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Long term intermittent high amplitude subcutaneous nerve stimulation reduces sympathetic tone in ambulatory dogs

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Abstract

Background—Reducing sympathetic efferent outflow from the stellate ganglia (SG) may be antiarrhythmic.

Objective—To test the hypothesis that chronic thoracic subcutaneous nerve stimulation (ScNS) could reduce SG nerve activity (SGNA) and control paroxysmal atrial tachycardia (PAT).

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COI: Indiana University has filed patent applications related to this work. Shien-Fong Lin and Thomas H. Everett IV have equity interest in Arrhythmotech, LLC.

Methods—We performed thoracic ScNS in 8 dogs while monitoring the SGNA, vagal nerve activity (VNA) and subcutaneous nerve activity (ScNA). An additional 3 dogs were used for sham stimulation as controls.

Results—We found that Xinshu ScNS (XScNS) and left lateral thoracic nerve ScNS (LLTNS) reduced heart rate (HR). XScNS at 3.5 mA for 2 weeks reduced the mean average SGNA (aSGNA) from 5.32 μV [95% CI, 3.89 to 6.75] at baseline to 3.24 μV [95% CI, 2.16 to 4.31] (P = 0.015) and the mean HR from 89 bpm [95% CI, 80 to 98] at baseline to 83 bpm [95% CI, 76 to 90] (P = 0.007). Bilateral SG showed regions of decreased tyrosine hydroxylase (TH) staining with increased terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive nuclei in 18.47% [95% CI, 9.68 to 46.62] of all ganglion cells, indicating cell death. Spontaneous PAT episodes were reduced from 9.83 [95% CI, 5.77 to 13.89] in controls to 3.00 [95% CI, 0.11 to 5.89] per day after ScNS (P = 0.027). LLTNS also led to significant bilateral SG neuronal death and significantly reduced aSGNA and HR in dogs.

Conclusions—ScNS at two different sites in the thorax led to SG cell death, reduced SGNA and suppressed PAT in ambulatory dogs.

Keywords

Autonomic nervous system; Arrhythmia; Neuromodulation; Nerve Recording; Stellate Ganglion

Neuromodulation methods that reduce sympathetic efferent outflow may be helpful in controlling cardiac arrhythmia.[1] However, other than the surgical resection of SGs to achieve permanent left sympathetic denervation, no less-invasive method is available to effectively inhibit sympathetic nerve output to the heart. We[2] previously reported that vagal nerve stimulation (VNS) could effectively induce left stellate ganglion (LSG) remodeling and suppress stellate ganglion nerve activity (SGNA). Nonetheless, clinical applications of VNS for arrhythmia control are often limited by the technical difficulties and potential complications associated with surgical implantation of the vagal nerve stimulators. Just like vagal nerves, subcutaneous nerves in dogs also contain sympathetic components.[3] The postganglionic sympathetic nerve fibers of the neck and thorax came primarily from the SG.[4] The thoracic subcutaneous nerve activity (ScNA) and superficial skin sympathetic nerve activity (SKNA) closely correlate with the SGNA,[3, 5, 6] further supporting a connection between thoracic subcutaneous nerves and the SG. We hypothesize that thoracic subcutaneous nerve stimulation (ScNS) can rapidly excite the SG and in turn cause neurotoxicity in the SG, which would reduce its sympathetic outflow. We first performed stimulation at the Xinshu acupoint (BL15, approximately 5 cm lateral to the spine at T5 level) because Xinshu acupoint stimulation has been reported to change mRNA expression in sympathetic ganglia in rats[7] and acupuncture at the Xinshu acupoint has prevented the atrial fibrillation recurrences after cardioversion in humans.[8] We also stimulated the left lateral thoracic nerve (LLTN) in additional dogs to determine if stimulation outside an acupoint can achieve the same antiarrhythmic effects. A sham control group was studied for comparison. We designed this study to test the hypothesis that long-term rapid ScNS may cause neuronal death in the SGs, leading to reduced sympathetic outflow and decreased spontaneous paroxysmal atrial tachyarrhythmias (PAT) in ambulatory dogs.

Methods

The animal protocol was approved by the Institutional Animal Care and Use Committee and conformed to the Guide for Care and Use of Laboratory Animals. The Figure 1 summarizes the study protocols and the location of electrode implantation. A detailed experimental method was included in an online supplement.

Protocol 1: The effect of Xinshu acupoint stimulation

A total of 6 mongrel dogs were studied. A radiotransmitter (D70EEE, Data Sciences International, St. Paul, MN) was implanted to record SGNA, vagal nerve activity (VNA) and ScNA. A Cyberonics of Model 304 bipolar vagal stimulating lead was implanted around subcutaneous nerves at Xinshu acupoint and connected to a Cyberonics Demipulse neurostimulator (Cyberonics Inc, Houston, TX). After baseline recording, the neurostimulator was turned on and programmed to 14-s ON and 1.1-min OFF (10 Hz, 500 μs pulse duration) in all dogs. The output current (mA) was progressively increased to 3.5 mA over 2 weeks (Figure 1A). We found that the dogs tolerated 3.5 mA ScNS without showing signs of discomfort or reduced appetite.

Protocol 2: The effect of left lateral thoracic nerve stimulation

We performed additional experiments in 2 dogs (dogs 7 and 8). The surgical preparation was the same as in Protocol 1 except that skin incision was lengthened to the ventral side to reach the left lateral thoracic nerve (LLTN) at the ventral border of the cutaneous trunci muscle. Figure 1B summarizes the study protocol. The dogs were then euthanized and the tissues harvested.

Protocol 3: Sham left lateral thoracic nerve stimulation

We performed additional experiments in 3 dogs (dogs 9, 10 and 11). The surgical preparation was the same as in Protocol 2. The output current was programmed to 0 mA (sham stimulation) during the 6 weeks recording period. The dogs were then euthanized and the tissues harvested (Figure 1C).

Data Analyses

Nerve activities were quantified by integrating the absolute value of the filtered signal over a 20-s window. In addition, we also compared the number of PAT episodes over 24 hours between baseline and different time points of the experiment. PAT was defined as a tachycardia with a rate of 200 bpm that lasted for ≥ 5 s, with either an abrupt onset (50 bpm increment) or an abrupt termination (50 bpm decrement) between 2 heart beats.[9] The data were reported as mean ± Standard deviation (SD) or 95% confidence interval (CI). Paired *t* test and Signed-rank test were performed to compare the differences between heart rate (HR), integrated nerve activities and the number of PAT episodes at different stages of experiments. A two-sided p value of ≤0.05 was considered as statistically significant.

Results

Protocol 1: Effects of Xinshu acupoint stimulation

At baseline, VNA and Xinshu ScNA (XScNA) could either activate simultaneously with SGNA (Figure 2A) or interactively with SGNA (Figure 2B). Increased SGNA is consistently associated with prolonged durations of HR increase and reduced HR variability (line segment, Figure 2B). ScNS at Xinshu acupoint could result in a transient termination of SGNA and reduction of HR (Figure 2C). Figure 2D shows that after 2 weeks of 3.5 mA Xinshu ScNS (XScNS), there was a significant reduction of spontaneous SGNA as compared with baseline. This example also shows activation of VNS during ScNS. Longer durations of bradycardia could be observed during XScNS ON-time, along with reduced HR and HR variability (line segment, Figure 2D) after 2 weeks of XScNS. For all dogs studied, XScNS reduced the mean average SGNA (aSGNA) from 5.32 μV [95% CI, 3.89 to 6.75] at baseline to 3.99 μV [95% CI, 3.11 to 4.88] at 3.5 mA sti_1 ($P = 0.030$), and then to 3.24 μV [95% CI, 2.16 to 4.31] at 3.5 mA sti_2 ($P = 0.015$) (Figure 2E-a) However, XScNS did not significantly change the average VNA (aVNA) or average XScNA (aXScNA) at either 3.5 mA sti_1 or 3.5 mA sti_2 versus baseline (Figure 2E-b, 2E-c, respectively). The mean HR reduced insignificantly ($P = 0.172$) from 89 bpm [95% CI, 80 to 98] at baseline to 87 bpm [95% CI, 79 to 95] at 3.5 mA sti_1, then reduced significantly ($P = 0.007$) to 83 bpm [95% CI, 76 to 90] at 3.5 mA sti_2 (Figure 2E-d).

SGNA recordings over a 24-hr period before (upper panel) and after (lower panel) XScNS shows an obvious reduction of the nerve activity after stimulation (Figure 3A).

Protocol 2: Effects of left lateral thoracic nerve stimulation

Stable baseline nerve activity in two dogs—At baseline (before LLTNS), simultaneous SGNA and LLTN activity (LTNA) were associated with a transient increase of HR and reduction of spontaneous HR variation (Figure 4A). We observed these 2 dogs over a prolonged period (6 weeks) without performing nerve stimulation (sham control). Weekly aSGNA showed no spontaneous changes of aSGNA, aVNA, aLTNA or HR reduction over the 6-week observational period (Figures 4C-a to 4C-d, respectively). In the first dog, the mean aSGNA increased from 3.47 μV [95% CI, 3.24 to 3.72] to 4.39 μV [95% CI, 4.04 to 4.68] at the 6th week (p=0.002). In a second dog, the aSGNA increased from 3.84 μV [95% CI, 3.62 to 4.06] to 4.70 μV [95% CI, 4.02 to 4.84] at the 6th week (p=0.229). These data are consistent with a previous study that showed no spontaneous reduction of SGNA over a period of a month.[10]

LLTNS started after Week 6 resulted in a reduction of aSGNA in both dogs. Figure 4B shows an example of LLTNS suppressing spontaneous nerve activity and HR. In the first dog, the aSGNA reduced from 4.39 μV [95% CI, 4.04 to 4.71] to 3.39 μV [95% CI, 3.12 to 3.64] after the first week of 3.5 mA LLTNS, and then to 3.19 μV [95% CI, 2.98 to 3.74] after 2 weeks of 3.5 mA LLTNS. In a second dog, the aSGNA reduced from 4.70 μV [95% CI, 4.02 to 4.84] to 3.56 μV [95% CI, 3.24 to 3.86] after the first week of 3.5 mA, and then to 3.29 μV [95% CI, 3.22 to 3.64] after 2 weeks of 3.5 mA LLTNS (Figure 4C-a). However, aVNA and aLTNA did not change during monitoring (Figure 4C-b, 4C-c, respectively). The

mean HR reduced from 81 bpm [95% CI, 81 to 83 bpm] to 76 bpm [95% CI, 74 to 77] (p<0.001) in the first dog and from 94 bpm [95% CI, 92 to 95] to 84 bpm [95% CI, 82 to 85] (p<0.001) in a second dog (Figure 4C-d).

SGNA recordings over a 24-hr period before (upper panel) and after (lower panel) LLTNS shows an obvious reduction of the nerve activity after stimulation (Figure 3B).

ScNS reduced PAT episodes

Figure 5A shows typical examples of PAT with sudden onset (Panel a) and sudden offset (Panel b). In Protocol 1, the mean episodes of PAT was 7.50 per day [95% CI, 3.82 to 11.18] at baseline, that was reduced to 2.50 per day [95% CI, 1.40 to 3.60] after 1 week of 3.5 mA XScNS, then to 0.67 [95% CI, 0 to 1.52] after 2 weeks of 3.5 mA XScNS ($P = 0.003$) (Figure 5B-a), along with a reduction of total duration from 63.17 s per day [95% CI, 30.04 to 96.30] at baseline to 18.50 s per day [95% CI, 8.27 to 28.73] after 1 week of 3.5 mA XScNS and then 4.00 s per day [95% CI, 0 to 8.92] after 2 weeks of 3.5 mA XScNS ($P = 0.004$) (Figure 5B-b). In 3 dogs, there was a complete suppression of PAT episodes after 2 weeks of 3.5 mA XScNS. Similarly, the PAT episodes and duration both reduced significantly in Protocol 2 (Figures 5C-a and 5C-b, respectively). In the first dog, PAT episodes decreased from 4 per day to a complete suppression after 2 weeks of LLTNS with the duration decreased from 27.5 s to 0 s per day. In a second dog, PAT episodes decreased from 2 per day to 1 per day after 2 weeks of LLTNS with the duration decreased from 13.5 s to 5.0 s per day.

ScNS caused bilateral SG Damage

In Protocol 1, six LSG and four RSG were successfully harvested for analyses. Large areas of damage, characterized by reduced or negative TH staining, pyknotic nuclei and shrinkage of cytoplasm, were visible in all LSG and RSG studied (Figure 6A). These damaged regions could be either confluent as a large abnormal area or multifocal. Within the damaged region, the percentage of TH-negative ganglion cells was 23.75% [95% CI, 15.88 to 31.62] in LSG and 15.78% [95% CI, 10.12 to 21.43] in RSG, both significantly higher than that of the normal region (10.46%, 95% CI, 7.46 to 13.46, p<0.001, and 8.84%, 95% CI, 7.34 to 10.33, p=0.030, respectively). The overall mean percentage of the TH negative ganglion cell was 16.77% [95% CI, 15.88 to 31.62] in LSG and 9.71% [95% CI, 7.55 to 11.89] in RSG (p=0.001). Tissue sections from the same specimens were then double stained for TH and TUNEL (Figure 7A). The ganglion cells in normal region mostly stained positive for TH (Red) and negative for TUNEL. In contract, TUNEL positive (Green) ganglion cell nuclei were found in the damaged regions of 5 LSG specimens (Figures 7A). TUNEL positive small non-ganglion cells could be also be found in the same region. The mean percentage of TUNEL-positive ganglion cells was 18.47% [95% CI 9.68 to 46.62]. Among them, 2 LSG showed a very high percentage of TUNEL positive cells (30.98% and 35.16%), TUNEL positive ganglion cells were found in 3 of 4 RSG specimens, with the mean percentage of 9.91% [95% CI 6.10 to 25.92]. One RSG showed a high percentage of TUNEL positive cells (23.75%).

Bilateral SG of both Protocol 2 dogs were available for analyses. All of them showed large areas of visible damage at low magnification (Figure 6B). Within the damaged region of LSG, 18.17% of the ganglion cells were negative for TH in the first dog, and 19.67% in the second dog. In the RSG, 17.74% ganglion cells were TH negative in the first dog and 16.27% in the second dog. The overall percentage of the TH (−) ganglion cells in LSG was 16.77% and 9.22% in RSG. The slides from the same specimens were then double stained for TH and TUNEL (Figure 7B). Confocal immunofluorescent images showed the ganglion cells in normal regions mostly stained positive for TH (red) and negative for TUNEL. In comparison, abundant TUNEL positive (green) ganglion cell nuclei could be found in the damaged regions of both specimens. In addition, small non-ganglion cells were also found to be TUNEL positive in the same region. The percentage of TUNEL positive cells of the first and second dogs were, respectively, 19.96% and 21.75% in LSG, while 4.98% and 18.85% in RSG.

ScNS activates SGNA

We selected the data window in which SGNA was quiescent to examine the effects of ScNS on SGNA. Figure 8A and 8B show the effects of Xinshu ScNS and LLTN ScNS, respectively, on SGNA. There was no SGNA response to 0.5 mA stimulus. Increasing the ScNS output resulted in greater SGNA and VNS responses in both protocols. Red arrows point to significant reduction of the HR during ScNS, indicating cardiac effects of ScNS. At 3.5 mA output, all dogs showed rapid VNA and SGNA activation when ScNS was given during quiescent periods of SGNA.

Protocol 3: Sham left lateral thoracic nerve stimulation

No changes of nerve activities were observed in 3 dogs with sham LLTNS. The aSGNA was at baseline was 2.48 μV [95% CI, 1.37 to 3.58] and 2.76 μV [95% CI, 2.07 to 3.45, p=0.558] after 6 weeks of sham LLTNS. The mean HR was 108 bpm [95% CI, 89 to 127 bpm] at baseline to 102 bpm [95% CI, 81 to 122 bpm] after 6 weeks of sham LLTNS (p=0.563). The average VNA (aVNA) was 1.22 μV [95% CI, 0.94 to 1.50] before and 1.20 μV [95% CI, 0.86 to 1.53] at 6 weeks (p=0.317). The average LLTNA (aLTNA) was 1.56 μV [95% CI, 0.94 to 2.18] before and 1.48 μV [95% CI, 0.72 to 2.24] at 6 weeks (p=0.475). Online Supplement Figure 1 shows the changes of nerve activity over time in this protocol. Histological studies showed no damaged areas in the SG. The TH negative cells was 6.17% [95% CI, 3.33 to 9.04] in LSG and 4.88% [95% CI, 0.66 to 9.10] in RSG, significantly lower than that of ScNS groups, respectively. No TUNEL positive cells was observed in any SGs. Examples of histological findings are shown in Online Supplement Figure 2.

Discussion

We showed in this study that ScNS from two different sites in the thorax can damage the SG, reduce SGNA and suppress PATs in ambulatory dogs. Dogs with sham stimulation had no changes of nerve activity or PAT episodes. These studies suggest that ScNS may be useful in reducing sympathetic tone and controlling cardiac arrhythmias.

SG damage

A major finding of this study was the presence of large and confluent regions of damages in both SG. These findings were not due to the irritation by the recording electrodes because these findings were also present in the RSG where no recordings were made. The sham stimulation group (Group 3) and a previous study from our laboratory[2] have also documented the absence of TUNEL-positive neurons in the normal SG. The TH-negative cells in the normal left SG usually accounts for about 5% of all ganglion cells.[11] A much higher percentage of TH-negative cells and a significant number of TUNEL-positive neurons were found in the LSG after VNS.[2] The large regions of SG damage, a high percentage of TH-negative ganglion cells than control, and numerous TUNEL-positive neuronal and non-neuronal cells document that SG damage can result from ScNS. In the central nervous system, prolonged electrical stimulation of the perforant pathway in the rat irreversibly damages hilar neurons.[12] The histological findings closely resemble the "excitotoxic" type of damage that the putative transmitters glutamate and aspartate are known to cause.[13] Therefore, investigators have proposed that sustained high frequency electrical stimulation of the perforant path results in excessive synaptic release and accumulation of glutamate (or aspartate) at numerous dendrosomal receptors in the hippocampus with consequent degeneration of the dendrosomal structures housing these receptors. It is possible that similar mechanisms may be responsible for SG damage induced by ScNS.

Acupuncture and transcutaneous nerve stimulation

Acupoints are richly innervated and contain catecholamine producing cells.[14, 15] Acupuncture, including electroacupuncture, are increasingly used in the Western world.[16, 17] It has been reported that acupuncture could reduce sympathetic tone in animal models and in humans.[18] A randomized clinical trial suggested that acupuncture at the Xinshu acupoint might prevent recurrent atrial fibrillation after cardioversion.[8] In addition to acupuncture, transcutaneous electrical stimulation has also been widely used in disease management, including potentially useful applications in cardiac arrhythmias.[19] We found that intermittent electrical stimulation near the Xinshu acupoint significantly reduced the incidence of PAT. However, these findings were not specific to the Xinshu acupoint, as the LLTN stimulation had similar effects on the histology of the SG and on arrhythmia suppression. These findings do not indicate that only acupoint stimulation can be used to suppress sympathetic tone. Rather, rapid stimulation of sympathetic nerves at various sites might reduce sympathetic tone through SG damage. Further investigation is needed to fully understand the mechanisms and risks of cutaneous and subcutaneous electrical stimulation.

Limitations of the study

We evaluated the antiarrhythmic effects of ScNS by using the spontaneous PAT episodes that are normally present in dogs.[20] Whether or not ScNS can be used to suppress more serious cardiac arrhythmias remain unclear. ScNS may cause pain and discomfort. However, our dogs did not show any change of appetite or behavior during the study. These observations suggest that both central habituating mechanisms and a decrease of excitability in thin fibers can occur during constant electrical stimulation, thus reduce pain.[21] We did not test the efficacies of transcutaneous nerve stimulation on arrhythmic control because it is very

difficult to determine how much current is actually given to the nerves through transcutaneous stimulation.

Conclusions

We conclude that ScNS at two different sites in the thorax can damage the SG, reduce SGNA and suppress PATs in ambulatory dogs. Because subcutaneous nerves are easily accessible, these methods may be useful in cardiac arrhythmia control in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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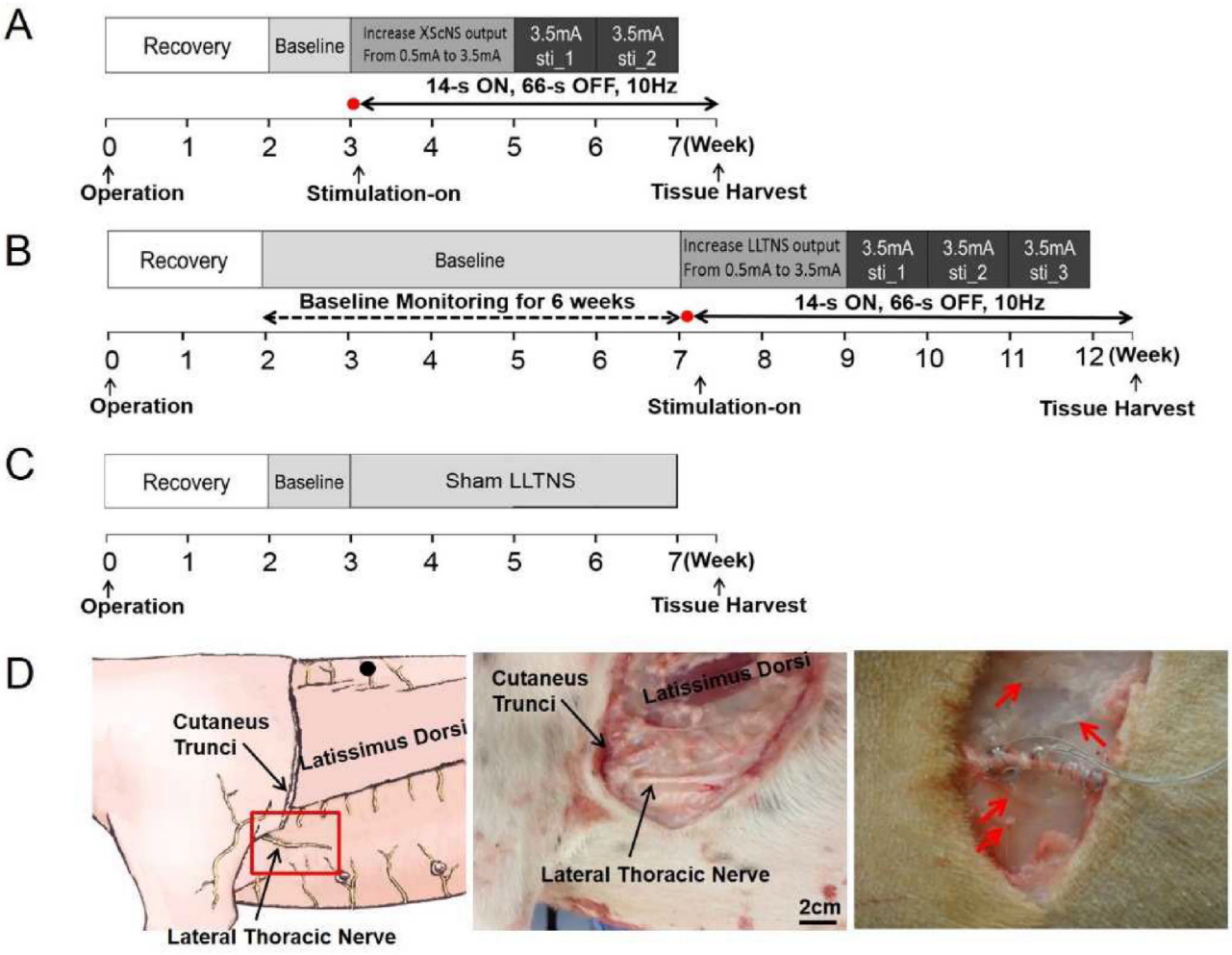

Figure 1.

The study protocols. **A.** Protocol 1, Xinshu subcutaneous nerve stimulation (n=6). After baseline recording, neurostimulator was turned on (red dot) and programmed 14-s ON (10 Hz, 500 μs pulse duration) and 66-s OFF. The output current was increased gradually from 0.5 mA to 3.5 mA in 2 weeks. After an additional 2 weeks of stimulation, the dogs were euthanized. **B.** Protocol 2, left lateral thoracic nerve stimulation (n=2). We extended the baseline recording to 6 weeks. The neurostimulator was turned on at week 8 and the output was gradually increased to 3.5 mA over a 2 week period. After an additional 3 weeks of stimulation at 3.5 mA, the dogs were euthanized. **C.** Protocol 3, left lateral thoracic nerve sham stimulation (n=3). **D.** Anatomy of Xinshu acupoint and left lateral thoracic nerve (LLTN). Black dot in left panel indicates site of Xinshu acupoint. Incision at the upper portion of the red box reveals LLTN beneath the cutaneus trunci (middle panel). The right panel shows the electrodes wrapped around the LLTN. Red arrows point to subcutaneous nerves, which are also found at Xinshu acupoint.

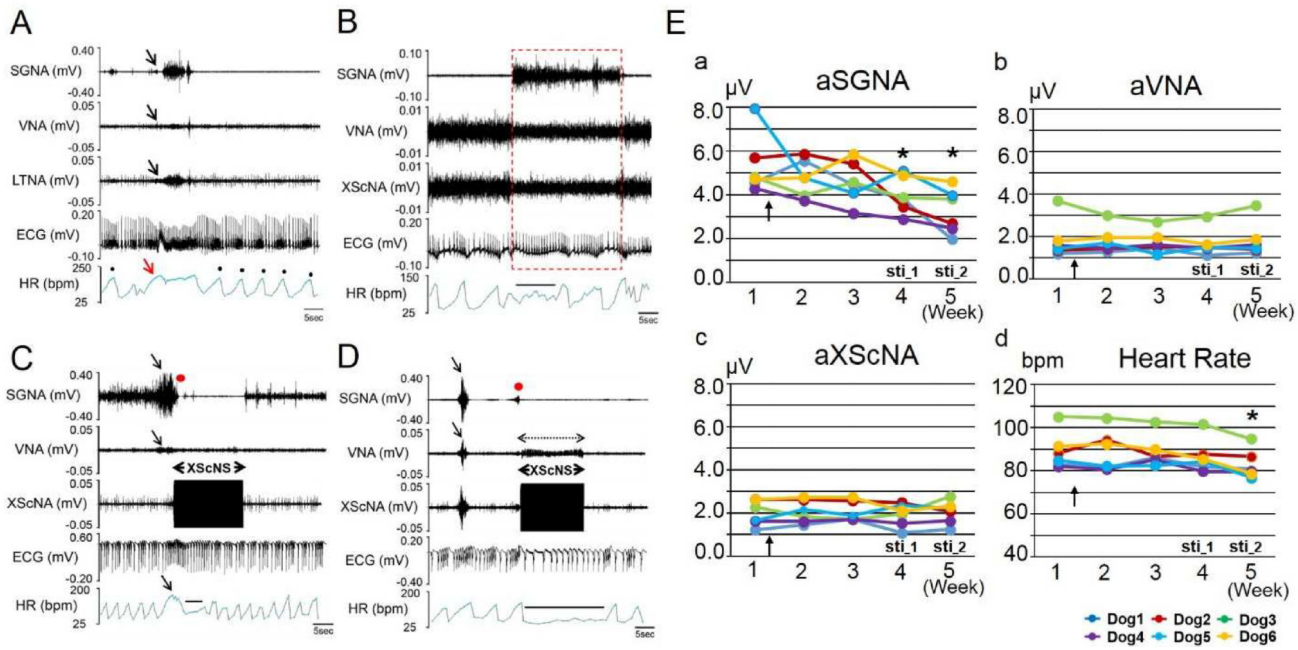

Figure 2.

Effects of Xinshu ScNS (XScNS). **A** shows nerve activities (black arrows) at baseline are associated with HR elevation (red arrows). **B** shows initial co-activation of left VNA and Xinshu subcutaneous nerve activity (XScNA), followed by activation of SGNA and reduced VNA and XScNA (red dotted box). These changes were associated with a transient reduction of HR variability (solid line). **C** shows an abrupt (red dot) reduction of SGNA (solid line) and HR during XScNS ON-time. When XScNS ended, SGNA abruptly resumed. **D** shows after 2 weeks of 3.5 mA XScNS, SGNA reduced significantly compared to baseline. The onset of XScNS further reduced SGNA (red dot), HR and HR variability (solid line). **E** shows the effects of XScNS on all 6 dogs. **E-a**: aSGNA reduced gradually after onset of XScNS (upward arrow). **E-b** and **E-c**: aVNA and average Xinshu subcutaneous nerve activity (aXScNA) did not change significantly. **E-d**: HR reduced gradually after XScNS. Asterisks indicate statistically significant reduction as compared with baseline. (ECG= electrocardiogram, SGNA= stellate ganglion nerve activity, VNA= vagal nerve activity, XScNA= Xinshu acupoint subcutaneous activity, aSGNA=average SGNA, aVNA=average VNA, aXScNA=average XScNA).

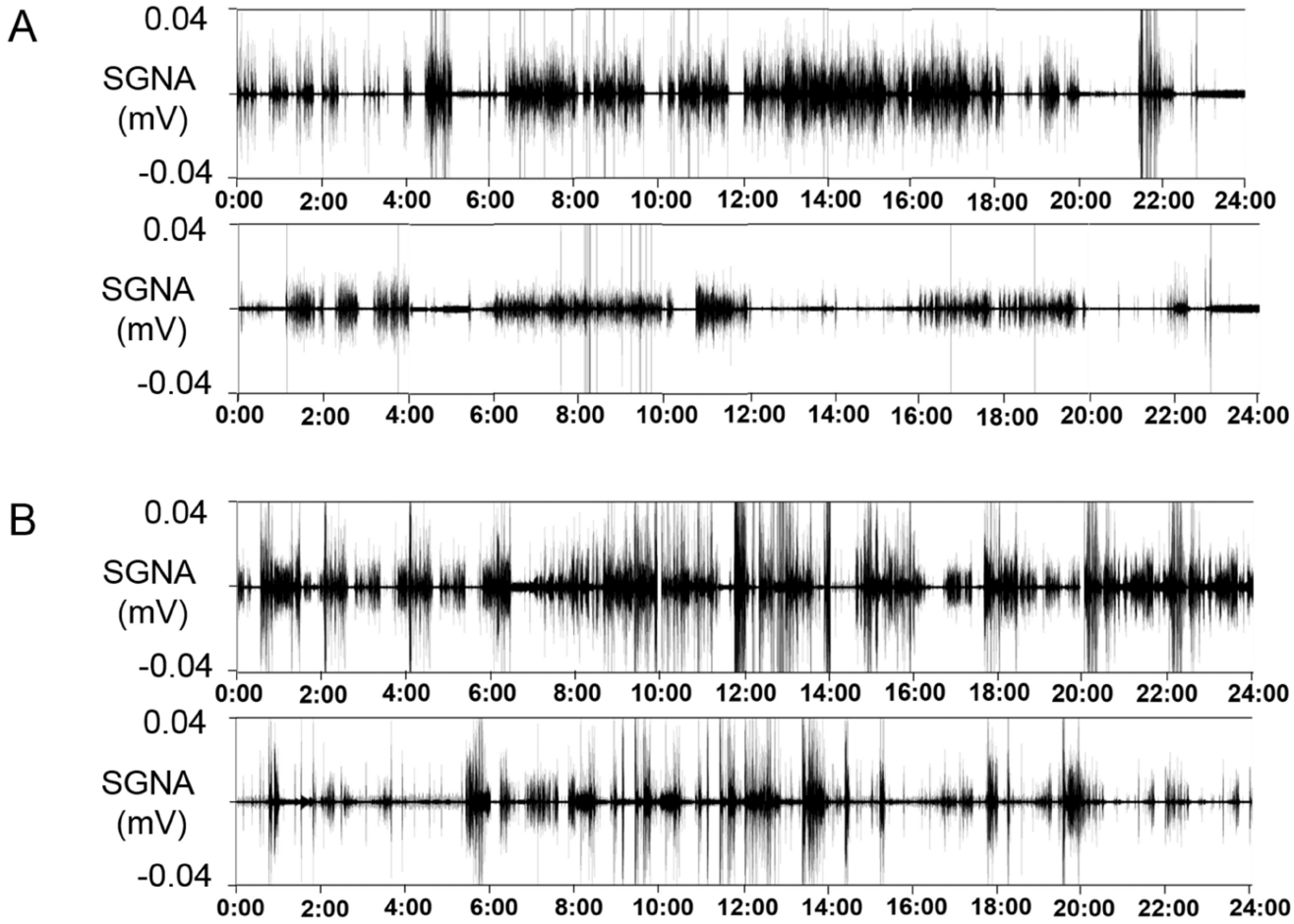

Figure 3.

ScNS reduces SGNA over a 24-hr period. **A:** SGNA before (upper panel) and 2 weeks after (lower panel) XScNS. **B:** SGNA before (upper panel) and 3 weeks after (lower panel) LLTNS.

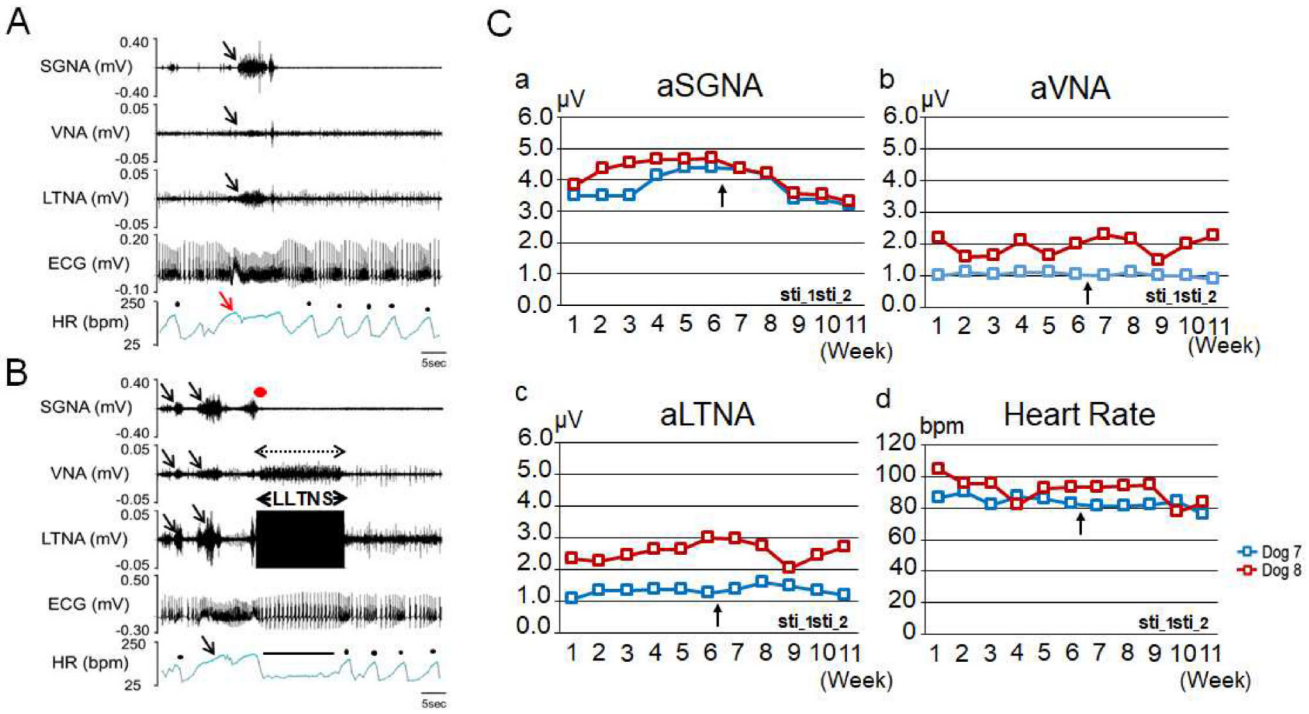

Figure 4.

Effects of left lateral thoracic nerve stimulation (LLTNS). **A** shows spontaneous nerve co-firing (black arrows) at baseline associated with an increased HR and a transient reduction of respiratory HR responses (red arrow). **B** shows LLTNS abruptly suppressed SGNA (red dot), HR and respiratory HR responses (solid line). **C** shows the results of both dogs. **C-a**: aSGNA was stable during prolonged (6 weeks) baseline recording. LLTNS (upward arrow) reduced aSGNA in both dogs. **C-b** and **C-c** show aVNA and aLTNA fluctuated but did not show a stable trend or increase or decrease after LLTNS. **C-d** shows reduction of HR after the onset of LLTNS. (LTNA= lateral thoracic nerve activity, aLTNA=average LTNA.)

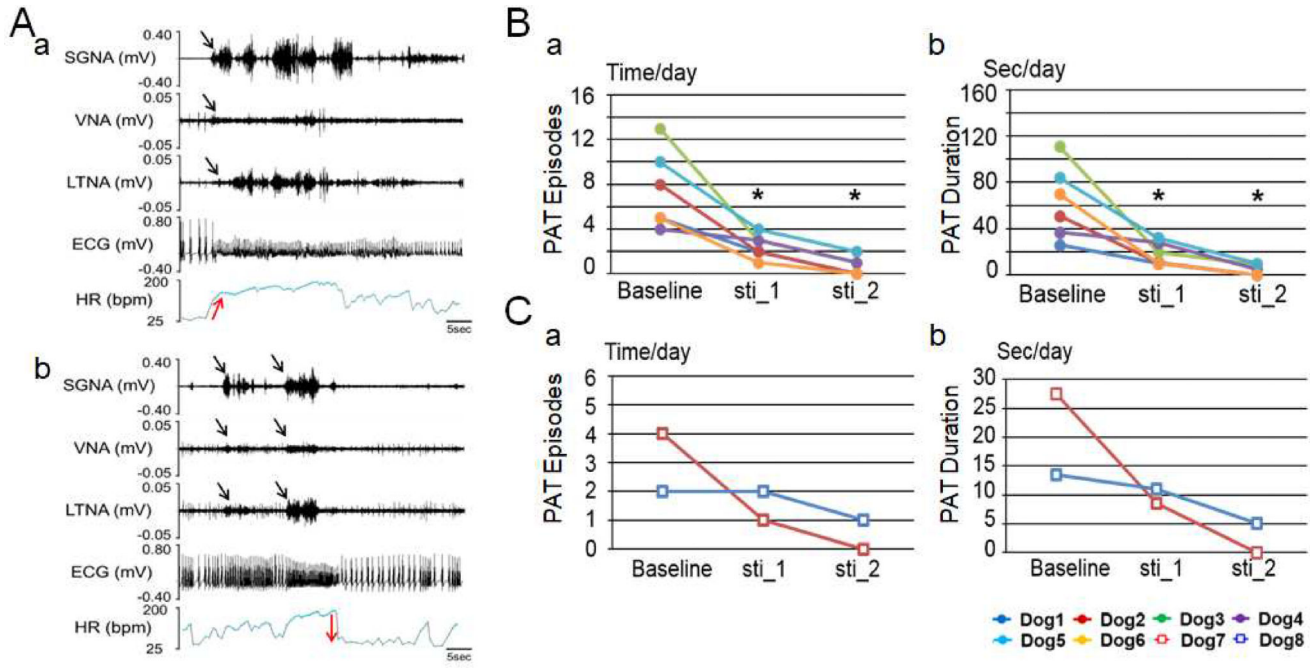

Figure 5.

Effects of ScNS on PAT episodes. **A** shows the typical onset (**Aa**) and offset (**Ab**) of PAT (red arrows), respectively, associated with significant nerve activities (black arrows) at baseline. These activities were rare after 3 weeks of LLTNS at 3.5 mA. **Ba** and **Bb** show a significant reduction of frequencies and durations, respectively, of the PAT episodes after 3.5 mA XScNS (N=6). **Ca** and **Cb** show a reduction of frequencies and durations, respectively, of the PAT episodes after 3.5 mA LLTNS (N=2).

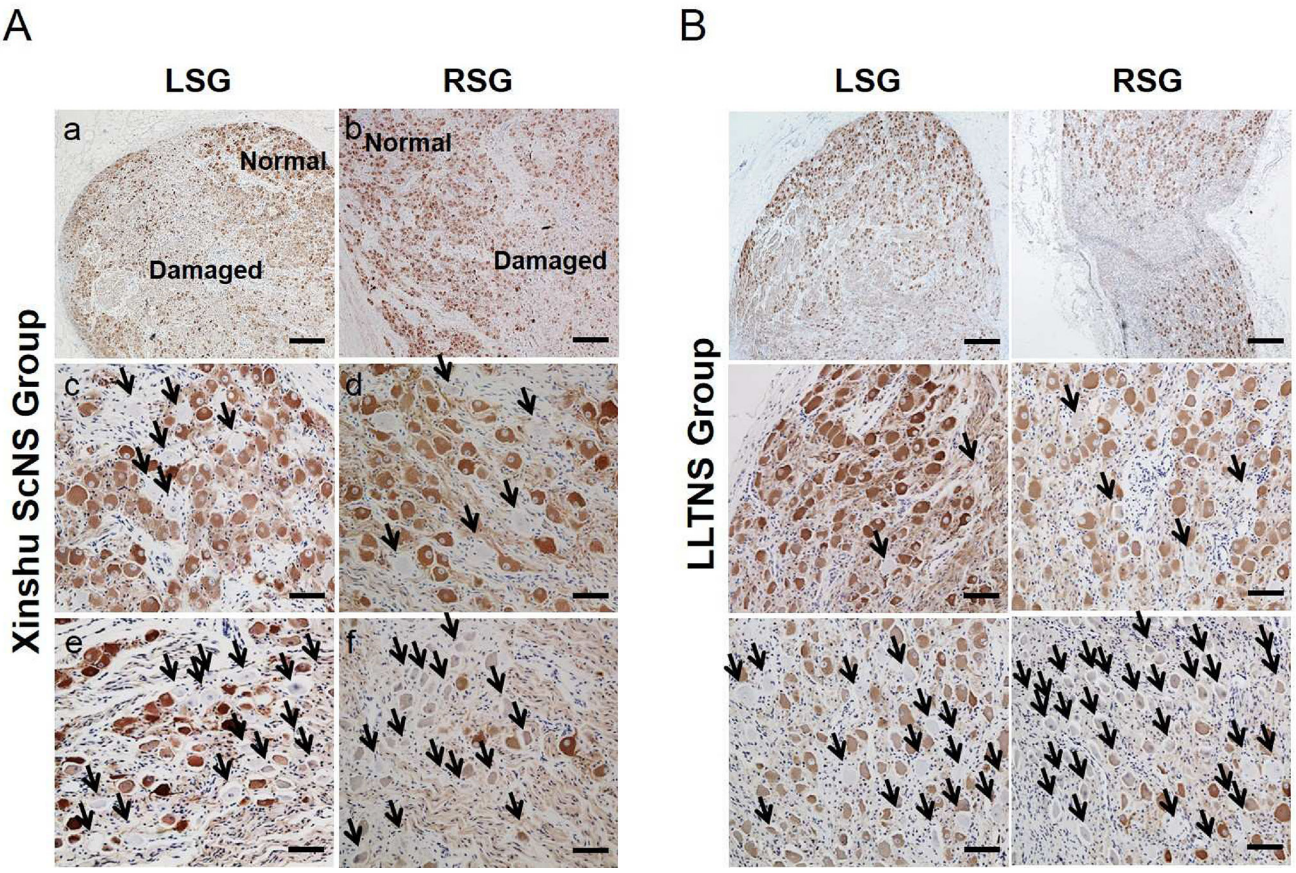

Figure 6.

Tyrosine hydroxylase (TH) staining in LSG and RSG of dogs with Xinshu (A) and LLTNS (B) ScNS. Panels a and b are low power views showing reduced TH staining in damaged region. (Calibration bar=200 μm). High power view of the normal regions (c and d) show normal morphology and rare TH-negative cells (arrows). In comparison, the ganglion cells in the damaged region (e and f) appeared small and had pyknotic nuclei. Many cells (arrows) stained negatively or weakly for TH. (Calibration bar=40 μm)

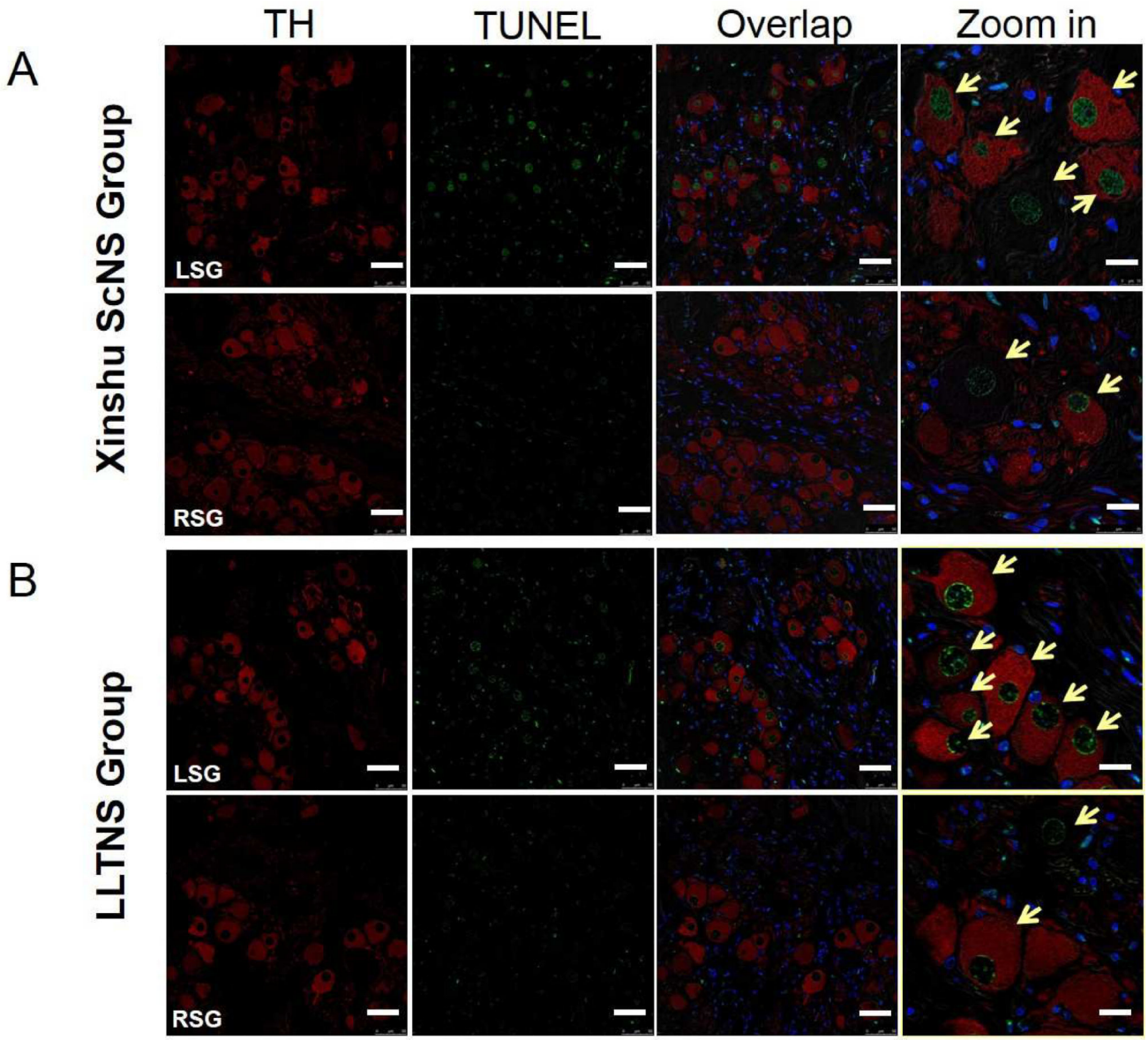

Figure 7.

Confocal microscope images of immunofluorescent Tyrosine hydroxylase (TH, red) and TUNEL (green) double staining after XScNS (**A**) and LLTNS (**B**). Blue is the DAPI stain of the nuclei. Arrows point to ganglion cells that stained positive for TUNEL. These cells could be either TH-positive or TH-negative. (Calibration bar=50 μm).

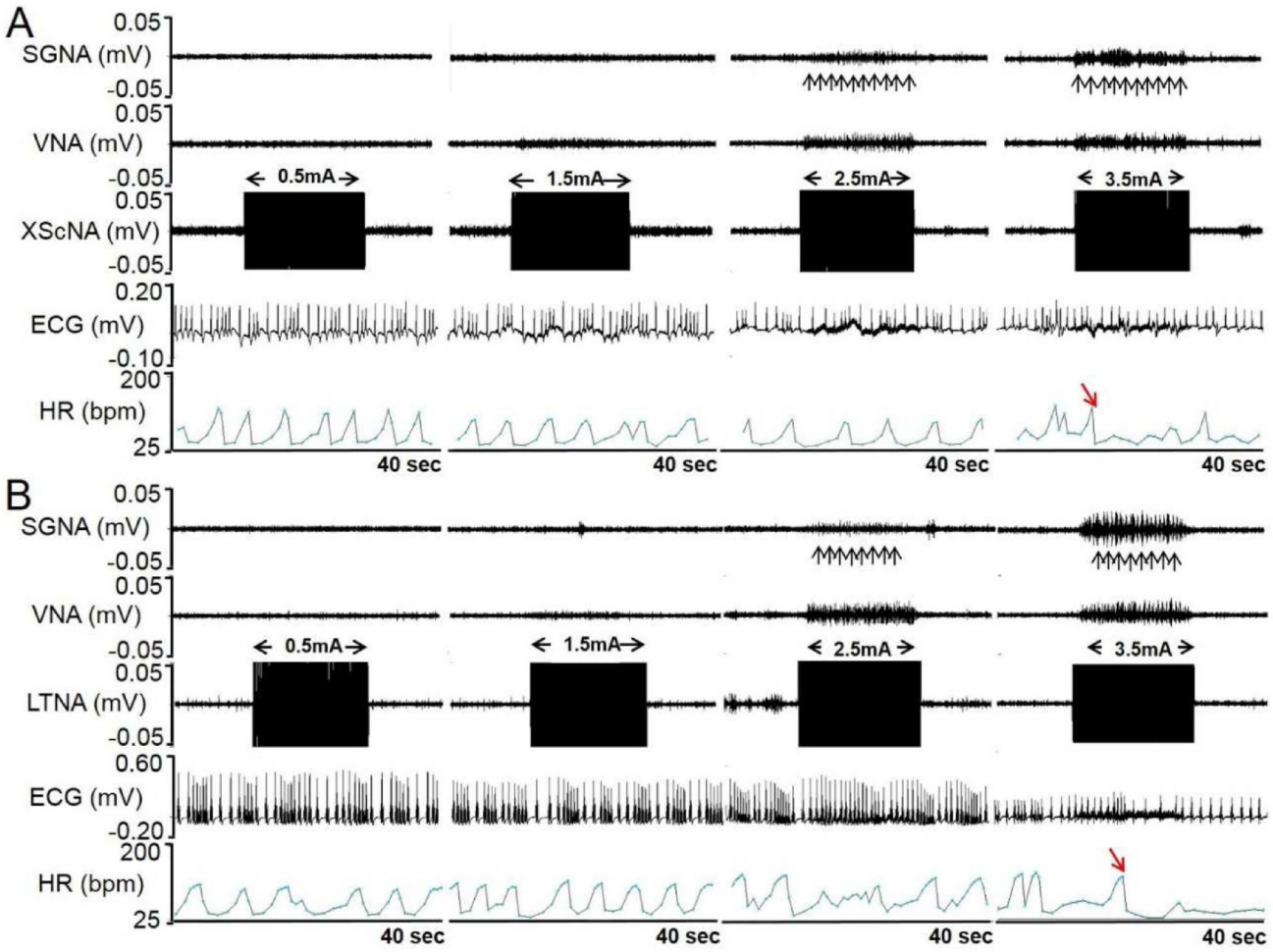

Figure 8.

The effects of ScNS on SGNA, VNA and HR. ScNS artifacts were clearly visible on XScNA and LTNA channels. There were no SGNA response at 0.5 mA in either A or B. Increasing the output to 1.5 mA, 2.5 mA and 3.5 mA resulted in increased responses along with reduced HR and HR variability (red arrows).