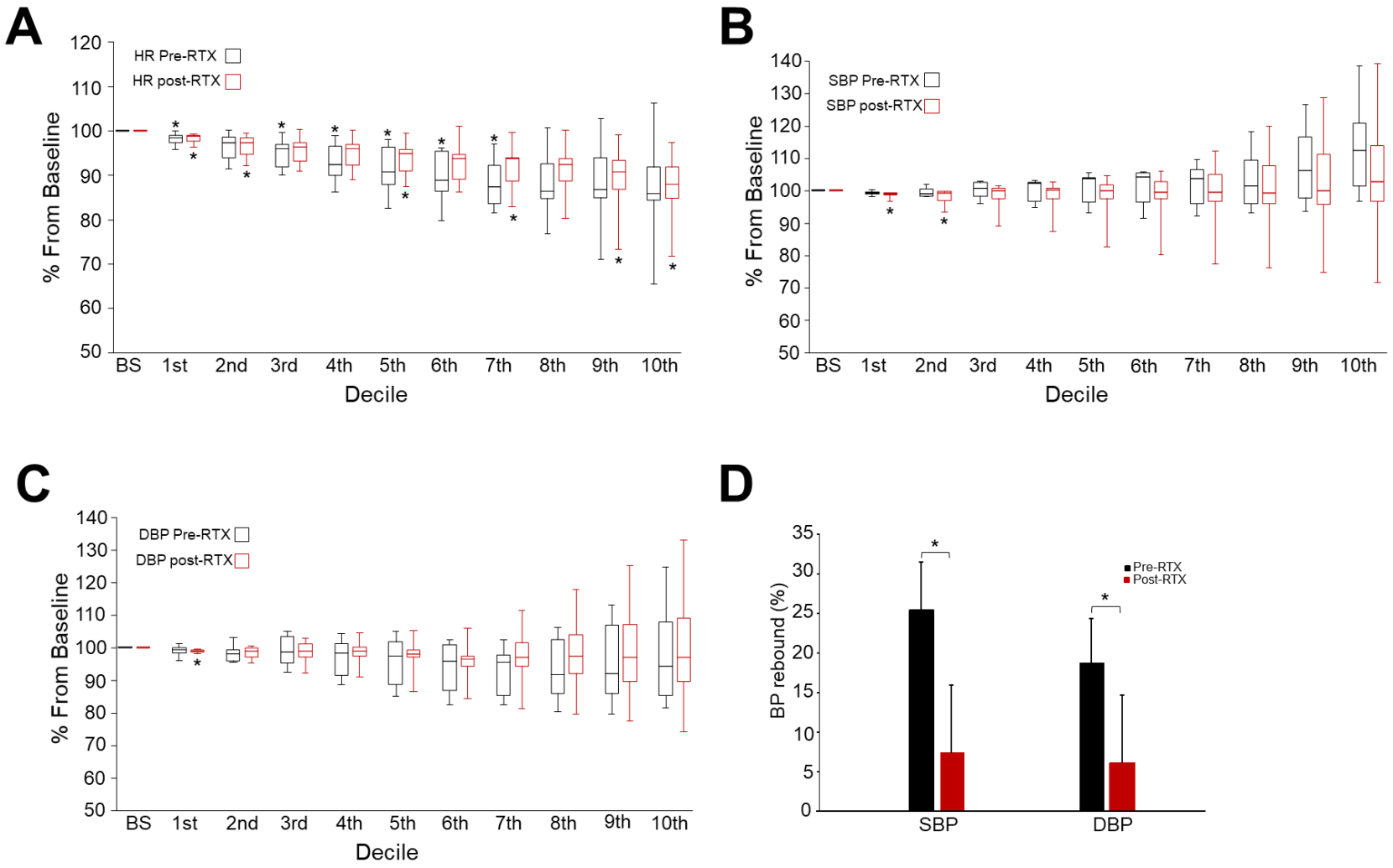
Acute RTX-induced hemodynamic changes

The increases in BP were similar to local anterior right GP injection (protocol 1). Figure 5 shows RTX-induced changes caused by pericardial injection, including electrocardiographic changes recorded. Eight dogs had T wave inversion and three developed ventricular fibrillation, successfully resuscitated in 2. Time from RTX administration to re-normalization of BP and HR was similar to protocol 1.

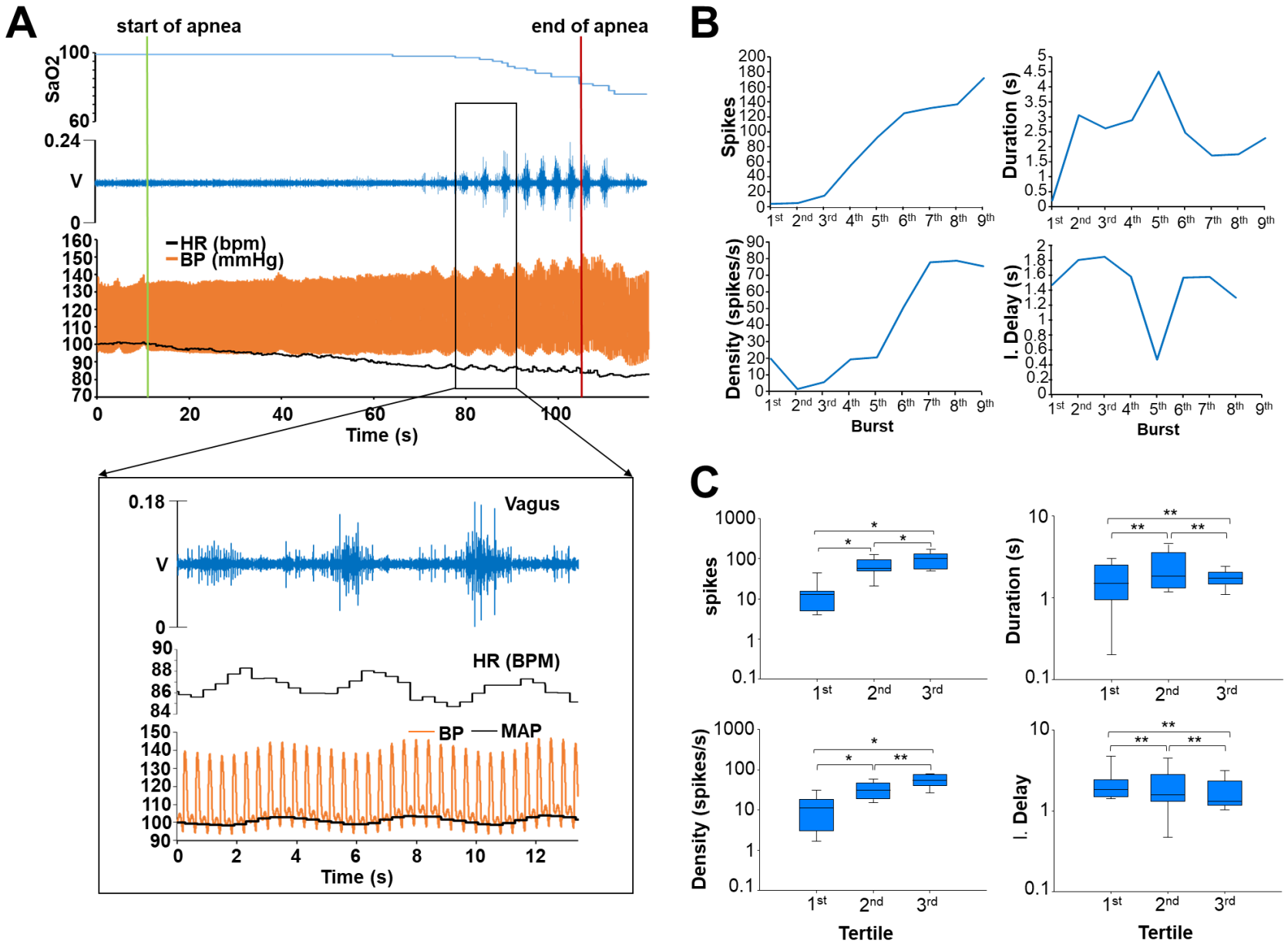
Hemodynamic parameter variation following RTX infusion is detailed in Figure 5.


A
Before RTX
After RTX
1
Start of apnea
End of apnea
SaO2
HR
(BPM)
Time (s)
2
Start of apnea
End of apnea
SaO2
HR
(BPM)
Time (s)
3
HR Pre-RTX
HR post-RTX
% From Baseline
BS
1st
2nd
3rd
4th
5th
6th
7th
8th
9th
10th
Decile
B
1
Start of apnea
End of apnea
SaO2
BP
(mmHg)
Time (s)
2
Start of apnea
End of apnea
SaO2
BP
(mmHg)
Time (s)
3
MAP pre-RTX
MAP post-RTX
% From Baseline
Decile
4
Start of apnea
End of apnea
SaO2
BP
(mmHg)
Time (s)
5
Start of apnea
End of apnea
SaO2
BP
(mmHg)
Time (s)
6
MAP pre-RTX
MAP post-RTX
%From Baseline
Decile

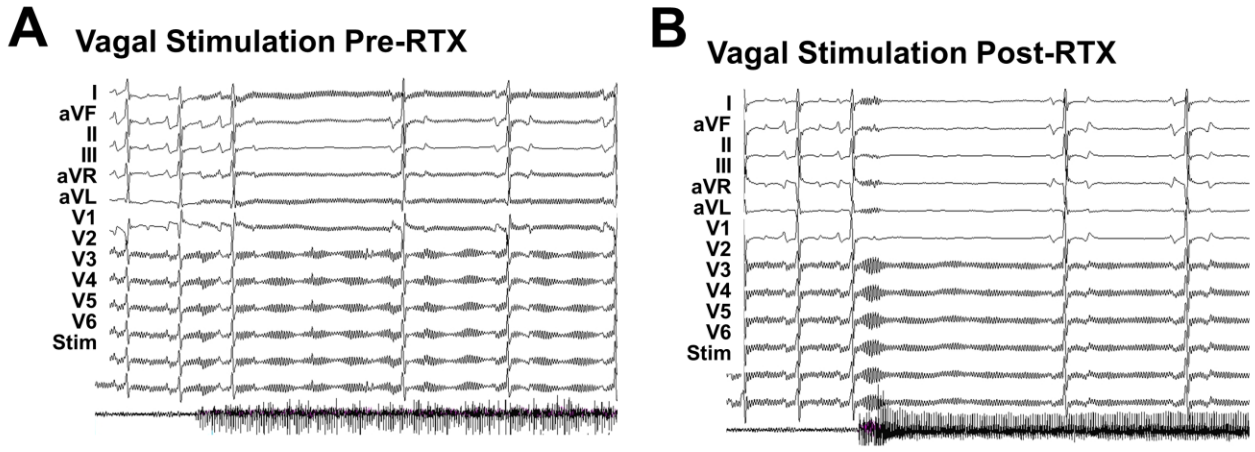
Supplemental Figure 1. Hemodynamic responses to apnea illustrating the inter-individual variability. A. An example of a HR change during apnea, before and after RTX. B. Examples of BP changes during apnea before and after RTX. BP, blood pressure; HR, heart rate; MAP, mean arterial pressure; RTX, resiniferatoxin; SaO2, oxygen saturation.


A
HR Pre-RTX
HR post-RTX
% From Baseline
120
110
100
90
80
70
60
50
BS
1st
2nd
3rd
4th
5th
6th
7th
8th
9th
10th
Decile
B
SBP Pre-RTX
SBP post-RTX
% From Baseline
140
130
120
110
100
90
80
70
60
50
BS
1st
2nd
3rd
4th
5th
6th
7th
8th
9th
10th
Decile
C
DBP Pre-RTX
DBP post-RTX
% From Baseline
140
130
120
110
100
90
80
70
60
50
BS
1st
2nd
3rd
4th
5th
6th
7th
8th
9th
10th
Decile
D
BP rebound (%)
35
30
25
20
15
10
5
0
Pre-RTX
Post-RTX
SBP
DBP

Supplemental Figure 2. Hemodynamic response to apnea divided by deciles before (black line) and after (red line) local RTX injection. A. Changes in HR. A decrease in HR during apnea was seen in all deciles. The highest decrease was seen at decile 7, 12% (P25 17.9, P75 6.2), p<0.001. B, C and D. Changes in BP. An abrupt elevation in BP occurred towards the end of the apneic episodes. The most salient apneic hemodynamic change after RTX injection was the decrease in both SBP and DBP rebound (SBP rebound: pre-RTX 25.39 ±6.07 % vs post-RTX 7.35 ±8.58 %, p=0.005; DBP rebound: pre-RTX 18.74 ±5.59 % vs post-RTX 6.12 ±8.52 %, p=0.02). HR, SBP and DBP percentage variation from baseline. A, B and C data expressed as median and interquartile range. *, statistical significance set at P<0.005 after the Bonferroni correction. D, data expressed as mean ± SD,* P<0.05. DBP, diastolic blood pressure; SBP, systolic blood pressure. Other abbreviations as in Supplemental Figure 1


A
start of apnea
end of apnea
SaO2
100
60
0.24
V
0
HR (bpm)
BP (mmHg)
160
150
140
130
120
110
100
90
80
70
0
20
40
60
80
100
Time (s)
0.18
V
0
Vagus
90
88
86
84
HR (BPM)
150
140
130
120
110
100
90
BP
MAP
0
2
4
6
8
10
12
Time (s)
B
Spikes
Duration (s)
Density (spikes/s)
I. Delay (s)
1st 2nd 3rd 4th 5th 6th 7th 8th 9th
Burst
C
spikes
Duration (s)
Density (spikes/s)
I. Delay
1st 2nd 3rd
Tertile

Supplemental Figure 3. Vagal activity during apnea after RTX injection. Vagal activity after RTX did not change. The crescendo phasic bursts of discharges during apnea were preserved as SaO2 declined. Data expressed as median and interquartile range. *, Statistical significance set at $p<0.017$ after the Bonferroni test. **, non-statistically significant. I. delay, interburst delay; other abbreviations as in Supplemental Figures 1 and 2.


A
Vagal Stimulation Pre-RTX
I
aVF
II
III
aVR
aVL
V1
V2
V3
V4
V5
V6
Stim
B
Vagal Stimulation Post-RTX
I
aVF
II
III
aVR
aVL
V1
V2
V3
V4
V5
V6
Stim

Supplemental Figure 4. Twelve-lead ECG showing the bradycardic response to high-frequency stimulation of the right and left vagal nerve (20 Hz, 0.1 ms, voltage 0.6 to 4.5 V) after RTX injection. Vagal response to apnea after RTX injection was unaltered. RTX, resiniferatoxin.

Supplemental References

1. Wang HJ, Wang W, Cornish KG, Rozanski GJ, Zucker IH. Cardiac sympathetic afferent denervation attenuates cardiac remodeling and improves cardiovascular dysfunction in rats with heart failure. *Hypertension*. 2014;64:745-55.
2. Liu P, Guo JH, Zhang HC, Wang MX, Li XB, Zhang P, Yi Z, Sun JL. Vagal effects on the occurrence of focal atrial fibrillation originating from the pulmonary veins. *Circ J*. 2009;73:48-54.