Chronic Intermittent Low-Level Stimulation of Tragus Reduces Cardiac Autonomic Remodeling and Ventricular Arrhythmia Inducibility in a Post-Infarction Canine Model

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ABSTRACT

OBJECTIVES This study investigated whether chronic low-level tragus stimulation (LL-TS) inhibits cardiac sympathetic remodeling and reduces ventricular arrhythmia inducibility in a post-infarction canine model.

BACKGROUND Low-level vagal stimulation has been shown to suppress cardiac sympathetic activity, which plays an important role in ventricular arrhythmia after myocardial infarction (MI). Our previous studies reported a noninvasive approach to deliver vagal stimulation by transcutaneous stimulation at the tragus, where the auricular branch of the vagus nerve is located.

METHODS Twenty-two beagles were randomized to the normal control (n = 6), MI (left anterior descending coronary artery ligation without LL-TS [n = 8]), and TS (MI plus LL-TS [n = 8]) groups. LL-TS was delivered 2 h each day at 80% below the threshold which slowed sinus rate.

RESULTS At 2-month follow-up, LL-TS was found to significantly reduce ventricular arrhythmia inducibility (arrhythmia score: 1.8 ± 0.8 vs. 3.6 ± 0.7, p < 0.01, compared to the MI group), decreased left stellate ganglion (LSG) activity (frequency: 32 ± 15 vs. 112 ± 29 impulses/s; and amplitude: 0.15 ± 0.12 mV vs. 0.38 ± 0.12 mV, compared to MI group), and attenuated cardiac sympathetic remodeling induced by chronic MI. The nerve growth factor (NGF) protein was down-regulated, whereas the small conductance calcium-activated potassium channel type2 (SK2) protein was up-regulated in the LSG by chronic LL-TS.


Ventricular arrhythmia remains a major cause of sudden cardiac death in patients with myocardial infarction (MI) (1). Cardiac sympathetic nerve sprouting, especially in the left stellate ganglion (LSG), significantly increased sudden cardiac death in dogs with MI (2–6). Cardiac sympathetic denervation by LSG resection was associated with a reduction in the incidence of ischemia-induced...
ventricular arrhythmia in both animal models and humans (7,8). Continuous vagal stimulation reduced ventricular arrhythmia and prevented sudden cardiac death in conscious dogs with healed MI (9). These data indicated that increased cardiac sympathetic tone played an important role in the generation of ventricular arrhythmia after MI and that inhibition of the cardiac sympathetic tone may prevent ventricular arrhythmia (10). Previous studies showed that low-level vagus nerve stimulation (11-13) at voltages that did not slow the sinus rate suppressed atrial fibrillation (AF) inducibility and shortened AF duration by inhibiting the activity of the cardiac autonomic nervous system and LSG. Similar antiarrhythmic effects can be accomplished by delivering low-level electrical stimulation to the tragus, where the auricular branch of the vagus nerve is located (14). Thus, we hypothesized that low-level tragus stimulation (LL-TS) may suppress cardiac sympathetic remodeling and reduce ventricular arrhythmia inducibility in a post-infarction canine model.

METHODS

ANIMAL PREPARATION AND STUDY PROTOCOL. Twenty-two beagles weighing 15 to 17 kg each were included in this study. The study was reviewed and approved by Wuhan University and conformed to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (number 85-23, revised 1996). The protocol was approved by the Animal Ethics committee of Wuhan University under approval number 2014-0256. All surgery was performed with the dogs under anesthesia by 3% sodium pentobarbital at an initial dose of 30 mg/kg and a maintenance dose of 60 mg/h. Body surface electrocardiograms were recorded during the entire procedure by using a computer-based Lab System (Lead 2000B, Jingjiang Inc., Chengdu Cidy, China), and the core body temperature of the dogs was maintained at 36.5 ± 1.5°C.

The MI model was established by ligating the left anterior descending coronary artery (LAD) below its first diagonal branch with a 4-0 proline suture. Twenty-two beagles were randomly allocated to 1 of 3 groups, as follows: the normal control (NC) group (LAD separation but without ligation [n = 6]), the TS group (LAD ligation followed by LL-TS for 2 h per day for 2 months [n = 8]), and the MI group (LAD ligation but without LL-TS [n = 8]).

CHRONIC INTERMITTENT LL-TS. Based on our previous studies (14,15), 2 alligator clips were attached to the tragus of the right ear, and incremental voltages were applied to the tragus (20 Hz, 1-ms square wave) until slowing of the sinus rate was achieved. The threshold for setting the LL-TS, which was defined as the voltage required to slow the sinus rate, was measured at baseline and at the end of every week for 2 weeks. After MI induction, LL-TS, set at 80% below the threshold with duty cycle of 5 s on and 5 s off, was delivered to the right tragus of dogs in the TS group for 2 h a day for 2 months (14,15).

NEURAL RECORDING. Neural activity from the LSG was recorded for 5 min at baseline during the first electrophysiology study and 2 months later during the second electrophysiology study, and then the best 1-min segment was analyzed at each time point. Tungsten-coated microelectrodes were inserted into the fascia of the LSG, and a ground lead was connected to the chest wall. Electrical signals generated by the LSG was recorded with a Power Lab data acquisition system (product 8/35, AD Instruments, Sydney, Australia) and amplified (product DP-304, Warner Instruments, Hamden, Connecticut) with band-pass filters set at 300 to 1 kHz and with an amplification range of 30 to 50 times. The neural activity, characterized by the recorded amplitude and frequency, was defined as deflections with a signal-to-noise ratio greater than 3:1 and manually determined as described in our previous studies (11,16).

MAP RECORDING AND CONSTRUCTION OF THE APD RESTITUTION CURVES. Monophasic action potential (MAP) was recorded at the remote zone and the peri-infarct zone, which was approximately 2 cm away from the infarct zone, at baseline and 2 months later, using a custom-made Ag-AgCl catheter. The MAP electrogram was filtered at 1 to 1,200 Hz. A dynamic steady-state pacing protocol (S1S1) was performed to determine the action potential duration (APD) alternant. The pulse train was delivered at an initial cycle length slightly shorter than the sinus cycle length, a drive train of stimuli was delivered for 30 s, interrupted by a 2-min pause between drive trains. Subsequently, another pulse train with the pacing cycle length shortened in a stepwise fashion by 10 ms was delivered until APD alternans occurred. APD alternans was defined as ΔAPD90 of ≥10 ms for ≥5 consecutive beats. The MAP recordings were analyzed by using LEAD 2000B workstation system (Lead 2000B, Jingjiang Inc., China). APD90 was defined as the...
90% repolarization duration, and the diastolic interval (DI) was defined as the time interval from the end of repolarization time of the current beat to the activation time of the following beat. The dynamic APD restitution curves were constructed from the DI and APD$_{90}$ by using Origin 8.0 (OriginLab, Co., Northampton, Massachusetts) as previously described (17,18). Slope of the shortest diastolic interval was defined as the maximal slope (S$_{\text{max}}$) of the restitution curve.

**PROGRAMMED VENTRICULAR STIMULATION.** A quadrripolar electrode catheter was sewed onto the right ventricular apex. Programmed ventricular stimulation at twice the diastolic threshold with a drive train (S1S1) of 8 beats at 350 ms followed by ≤3 extra-stimulus (19) was performed using a programmable stimulator (Lead 2000B, Jingjiang Inc., China). Initial extra-stimulus was conducted with an S1-S2 coupling interval of 30 ms longer than the ventricular effective refractory period and then decreased in 10 ms steps to ventricular refractoriness. Another extra-stimulus (S3) was delivered if single extra-stimuli did not induce sustained monomorphic ventricular tachycardia. Ventricular arrhythmia, including ventricular tachycardia and ventricular fibrillation, was considered sustained when it lasted >15 beats. An arrhythmia scoring system was modified as previously described (20,21). Arrhythmia was scored as follows: 0, noninducible preparations; 1, nonsustained tachyarrhythmias induced with three extrastimuli; 2, sustained tachyarrhythmias induced with three extrastimuli; 3, nonsustained tachyarrhythmias induced with 2 extrastimuli; 4, sustained tachyarrhythmias induced with 2 extrastimuli; 5, nonsustained tachyarrhythmias induced with 1 extra-stimulus; 6, sustained tachyarrhythmias induced with one extrastimulus; and 7, tachyarrhythmias induced during the eight paced beats. If the heart stopped before the pacing, the arrhythmia score assigned to that heart was 8. When multiple forms of arrhythmias occurred in 1 heart, the highest score was used. The experimental protocols were typically completed within 10 min.

**HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDIES.** In all animals, fresh tissues from the left ventricular peri-infarct myocardium and the cranial half of LSG were quickly excised and fixed in 4% formalin for 1 h, followed by storage in 70% alcohol for immunohistochemical studies. Subsequently, multiple tissue paraffin blocks were sampled and processed. The immunohistochemical avidin-biotin complex method was used for immunostaining with antibodies for tyrosine hydroxylase (TH) (1:100 dilution, Abcam, Cambridge, Massachusetts), growth-associated protein-43 (GAP43) (1:300 dilution, Life Technologies, Grand Island, New York) and synaptophysin (SYN) (1:50 dilution, Life Technologies). All immunoreactivity was quantified using commercial software (ImagePro, Media Cybernetics, Inc., Rockville, Maryland). The nerve density based on the immunoreactivity of each slide was determined by the average of 3 fields with the highest nerve density. Nerve density was expressed as the total area of positive staining per square millimeter (μm$^2$/mm$^2$) (22).

**WESTERN BLOTTING.** Peri-infarct cardiac tissues and the caudal half of LSG were used for Western blotting to quantify the amount of protein. Equal amounts (60 μg) of homogenate proteins were loaded on 4 to 20% gradient Minigels (CPL, Austin, Texas) and transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, California). Membranes were blocked with 5% nonfat dry milk in PBST (containing 0.05% Tween 20) and incubated overnight at 4°C with the primary antibody (NGF: 1:200 dilution; Santa Cruz Biotechnology, Dallas, Texas; antisubtype 2 of small conductance calcium-activated potassium channels [SK2]; 1:600 dilution; Abcam, Cambridge, Massachusetts) polyclonal antibodies. Antibody-binding protein bands were visualized by iodine-125–labeled protein and quantified with a Personal FX phosphorimager (Bio-Rad), not by film-based densitometry. We adjusted the exposure to ensure that the protein bands were not saturated. The same Western blots were reprobed with anti-GAPDH (1:5,000 dilution; Research Diagnostics Inc., Flanders, New Jersey) to normalize gel loading. Every blot was conducted in triplicate, and the mean of the intensity value was seen as the value for the tissue.

**STATISTICAL ANALYSIS.** All continuous data are mean ± SD. Parameters were compared among the NC, MI, and TS groups, using 1-way analysis of variance (ANOVA) followed by Dunnnett’s t test. Prism version 5.0 software (GraphPad Software, Inc., San Diego, California) was used to evaluate the data. A 2-tailed p value of ≤0.05 was considered significant.

**RESULTS**

The average stimulation threshold, which was required to induce any slowing of the sinus rate, was 10.2 ± 3.2 V. The heart rate was maintained at a stable...
level without significant change during 2 months of LL-TS. There was no significant change in voltage levels of tragus stimulation over 2 months, indicating that there was no injury of the tragus. There were also no significant differences in sinus rate, PR intervals, QRS durations, and corrected QT (QTc) intervals among the 3 groups at baseline and after 2-month follow-up (Table 1).

**LL-TS REDUCED THE INDUCIBILITY OF VENTRICULAR ARRHYTHMIA.** After 2 months of follow-up, as shown in Figure 1A and 1B, $S_{\text{max}}$ in the MI group was $>1$

### Table 1: Electrocardiogram Parameters in the 3 Groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>2-Month Follow-up</th>
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<tbody>
<tr>
<td></td>
<td>NC</td>
<td>MI</td>
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<tr>
<td>Sinus rate, beats/min</td>
<td>139 ± 9</td>
<td>135 ± 7</td>
</tr>
<tr>
<td>PR interval, ms</td>
<td>80 ± 5</td>
<td>82 ± 6</td>
</tr>
<tr>
<td>QRS duration, ms</td>
<td>59 ± 5</td>
<td>57 ± 8</td>
</tr>
<tr>
<td>QTc interval, ms</td>
<td>352 ± 10</td>
<td>350 ± 8</td>
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Values are mean ± SD.

MI = myocardial infarction; NC = normal control; TS = tragus stimulation.
both in the peri-infarcted zone and in the infarcted zone (1.24 ± 0.43 and 1.31 ± 0.36, respectively), whereas it was <1 in the NC group (0.63 ± 0.21 and 0.69 ± 0.25, respectively) and in the TS group (0.71 ± 0.32 and 0.78 ± 0.34, respectively). The duration of the alternans recordings was 175 ± 5.5 ms, 226.3 ± 7.4 ms, and 180 ± 7.6 ms in the NC group, the MI group, and the TS group, respectively. In addition, APD alternans occurred at a significantly longer pacing cycle length in the MI group than in the NC or the TS group (Figures 1C and 1D). Furthermore, Figure 1E shows the representative examples of programmed ventricular stimulation-induced ventricular arrhythmias, and Figure 1F shows that the ventricular arrhythmia inducibility score was significantly increased by MI but attenuated by chronic LL-TS.

**LL-TS ATTENUATED THE ACTIVATION OF LSG NEURAL ACTIVITY.** Figure 2 shows typical examples of LSG nerve activity recorded at baseline (Figure 2A) and at 2 months follow-up (Figure 2B) in these 3 groups. No significant differences were shown among groups at baseline. Compared with group baseline measurements, no significant changes were shown at 2 months later in the NC group (29 ± 12 impulses/min vs. 32 ± 17 impulses/min; amplitude: 0.08 ± 0.04 mV vs. 0.09 ± 0.04 mV; both: p < 0.05), whereas a significant increase was shown in MI group frequency (32 ± 15 impulses/min vs. 112 ± 29 impulses/min; amplitude: 0.09 ± 0.04 mV vs. 0.38 ± 0.12 mV; both: p < 0.05), and a slight but not significant change was shown in the TS group (frequency: 30 ± 14 impulses/min vs. 42 ± 14 impulses/min; amplitude: 0.09 ± 0.03 mV vs. 0.15 ± 0.09 mV, both p > 0.05).

**LL-TS SUPPRESSED CARDIAC SYMPATHETIC NERVE SPROUTING AND DECREASED SYNAPTIC DENSITY.** Figure 3 shows typical examples of TH and GAP43 staining in the peri-infarct myocardium (Figure 3A) and LSG (Figure 3B). Compared with the NC group, the immunoreactivity levels of both TH and GAP43 in the peri-infarct myocardium and LSG increased in the MI group, which was attenuated in the TS group (Figures 3C and 3D). Similarly, the synaptic density represented by SYN was also increased in the MI group and attenuated in the TS group (Figure 4). The density of SYN immunoreactivity in the peri-infarct myocardium was 1,523.4 ± 235.7, 819.8 ± 214.5, and 1,021.3 ± 312.3 μm²/mm² in the MI, NC, and TS group, respectively. The density of SYN immunoreactivity in the LSG was 35,789.6 ± 8,714.2, 19,232.3 ± 5,612.7, and 23,123.7 ± 5,113.2 μm²/mm² in the MI, NC, and TS group, respectively.
**DISCUSSION**

**MAJOR FINDINGS.** The present study showed that chronic LL-TS produced the following significant effects: 1) decreased the slope of APD restitution curves and suppressed APD alternant and reduced ventricular arrhythmia inducibility; 2) suppressed LL-TS MODULATED NGF AND SK2 EXPRESSION. Representative examples of Western blots obtained from the LSG in the NC, MI, and TS groups are shown in Figures 5A and 5B. No significant differences in GADPH density were found among groups. Corresponding mean values normalized to GADPH indicated that the protein level of NGF in the TS group was significantly lower than that in the MI group (1.14 ± 0.29 vs. 2.45 ± 0.50, p < 0.01) (Figure 5C). However, the level of SK2 protein in the TS group was significantly higher than that in the MI group (0.89 ± 0.20 vs. 0.54 ± 0.15, respectively, p < 0.01) (Figure 5D).
Synaptic density is shown within the peri-infarcted myocardium and LSG (brown stain, black arrows) at ×20 magnification. (A) Representative images of SYN in peri-infarcted myocardium (upper) and LSG (lower) are shown. (B) Quantitative differences are shown between SYN in the peri-infarcted myocardium and (C) SYN in LSG. *p < 0.05 compared to the NC group; §p < 0.05 compared to the MI group. SYN = synaptophysin; other abbreviations as in Figures 1 and 2.

A and B show representative Western blot images of NGF and SK2; C and D show corresponding quantification analyses. Compared to the NC group, there was a significant downregulation of NGF and upregulation of SK2 in the TS group. *p < 0.05 compared to the NC group; §p < 0.05 compared to the MI group. AU = arbitrary unit; GAPDH = glyceraldehydes-3-phosphate dehydrogenase; NGF = nerve growth factor; SK2 = small conductance calcium-activated potassium channel type 2.
LSG activity; 3) ameliorated sympathetic neural sprouting within myocardium and LSG; and 4) down-regulated NGF and up-regulated SK2 protein expression in LSG.

**LL-TS Suppressed Susceptibility to Ventricular Arrhythmia After MI.** It is well known that a decrease in sympathetic tone or an increase in parasympathetic tone protects MI-related ventricular arrhythmia and mortality (9). The APD restitution hypothesis asserts that a steep restitution slope (>1) facilitates, whereas a flattened restitution slope (<1) inhibits the initiation and maintenance of malignant ventricular arrhythmia (e.g., ventricular fibrillation) (23). Moreover, APD alternant, which occurs more easily when the restitution slope is >1, is also a predictor of ventricular fibrillation (24,25). A reduction of the slope of APD restitution curves and APD alternant by LL-TS suggested that LL-TS may increase the ventricular electrophysiological stability and exert a protective effect on ventricular arrhythmia.

**LL-TS Attenuated Autonomic Remodeling in the Ventricle and LSG.** It is well known that there was a positive correlation between myocardial sympathetic nerve sprouting and the incidence of ventricular arrhythmia, indicating that increased myocardial sympathetic innervation was indeed an important factor contributing to ventricular arrhythmogenesis (26). Autonomic remodeling after MI often presents as cardiac sympathetic nerve sprouting and hyperinnervation as well as postganglionic adrenergic axons and preganglionic cholinergic axons hyperinnervation in the stellate ganglion. Increased LSG neural activities, resulting in greater intramyocardial catecholamine release, were immediate triggers of malignant ventricular arrhythmia (26–28). Previous studies demonstrated similar antiarrhythmic effects between low-level vagal stimulation and LL-TS (11–13,16). Continuous low-level vagal stimulation reduced LSG activity and paroxysmal atrial tachyarrhythmia in ambulatory canines, the primary antiarrhythmic effects of which resulted from inhibition of the sympathetic neural activity (13). In the present study, a reduced LSG activity as well as reduced cardiac sympathetic sprouting both in the myocardium and in the LSG in the LL-TS group may translate into a reduced incidence of ventricular arrhythmia.

**Possible Mechanisms Underlying the Inhibitory Effect of LL-TS on LSG Activity and Neural Remodeling.** To further explore the neuromodulatory mechanisms of chronic LL-TS, we measured expression levels of the SK2 and NGF proteins. SK2, an ion channel molecule responsible for after-hyperpolarization that suppresses nerve discharges, plays a vital role in regulating sympathetic nerve activities (29). Shen et al. (13,29) showed that a reduction in LSG activity by chronic low-level vagal stimulation was consistent with the time course of up-regulating SK2, suggesting that SK2 contributes to the reduced sympathetic tone caused by low-level vagal stimulation. The present study also showed up-regulation of SK2 and a reduction in neural activity of LSG after chronic LL-TS, indicating that up-regulation of SK2 by chronic LL-TS may be responsible for the reduced LSG activity.

As a member of the neurotrophin family, NGF is recognized as a novel biomarker in MI for its critical role in the differentiation, survival, and synaptic activity of sympathetic nerves after MI. Suppression of NGF significantly decreased GAP43- and TH-positive nerve fiber density, thereby inhibiting sympathetic nerve remodeling (30). In our study, down-regulation of NGF induced by chronic LL-TS may partially explain the attenuated sympathetic nerve sprouting and hyperinnervation in the TS group. Our findings indicate that up-regulation of the SK2 protein and down-regulation of the NGF protein may underlie the inhibitory effect of chronic LL-TS on LSG nerve activity, neural remodeling, and antiarrhythmic effects.

It is of great significance that long-term neuromodulation and antiarrhythmia effects can be achieved by daily LL-TS for only 2 h. Long-term depression induced by a short period of repeated stimulation of the preganglionic nerves is a form of synaptic plasticity and an activity-dependent weakening of synaptic strength widely observed at many regions of the nervous system (31). Alkadhi et al. (32) also showed that long-term depression can be induced in autonomic ganglia by electrical stimulation (3 to 5 Hz/15 min) of the preganglionic nerve. Zhou et al. showed that electroacupuncture for minutes can reduce ischemia-reperfusion-related ventricular arrhythmia by mitigating structural remodeling following MI, and the effect outlasts the duration of conditioning stimulation by hours. Therefore, we hypothesized that long-term depression may be a potential mechanism for the long-lasting neuromodulatory and antiarrhythmic effects of LL-TS delivered only 2 h a day. However, the long-term effects of LL-TS are unknown, and an evaluation of more extended periods of LL-TS is warranted.
STUDY LIMITATIONS. Because of the limit in neural recording instruments, we only recorded nerve activity in the dogs under anesthesia for 5 min without continuously recording the neural activity over 2 months. Progressive changes in the cardiac autonomic nervous system can only be extrapolated by the 2 data points (baseline and 2 months). Spontaneous arrhythmias were not recorded after MI though it will be more persuasive than programmed stimulation induced arrhythmia. We only used right tragus for stimulation. Further studies are warranted to evaluate the potential differences in the inhibition of LSG activity and antiarrhythmic effects for left-sided versus right-sided or bilateral stimulation.

CONCLUSIONS

Chronic LL-TS was capable of suppressing ventricular arrhythmia inducibility, LSG activity, sympathetic remodeling, and inflammation in a canine model of chronic MI. Up-regulating SK2 protein and down-regulating NGF protein within the LSG may be one of the mechanisms underlying the neuromodulatory effects of chronic LL-TS. LL-TS may serve as a noninvasive approach to reduce the incidence of ventricular arrhythmia in patients with prior MI.

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REFERENCES


PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE:

Malignant VA is the main cause of sudden cardiac death in post-infarction patients. Cervical vagus nerve stimulation is an emerging neuromodulatory therapy to treat heart failure patients, but the invasiveness of this procedure and its complications may limit its application. This disadvantage, however, may be overcome by a noninvasive approach such as LL-TS that may serve as a new approach to prevent VA and possibly sudden cardiac death in patients who have had MI. Notably, LL-TS exerted powerful antiarrhythmic effects when delivered to each animal for only 2 h a day at a substantially low voltage (80% below the threshold of slowing sinus rate). This approach is potentially tolerable by ambulatory patients.

TRANSLATIONAL OUTLOOK: The present study and our previous work indicate that LL-TS could attenuate cardiac sympathetic remodeling; suppress ventricular arrhythmia inducibility; and improve cardiac fibrosis, inflammation response, and cardiac dysfunction in conscious dogs with healed MI. There is also a clinical study showing that LL-TS could suppress atrial fibrillation and decrease inflammation cytokines in patients with paroxysmal atrial fibrillation. In consideration of the ability of LL-TS to modulate autonomic nervous system and inflammatory response, several other cardiac conditions, such as myocardial ischemia-reperfusion injury, resistant ventricular electrical storm, and long QT type 1, may also benefit from this technique. In addition, several noncardiologic conditions associated with autonomic dysfunction, such as metabolism syndrome, obstructive sleep apnea, and renal failure, may also be treated using LL-TS, which might help to restore autonomic imbalance and reduce the unacceptably high cardiovascular risk among this group of patients.

KEY WORDS left stellate ganglion, neural remodeling, tragus stimulation, ventricular arrhythmia