Antiarrhythmic and proarrhythmic effects of subcutaneous nerve stimulation in ambulatory dogs

Short title: Stimulus strength and subcutaneous nerve stimulation

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Abstract

Background: High output subcutaneous nerve stimulation (ScNS) remodels the stellate ganglia and suppresses cardiac arrhythmia.

Objective: To test the hypothesis that long duration low output ScNS causes cardiac nerve sprouting, increases plasma norepinephrine concentration and the durations of paroxysmal atrial tachycardia (PAT) in ambulatory dogs.

Methods: We prospectively randomized 22 dogs (11 males and 11 females) into 5 different output groups for 2 months of ScNS: 0 mA (sham) (N=6), 0.25 mA (N=4), 1.5 mA (N=4), 2.5 mA (N=4) and 3.5 mA (N=4).

Results: As compared with baseline, the changes of the durations of PAT episodes per 48 hours were significantly different among different groups (sham, -5.0±9.5 s; 0.25 mA 95.5±71.0 s; 1.5 mA, -99.3±39.6 s; 2.5 mA, -155.3±87.8 s and 3.5 mA, -76.3±44.8 s, p<0.001). The 3.5 mA group had greater reduction of sinus heart rate than the sham group (-29.8±15.0 bpm vs -14.5±3.0 bpm, p=0.038). Immunohistochemical studies showed that the 0.25 mA group had a significantly increased while 2.5 mA and 3.5 mA stimulation had a significantly reduced growth-associated protein 43 nerve densities in both atria and ventricles. The plasma Norepinephrine concentrations in 0.25 mA group was 5063.0±4366.0 pg/ml, which was significantly higher than other groups of dogs (739.3±946.3, p=0.009). There were no significant differences in the effects of simulation between males and females.

Conclusions: In ambulatory dogs, low output ScNS causes cardiac nerve sprouting, increases plasma norepinephrine concentration and the duration of PAT episodes while high output ScNS is antiarrhythmic.

Key words: Cardiac nerve sprouting, Immunostaining, neuromodulation, neurotoxicity

Introduction

The autonomic nervous system is important in cardiac arrhythmogenesis.[1] Neuromodulation methods have been used to control cardiac arrhythmias both in animal models and in humans.[2, 3] However, because of the invasiveness of many neuromodulation procedures, clinical translation has been difficult. To overcome the problems associated with invasive procedures, Po's laboratory invented a method to reduce atrial fibrillation (AF) inducibility by non-invasive transcutaneous electrical nerve stimulation (TENS) of the tragus of dogs and humans.[4, 5] Those studies showed the beneficial effects of acute AF suppression, but the chronic effects of TENS remain difficult to study in ambulatory animals in part due to the absence of appropriate equipment. We recently reported that subcutaneous nerve stimulation (ScNS) in ambulatory dogs can lead to stellate ganglion (SG) cell death, reduction of SG nerve activity (SGNA) and suppression of atrial fibrillation and spontaneous paroxysmal atrial tachycardia (PAT) episodes.[6, 7] Because skin is easily accessible, these methods can be readily translated to humans. Antiarrhythmic interventions have the potential of being proarrhythmic. Chronic low amplitude subthreshold electrical stimulation of the left SG in canine models can cause nerve sprouting and sudden cardiac death.[8] It is possible that subthreshold low output ScNS can have similar effects. We hypothesize that ScNS can remodel not only the SG but also cardiac innervation. While high output ScNS is antiarrhythmic, low output ScNS may be proarrhythmic by inducing cardiac nerve sprouting and sympathetic hyperinnervation. To test that hypothesis, we performed a prospective randomized study in normal dogs undergoing chronic ScNS. The dogs were randomized into 5 groups: sham, 0.25 mA (low output), 1.5 mA, 2.5 mA and 3.5 mA stimulation groups. The subcutaneous nerve activity (ScNA) and cardiac arrhythmias were chronically monitored through implanted radiotransmitters with electrodes under the skin.[9] The data were analyzed to test the hypothesis that very low output ScNS can cause cardiac nerve sprouting and increase the duration of PAT while high output stimulation has opposite effects.

Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Indiana University and the Methodist Research Institute and were conducted in compliance with the Guide for the Care and Use of Laboratory Animals. Detailed methods are included in the Supplement. The study protocols are presented in Figure 1. The stimulation parameters mimics that used in the ANTHEM-HF Trial.[10] Blood samples were obtained to determine plasma norepinephrine concentrations by a commercial ELISA kit (Alpco, Salem, NH). The dogs then underwent non-survival surgery under general anesthesia. The tissues were harvested for immunostaining and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. The nerve activities and heart rate were manually analyzed using custom-written software. In addition, we also compared the number of PAT episodes over 48 hours between baseline and final recording of the experiment. PAT was defined as an abrupt (>50 bpm/s) increase or decrease in the atrial rate to >200 bpm that persisted for at least 5 s. All values are expressed as mean ± Standard deviation (SD) or 95% confidence interval (CI). Analyses of variance with posthoc testing was performed to compare the means of multiple groups. A two-sided p value of ≤0.05 was considered as statistically significant.

Results

Stimulus strength and response

The strength of stimulation determined the heart rate and ScNA responses (Figure 2). Figure 2A shows a typical example of 0.25 mA stimulation, which did not change either the heart rate or the nerve activity. The absence of response is also typical for 1.5 mA stimulation (Figure 2B). In contrast, the 2.5 mA and 3.5 mA output stimulation may interrupt the ScNA and transiently reduce the heart rate in the immediate post-stimulation period (Figures 2C and 2D, respectively). Furthermore, the ScNA of channel 3 in 3.5 mA group (Figure 2D) typically

registered very low level of nerve activity after the second week of the stimulation, suggesting profound suppression of nerve activity in the stimulated nerve.

The effects of stimulus strength on weight (Panel A), heart rate (Panels B-D) and ScNA (Panel E) are shown in Figure 3. Figure 3A shows that all dogs except one in 0.25 mA group showed an increased body weight during the study, suggesting that ScNS did not suppress appetite in a vast majority of dogs. The body weight of all dogs increased from 25.55±4.58 Kg before surgery to 33.32±4.31 Kg by the end of the study. There were no significant differences of delta body weights among these five groups of dogs (sham, 6.95±3.97 Kg; 0.25 mA, 9.53±7.93 Kg; 1.5 mA, 6.88±3.32 Kg; 2.5 mA, 5.73±4.14 Kg and 3.5 mA, 10.23±2.01 Kg, p=0.599). Figure 3B shows that all dogs except one in 1.5 mA group demonstrated a reduced heart rate at the end of the study. The heart rate (bpm) reduced from 104.7±9.6 to 90.2±7.1 in sham ($p<0.001$), from 113.5±13.2 to 96.3±13.8 in 0.25 mA ($p<0.001$), from 94.5±17.4 to 81.0±5.0 in 1.5 mA (p=0.141), from 94.3±14.1 to 79.0±8.4 in 2.5 mA (p=0.043) and from 116.5±11.9 to 86.8±7.7 in 3.5 mA (p=0.029) groups. The heart rate (bpm) reduced more in 3.5 mA group (29.8±15.0) than in sham group (14.5±3.0, p=0.038) (Figure 3C). The percent heart rate reduction in 3.5 mA group was significantly larger than the sham group (24.95±10.00% vs 13.75±1.88%, p=0.025) (Figure 3D). There were no significant differences between the baseline and final recording of the aScNA of channel 1, channel 2 and channel 3 (Figure 3E).

Effects of stimulation strength on PAT episodes

Consistent with our previous studies, all dogs had spontaneous PAT episodes at baseline.[11] Figure 4A shows a typical example of PAT with heart rate reaching > 200 bpm and sudden offset. Both onset and offset are preceded by bursts of ScNA, which is consistent with that observed in PAT of human patients.[12] The episodes of PAT per 48 hours at baseline for sham, 0.25 mA, 1.5 mA, 2.5 mA and 3.5 mA were 10.8±6.7, 16.0±12.6, 11.5±4.0, 22.8±8.8 and 22.8±20.1, respectively. They were changed to 11.3±7.4 (p=0.296), 23.8±18.2 (p=0.141),

1.8±1.0 (p=0.019), 6.8±5.9 (p=0.022), and 14.8±18.4 (p=0.024) (Figure 4B). Figure 4C shows that the total durations of PAT episodes per 48 hours were 109.0±70.0 s, 178.3±114.0 s, 118.8±37.0 s, 211.5±100.9 s and 211.0±204.8 s for sham, 0.25 mA, 1.5 mA, 2.5 mA and 3.5 mA groups, respectively. Following stimulation, the PAT durations changed to 104.0±67.9 s (p=0.254), 273.8±183.5 s (p=0.047), 19.5±12.9 s (p=0.015), 56.3±52.9 s (p=0.038) and 134.8±165.2 s (p=0.042). These data show that the total durations of PAT were significantly increased in 0.25 mA group, but significantly reduced in 1.5 mA, 2.5 mA and 3.5 mA groups as compared with baseline.

Figure 4D shows that the differences (delta) of PAT episodes per 48 hours among the five groups were significantly different (sham, 0.5±1.0; 0.25 mA, 7.8±9.2; 1.5 mA, -9.8±4.2; 2.5 mA, -16.0±7.3; 3.5 mA, -8.0±3.8; p<0.001). There were significant differences between sham and 1.5 mA (p<0.001), 2.5 mA (p<0.001) and 3.5 mA (p<0.001) groups, consistent with antiarrhythmic effects. There was no significant difference between sham and 0.25 mA, (p=0.084).

Figure 4E shows the changes (delta) of total duration of PAT episodes in all groups of dogs. As compared with baseline, the delta per 48 hours were significantly different among different groups (sham, -5.0±9.5 s; 0.25 mA 95.5±71.0 s; 1.5 mA, -99.3±39.6 s; 2.5 mA, -155.3±87.8 s and 3.5 mA, -76.3±44.8 s, p<0.001). There was significant longer total duration of PAT episodes at 0.25 mA as compared with sham (p=0.008). In comparison, a statistically significant reduction of PAT durations was noted for 1.5 mA (p<0.001), 2.5 mA (p=0.003) and 3.5 mA (p=0.005) groups.

We have plasma available in 4 dogs in 0.25 mA group and in 9 dogs of all other groups (Figure 4F). The plasma norepinephrine levels were analyzed by a technician unaware of the grouping of the dogs. Because we do not have data on all dogs, we performed non-paired t tests between norepinephrine levels of 0.25 mA group versus all other dogs. The results

showed the norepinephrine levels were 5063.0±4366.0 pg/ml in 0.25 mA group (N=4), higher than 739.3±946.3 pg/ml in the other dogs (N=10, p=0.009). In the latter group of dogs, only two dogs in 1.5 mA group had concentrations > 1,000 pg/ml (1633.3 pg/ml and 3133.3 pg/ml).

Effects of stimulus strength on nerve sprouting

As shown in Figure 5A, there were growth-associated protein 43 (GAP43) positive nerves in left atrium (LA) and right atrium (RA) in all dogs studied. However, the GAP43 positive nerves were more abundant in 0.25 mA while less abundant in 3.5 mA groups than sham group. There were significant differences of GAP43 positive nerve structures as percentages of total LA tissue (sham, 0.60±0.11%; 0.25 mA, 1.03±0.43%; 1.5 mA, 0.46±0.07%; 2.5 mA, 0.44±0.10%; 3.5 mA, 0.35±0.09%, p=0.001). The 0.25 mA stimulation caused a significant (p=0.044) increase, while 2.5 mA (p=0.047) and 3.5 mA stimulation (p=0.005) caused significant reduction of nerve densities compared with sham control. GAP43 expression in the 1.5 mA stimulation group did not differ from sham (p=0.053, Figure 5B). Similarly, the relative GAP43 positive nerve densities of the RA were significantly different among groups (sham, 0.60±0.11%; 0.25 mA, 1.27±0.43%; 1.5 mA, 0.54±0.10%; 2.5 mA, 0.41±0.12% and 3.5 mA, 0.28±0.06%, p<0.001). Among them, 0.25 mA stimulation caused a significant (p=0.005) increase while the 2.5 mA stimulation (p=0.031) and the 3.5 mA stimulation (p<0.001) caused a significant reduction in GAP43 nerve density. No difference was detected between the 1.5 mA stimulation and sham control (p=0.363) (Figure 5C).

As shown in Figure 6A, there were GAP43 positive nerve structures in the left ventricle (LV) and the right ventricle (RV) in all dogs. The relative GAP43 positive nerve densities of the LV were significantly different among the five groups (sham, 0.33±0.14%; 0.25 mA, 0.56±0.07%; 1.5 mA, 0.32±0.04%; 2.5 mA, 0.22±0.07% and 3.5 mA, 0.13±0.05%, p<0.001). The 0.25 mA stimulation significantly (p=0.014) increased whereas the 3.5 mA stimulation significantly (p=0.024) reduced the GAP43-positive nerve twigs. The 1.5 mA stimulation

(p=0.928) and 2.5 mA stimulation (p=0.170) did not significantly change the nerve densities (Figure 6B). The relative GAP43 positive nerve structures when considered as a percentage of the RV were also significantly different among the five groups (sham, 0.30±0.08%; 0.25 mA, 0.44±0.10%; 1.5 mA, 0.22±0.06%; 2.5 mA, 0.27±0.06% and 3.5 mA, 0.16±0.03%, p<0.001). The 0.25 mA stimulation significantly (p=0.036) increased while the 3.5 mA stimulation significantly (p=0.014) decreased the nerve densities. No changes were observed for 1.5 mA (p=0.174) or 2.5 mA (p=0.582) groups (Figure 6C). As shown in Figures 5 and 6, the overall percentage of GAP43 in 3.5 mA group was less than that of the sham group. However, some parts of the ventricles do have GAP43-positive nerve fibers. These findings are consistent with heterogeneous responses to the electrical stimulation.

Stellate ganglion remodeling

Bilateral SG specimens were double stained for tyrosine hydroxylase (TH) (red) and TUNEL (green) (Figure 7A). No TUNEL positive cells were observed in sham group. TUNEL positive interstitial cells and neurons were observed in both SG in one dog (25%) of the 1.5 mA group and in two dogs (50%) of 3.5 mA group. A significant difference of delta heart rates among sham (N=6), TUNEL+ (N=3) and TUNEL- (N=13) dogs (sham, -14.5±3.0 bpm; TUNEL+, -36.7±12.5 bpm; TUNEL-, -14.9±7.4 bpm, p<0.001) was noted. Figures 7B-D show the relationship between TUNEL positivity and heart rate (B), changes (delta) heart rate (C) and % change of heart rate compared with baseline. There was a significant difference between sham and TUNEL+ group but no difference between sham and TUNEL- group.

Sex and the effects of ScNS

There were no significant differences in the effects of simulation between males and females regarding heart rate reduction (p=0.228), the percent heart rate reduction (p=0.162), the delta episodes of PAT per 48 hours (p=0.281), the percent changes of PAT frequencies per 48 hours

(p=0.744), the delta durations of PAT per 48 hours (p=0.078), the delta percentages of PAT duration changes per 48 hours (p=0.082) or the relative GAP43 positive nerve densities of LA (p=0.943), RA (p=0.835), LV (p=0.254) and RV (p=0.258).

Discussion

The salient findings of this study are that in ambulatory dogs, low output ScNS causes cardiac nerve sprouting, increases the plasma norepinephrine and increases the duration of PAT episodes. In comparison, high output ScNS suppresses PATs and reduces heart rate. Similar effects were observed in males and females.

Chronic stimulation and neural remodeling

High output electrical stimulation of the perforant path in the rat brain can cause epileptic brain damage. The ultrastructural changes are similar to glutamate-induced excitotoxicity.[13] Very low output electrical current too low to induce immediate physiological responses can cause nerve sprouting and the kindling model of epilepsy.[14] A possible molecular mechanism is that electrical stimulation can increase the expression of neurotrophic factors and their receptors.[15] The present study showed that there is significant atrial and ventricular nerve sprouting in the 0.25 mA group, which was accompanied by the increased plasma norepinephrine concentration and duration of PATs. The source of the sympathetic nerves of the thorax come primarily from the middle cervical ganglion and SG.[16, 17] The low output subthreshold electrical stimulation of the axons induced nerve sprouting in the SG, which then induced nerve sprouting in the atria and ventricles. Transneuronal induction of nerve sprouting is a well-documented phenomenon in the peripheral nervous system[18] and could explain the findings of the present study. There are several significant implications. First, low output ScNS might be proarrhythmic especially in patients with preexisting heart diseases. Clinical trials of electrical neuromodulation for cardiac arrhythmias need to consider the possible proarrhythmic outcomes. Second, these findings may

have implications on TENS, a technique widely used for pain management. Because chronic norepinephrine infusion can create heart failure in dogs,[19] very low levels of TENS or ScNS might have similar detrimental effects if used on a long-term basis. However, we do not have data in the present study to test that hypothesis.

Subcutaneous and Transcutaneous electrical nerve stimulation

Recent studies showed that electroacupuncture can result in unique immunomodulation possibly via stimulation of the autonomic nervous system[20] to release mesenchymal stem cells.[21] Multiple studies have demonstrated that the potential antiarrhythmic effects of acupuncture.[22] However, there have been no reports showing cardiac nerve sprouting after cutaneous electrical stimulation. The results of the present study suggest that the mechanisms of chronic intermittent cutaneous electrical stimulation may result in neural remodeling in the heart. The physiological responses to cutaneous electrical stimulation should be measured chronically in multiple organs to determine their impact.

Subcutaneous nerve stimulation for arrhythmia control

Dogs randomized to chronic high output stimulation had a reduced incidence and duration of spontaneous PAT episodes. The SG of three dogs showed TUNEL positivity consistent with our previous studies,[6, 7] with the exception that the present study did not show large confluent areas of damage in the SG. These differences could be due to the different devices used. The previous studies used wrap around electrodes insulated from the outside. They might have delivered more current to a specific peripheral nerve. The catheter electrodes used in the present study were not insulated from outside and thus the current may be distributed to multiple peripheral nerves in the region leading to diffuse remodeling. While most SG in the high output groups showed no TUNEL positive neurons, those with TUNEL positivity had more reduction of heart rate than those without TUNEL positivity. The effects on PAT, however, were

not determined by TUNEL positivity. For clinical application, the catheter electrodes used in the present study would be easier to implant and remove than the wrap-around electrodes. We found that 1.5 -3.5 mA ScNS all reduced the PAT duration but only 3.5 mA reduced heart rate. These data suggest that 1.5 mA and/or 2.5 mA output may be used to suppress PAT without inducing bradycardia, a potential side effect of the ScNS. On the other hand, if the purpose of stimulation is to significantly reduce the heart rate, then 3.5 mA output is appropriate. This technique can be practiced by inserting electrodes under the skin and connecting the exteriorized electrodes to a neurostimulator for chronic ScNS. The stimulus output should be programmed to 3.5 mA. The low output stimulation (0.25 mA) should be avoided. The electrodes is removed after achieving desired therapeutic effects, thus reducing the risk of infection.

Discomfort associated with subcutaneous stimulation

Stimulating subcutaneous nerves may cause pain or discomfort, especially at high output when C fibers are activated.[23] However, on continued stimulation with constant intensity, the burning pain has been reported to diminish markedly concomitant with a reduction of the C-fiber response.[24, 25] These observations suggest that both central habituating mechanisms and a decrease of excitability in thin fibers can occur during constant electrical stimulation, thus reducing pain. Consistent with these results, the dogs that underwent chronic ScNS studies behaved normally, had good appetite and all but one gained weight.

Limitations

Autonomic nerve fibers in the skin almost completely derive from sympathetic neurons.[26] Therefore, our data cannot be used to determine whether or not there exists a neural fulcrum as observed in vagal nerve stimulation.[27] We do not have long term follow up to detect chronic side

effects of high output ScNS. We did not perform sternotomy or place electrodes on the stellate ganglion in the present study. Therefore, the histological findings of the stellate ganglion cannot have been caused by electrode irritation. However, this approach also prevented us from determining the effects of stimulus strength on the SGNA.

Conclusions

Very low output ScNS (0.25 mA) can cause cardiac nerve sprouting, increase plasma norepinephrine concentration and increase the duration of PAT. High output electrical stimulation may remodel the SG and reduce sympathetic outflow, leading to the decreased duration of PAT.

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Disclosures

Dr Everett has equity interest in Arrhythmotech LLC.

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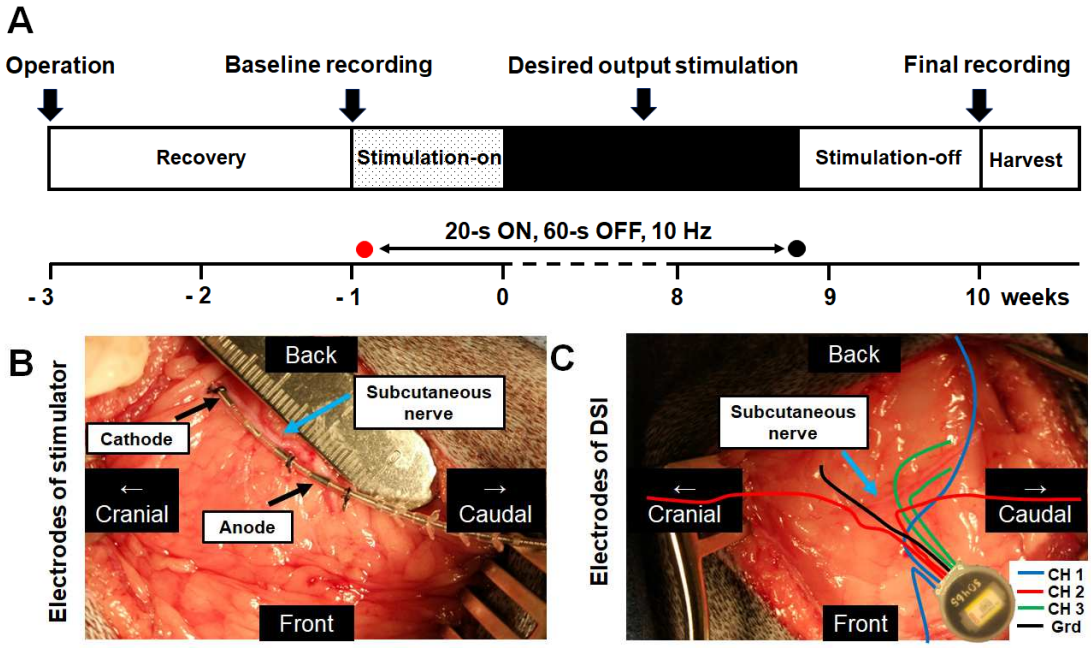

Figure 1. Study protocol and the surgical procedure. A. The DSI radiotransmitter was turned on two weeks after surgery to record baseline heart rhythm and subcutaneous nerve activity (ScNA). After baseline recording, the neurostimulator was turned on (red dot) and programmed to 20-s ON and 1 min OFF (10 Hz, 450 μs pulse width) for ScNS. The sham group had 0 mA output. All others had the output progressively increased until the desired output. Because the stimulator is a constant voltage stimulation, the impedance was measured weekly and the voltage output was adjusted to maintain the desired current output for 8 weeks. The final DSI recording was collected 1 week after the neurostimulator was turned off (black dot). The dogs were then euthanized, and the tissues were harvested. **B.** Surgical approaches for subcutaneous nerve stimulation. **C.** The location of DSI leads. CH = channel, Grd = ground.

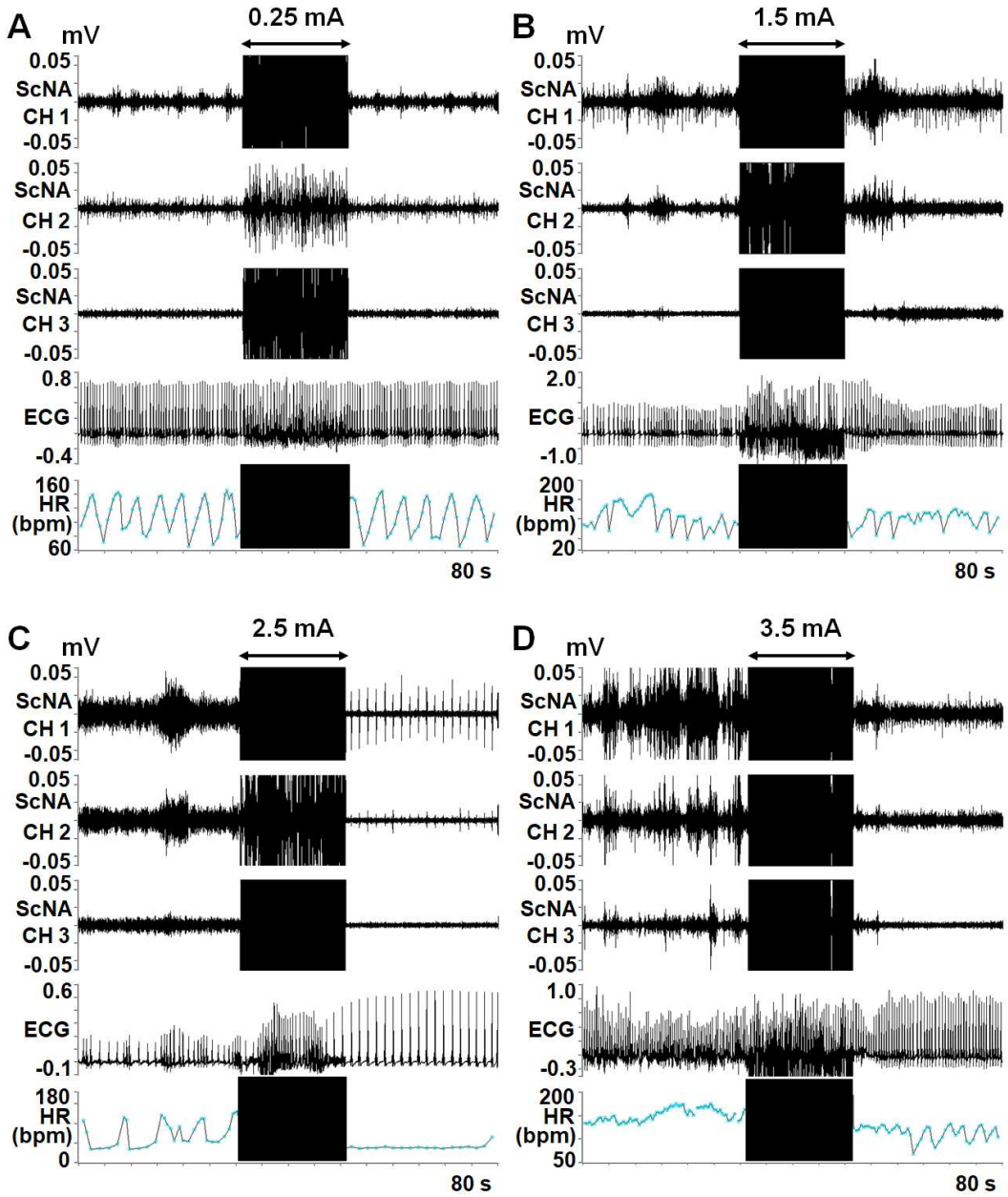

Figure 2. Stimulus output and cardiac response. A and **B** showed that stimulation with 0.25 mA and 1.5 mA, respectively did not change ScNA or heart rate. **C** and **D** showed stimulation with 2.5 mA and 3.5 mA, respectively resulted in transient suppression of the ScNA and heart rate. ECG = Electrocardiogram, HR = heart rate, bpm = beats per minute.

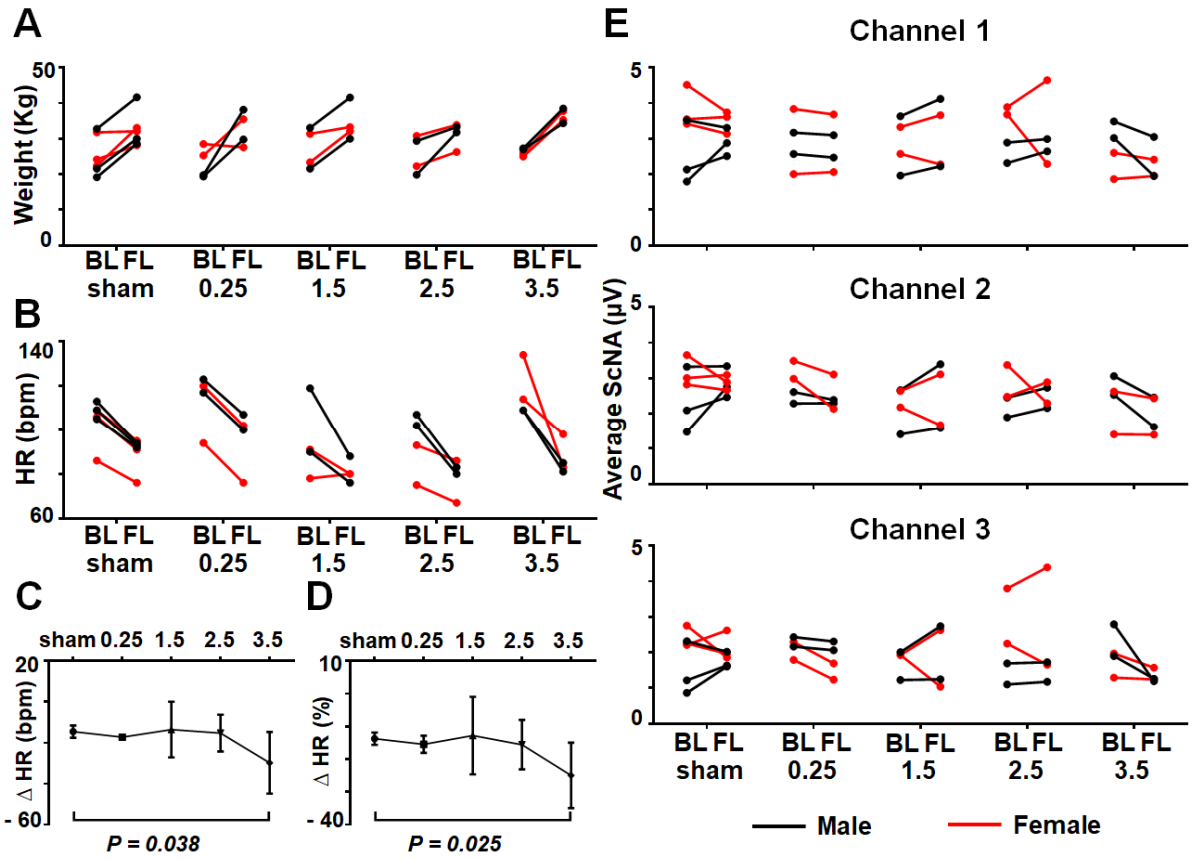

Figure 3. Effects of ScNS. A. The weight of all but one dog increased from baseline (BL) to final week (FL) of the study. **B.** All but one dog showed a reduction of heart rate (HR). **C.** The delta HR (bpm) among the five groups, showing significantly more HR reduction in 3.5 mA group than sham group. **D.** The percentage of HR reduction, showing the same finding. **E.** The ScNA of 3 subcutaneous channels showing no significant changes overall. There are no apparently changes between male and females. BL = baseline recording, FL = final recording.

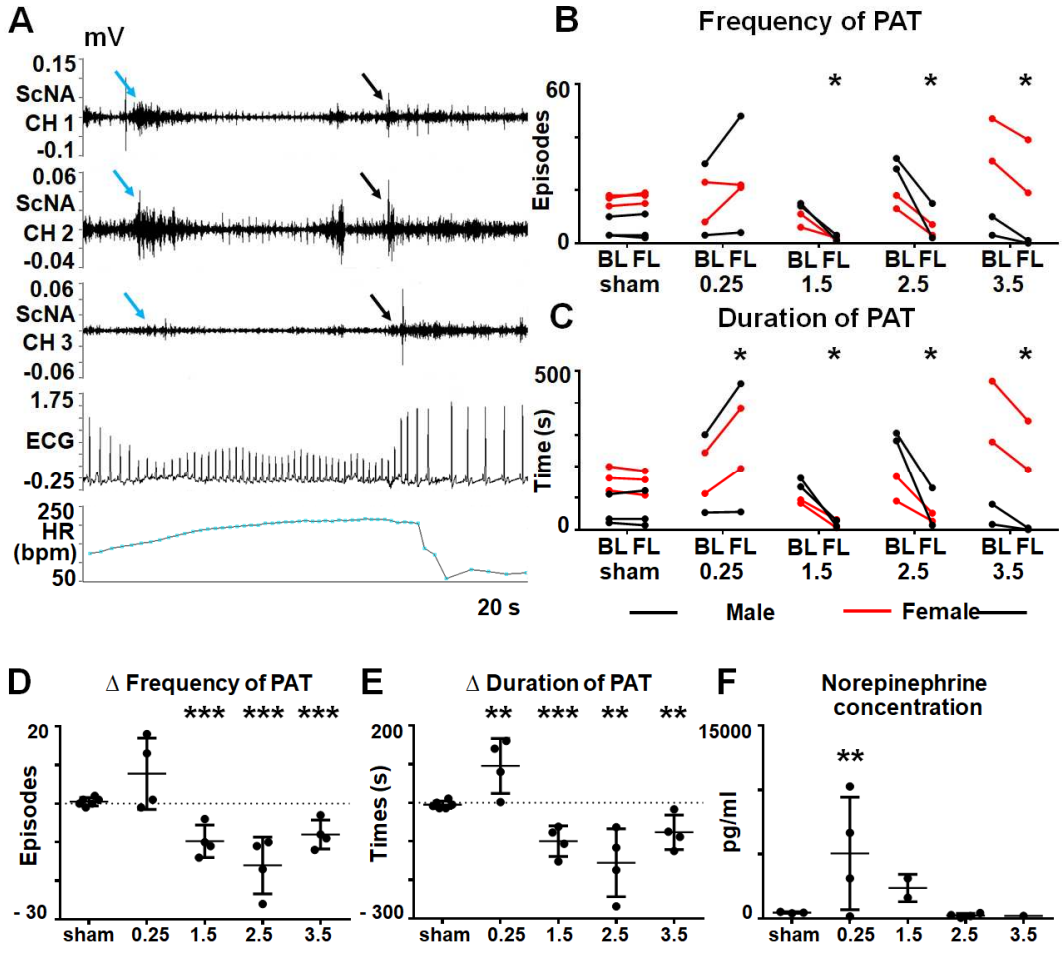

Figure 4. Effects of ScNS on PAT episodes. A. A typical example of PAT. There were ScNA bursts before the onset (blue arrows) and termination (black arrows) of the PAT. Abrupt offset of PAT was noted, with rate reduction of > 50 bpm within one beat. **B.** The frequency of PAT (episodes per 48 hours) of all dogs, showing significant reduction in 1.5 mA-3.5 mA groups. **C.** The durations of PAT per 48 hours of all dogs, showing significant increase in 0.25 mA group and significant reduction in 1.5-3.5 mA groups, as compared with baseline. **D and E** show the delta PAT frequency/48 hrs and PAT durations, respectively among the five groups of dogs. **F** shows the plasma norepinephrine concentrations of different groups of dogs. Asterisks indicates significantly (p=0.009) higher concentrations in 0.25 mA group (N=4) than all other dogs (N=10). * p<0.05, ** p<0.01, *** p<0.001.

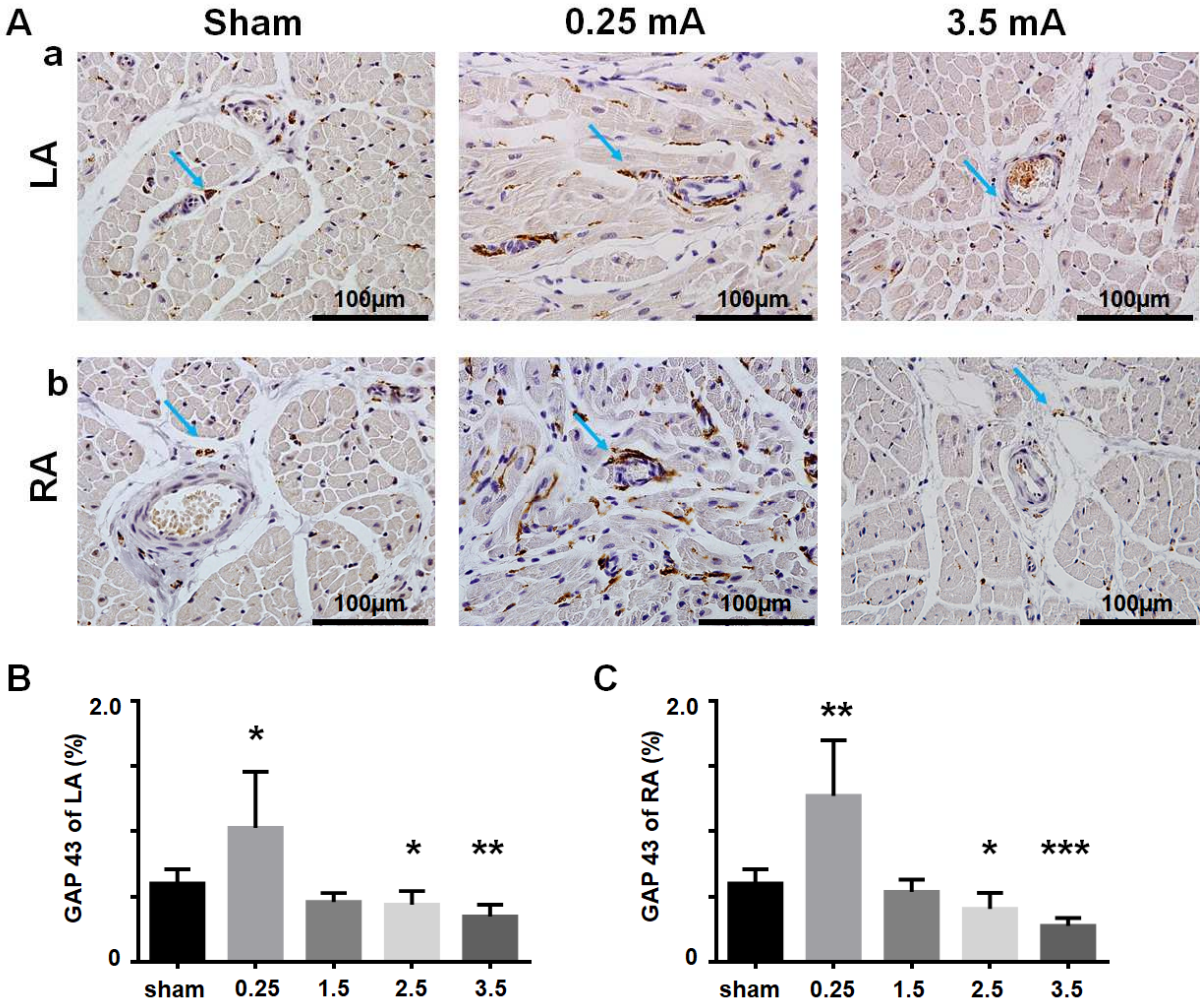

Figure 5. Growth associated protein 43 (GAP43)-positive nerve structures of the left atrium (LA) and right atrium (RA). A. a. Examples of the GAP43 positive fibers (blue arrows) of LA in sham, 0.25 mA and 3.5 mA groups. **b.** Examples of the GAP43 positive fibers (blue arrows) of RA in sham, 0.25 mA and 3.5 mA groups. The GAP43 positive nerve densities were significantly higher in 0.25 mA group and significantly lower in 2.5 mA and 3.5 mA groups as compared with sham group in LA (**B**) and RA (**C**). * p<0.05, ** p<0.01, *** p<0.001.

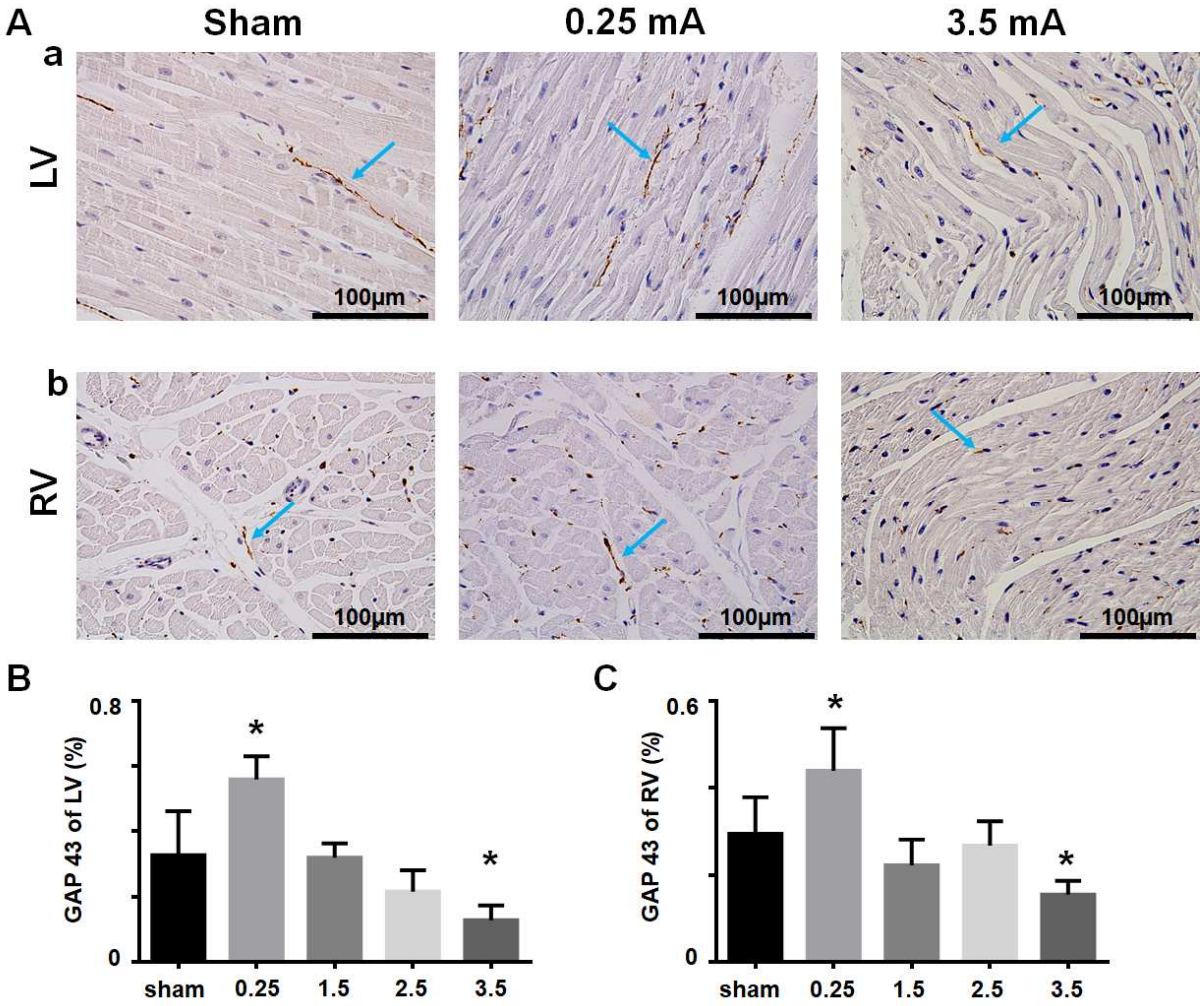

Figure 6. Growth associated protein 43 (GAP43)-positive nerve structures of the left ventricle (LV) and right ventricle (RV). A. a. Examples of the GAP43 positive fibers (blue arrows) of LV in sham, 0.25 mA and 3.5 mA groups. **b.** Examples of the GAP43 positive fibers (blue arrows) of RV in sham, 0.25 mA and 3.5 mA groups. The GAP43 positive nerve densities was significantly higher in 0.25 mA group and significantly lower in 3.5 mA group as compared with sham group in LV (**B**) and RV (**C**). * $p<0.05$.

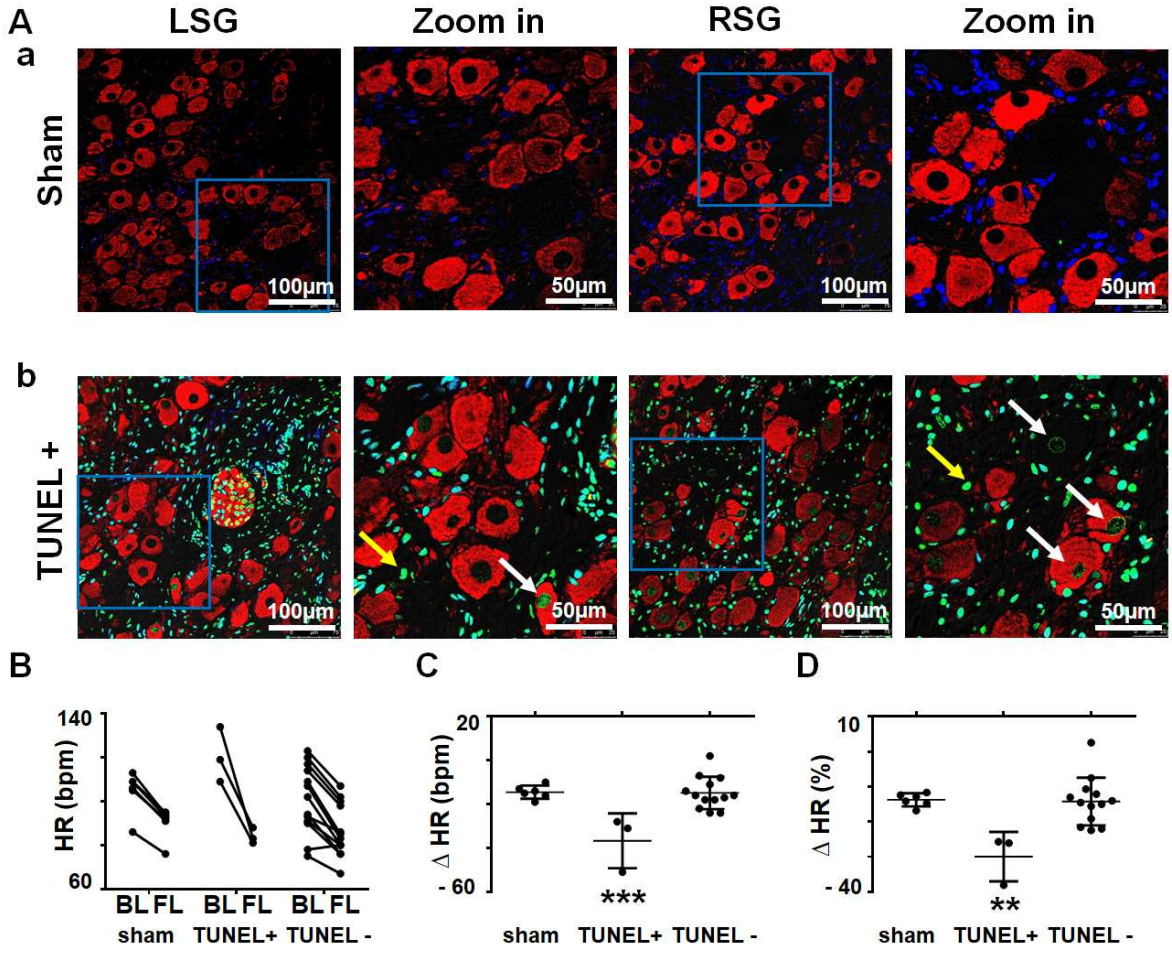

Figure 7. TUNEL staining of the SG. A. a. The TH and TUNEL staining of sham group. The neurons were mostly stained positive for tyrosine hydroxylase (red). **b.** The LSG and RSG from a 1.5 mA group were stained positively for both tyrosine hydroxylase (red) and TUNEL (green). The TUNEL positive cells include both neurons (white arrows) and non-neuronal cells (yellow arrows). **B.** The heart rate (HR) of all dogs studied, including the sham group, TUNEL-positive and TUNEL-negative groups. **C.** There was a greater reduction of HR in TUNEL+ group compared with sham group and TUNEL- group. **D.** The percentage reduction of HR was greater in TUNEL+ group than sham and TUNEL- group. BL, baseline; FL, final (before euthanasia). LSG = left stellate ganglion, RSG = right stellate ganglion. ** p<0.01, *** p<0.001.