PRECLINICAL INVESTIGATION

Long-Term Subthreshold Electrical Stimulation of the Left Stellate Ganglion and a Canine Model of Sudden Cardiac Death

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OBJECTIVES
We sought to develop a high-yield canine model of sudden cardiac death (SCD).

BACKGROUND
Because electrical stimulation is a powerful means to elicit nerve sprouting, we hypothesize that subthreshold electrical stimulation is more effective than nerve growth factor (NGF) infusion in inducing nerve sprouting and SCD in dogs with myocardial infarction (MI) and complete atrioventricular block (CAVB).

METHODS
We gave subthreshold electrical stimulation to the left stellate ganglion (LSG) in six normal dogs for 41 ± 9 days (protocol 1) and to six dogs with MI and CAVB for 41 ± 29 days, while continuously monitoring their cardiac rhythm (protocol 2). We also monitored the rhythm of two dogs with MI, CAVB, and NGF infusion to the LSG and determined the ventricular nerve density in six healthy control dogs.

RESULTS
In protocol 1, the hearts from dogs with LSG electrical stimulation had a higher density of nerve fibers immunopositive to tyrosine hydroxylase, synaptophysin, and growth-associated protein-43 than those of normal control dogs (p < 0.01). In protocol 2, there was a high magnitude of cardiac nerve sprouting in all dogs studied. Ventricular tachycardia ≥8 beats and ≥20 beats was more frequent in dogs with electrical stimulation than in dogs with NGF infusion to the LSG (36 ± 60 and 11 ± 17 vs. 4.7 ± 6.1 and 0.1 ± 0.33 episodes per day, p < 0.05 and p < 0.03, respectively). Four of six dogs in protocol 2 had SCD.

CONCLUSIONS
Subthreshold electrical stimulation of the LSG induces cardiac nerve sprouting and sympathetic hyperinnervation and facilitates the development of a high-yield canine model of ventricular arrhythmia and SCD. (J Am Coll Cardiol 2004;43:858–64) © 2004 by the American College of Cardiology Foundation

Sudden cardiac death (SCD) continues to be a major public health problem. It is highly desirable to develop a reliable animal model to study the mechanisms of SCD and to test new drugs or devices for SCD prevention. Sympathetic activity plays an important role in the generation of spontaneous ventricular ectopy leading to SCD after myocardial infarction (MI). We have developed a canine model of SCD by long-term infusion of nerve growth factor (NGF) into the left stellate ganglion (LSG) of dogs with MI and complete atrioventricular block (CAVB) (1). This SCD model demonstrates spontaneous onset of ventricular fibrillation without investigator intervention, a diurnal variation of ventricular arrhythmia, short–long–short coupling before the onset of ventricular fibrillation, T-wave alternans (2), and a prolonged QT interval (3). However, the average daily episodes of ventricular tachycardia (VT) were low (approximately 2 per day), and the effects of NGF infusion might be short-lived (4). Subthreshold electrical stimulation to the central nervous system effectively elicits nerve sprouting, resulting in a kindling model of epilepsy (5,6). These findings led us to hypothesize that subthreshold electrical stimulation to the LSG of dogs with CAVB and MI would induce sympathetic nerve sprouting and an increased incidence of ventricular arrhythmia. If so, using a long-life cardiac pacemaker to induce nerve sprouting from the LSG may create a more useful model of ventricular arrhythmia and SCD. In this study, we intended to test the hypothesis that subthreshold electrical stimulation is more effective than NGF infusion to induce nerve sprouting and ventricular arrhythmia and to determine whether 24-h continuous arrhythmia monitoring is feasible in this animal model.

METHODS
The research protocol was approved by the Institutional Animal Care and Use Committee at Cedars-Sinai Medical
Abbreviations and Acronyms

CAVB = complete atrioventricular block
DSI = Data Sciences International
GAP43 = growth-associated protein-43
ICD = implantable cardioverter-defibrillator
LSG = left stellate ganglion
LTP = long-term potentiation
MI = myocardial infarction
NGF = nerve growth factor
SCD = sudden cardiac death
SYN = synaptophysin
TH = tyrosine hydroxylase
VT = ventricular tachycardia

Center and followed the guidelines of the American Heart Association. We used adult female mongrel dogs for the study.

Animals and surgery. PROTOCOL 1: SUBTHRESHOLD ELECTRICAL STIMULATION OF THE LSG IN NORMAL DOGS. The chests of six dogs (23.6 ± 2.6 kg [range 20.5 to 27.3]) were opened via the left fourth intercostal space under isoflurane anesthesia. We first defined the stimulation threshold for each dog by stimulating the LSG at 20 Hz (5-ms pulse width). The electrical current (mA) strength that elicited an abrupt increase in systolic blood pressure and a heart rate of >20% from baseline was defined as a stimulation threshold. An active fixation pacemaker lead was then secured to the LSG and connected to a subcutaneous Medtronic Itrel neurostimulator (n = 3) or a modified pacemaker (n = 3; model 1275, Guidant Corp., St. Paul, Minnesota) to give rapid stimulation at 20 Hz (0.45-ms pulse width) or 5 Hz (1.9-ms pulse width), respectively. The actual pacing time was roughly the same for the 20-Hz and 5-Hz pacing methods (9 vs. 9.5 ms/1 s, respectively). We then programmed the pacemaker output to 25% of the calculated voltage threshold and confirmed that this stimulation voltage did not cause any change of heart rate or blood pressure. The chest was closed, and the animals, while ambulatory, were stimulated with subthreshold (25% threshold) voltage to the LSG continuously for 41 ± 9 days (range 30 to 53) before euthanasia.

PROTOCOL 2A: SUBTHRESHOLD ELECTRICAL STIMULATION OF THE LSG IN DOGS WITH MI AND CAVB. Six dogs (24.1 ± 2.1 kg [range 20.5 to 26.3]) underwent general anesthesia. Complete AV block was induced by radiofrequency ablation of the AV junction, and the ventricles were temporarily paced at 70 beats/min. We then performed a thoracotomy to determine the subthreshold stimulation current, according to the method described in protocol 1. Modified pacemakers (Guidant model 1275) were used to perform subthreshold stimulation of the LSG at 5 Hz. A Guidant defibrillation lead (model 0144 or 1047) was implanted in the right ventricle. In two dogs, the defibrillation leads were connected to an implantable cardioverter-defibrillator (ICD) programmed to the monitor-only mode for backup pacing at 40 beats/min and for rhythm monitoring (1). In the remaining four dogs, we placed an additional bipolar lead in the right atrium. Both atrial and ventricular leads were connected to a Data Sciences International (DSI) transmitter in a submuscular chest pocket for continuous recording, with a sampling rate of 1,000/s. A Guidant pacemaker (model 1275) was used for continuous backup pacing at 40 beats/min. We then ligated the left anterior descending coronary artery below the first diagonal branch to create MI (1). The chests were closed, and the dogs were allowed to recover. In dogs 1 through 4, subthreshold LSG stimulation continued until euthanizing or sudden death. To rule out the possibility that subthreshold electrical stimulation of the LSG directly caused arrhythmia, we discontinued LSG stimulation in dogs 5 and 6 after 30 days, but continued arrhythmia monitoring until euthanizing or sudden death.

PROTOCOL 2B: NGF INFUSION TO THE LSG IN DOGS WITH MI AND CAVB. To compare the incidence of ventricular arrhythmia in dogs with NGF infusion, we performed two additional studies. These two dogs (body weight 25 and 24 kg, respectively) had MI, CAVB, and NGF infusion to the LSG via an osmotic pump (1). In addition, a modified DSI transmitter was implanted for continuous electrographic telemetry.

Electrophysiologic monitoring and data analyses. The ICD was interrogated at weekly intervals to determine the incidence of ventricular arrhythmia. The ICD declared VT episodes once the ventricular rate exceeded 100 beats/min for 8 of 10 beats. The data telemetered by the DSI transmitters were stored in the computer. Among them, 25% of the data were randomly selected for manual analyses of the number of phase 2 VT episodes (1).

Immunocytochemistry. IMMUNOSTAINING. After euthanasia, six hearts from protocol 1, four hearts (2 with LSG stimulation and 2 with NGF infusion to the LSG) from protocol 2, and six hearts from normal control dogs that did not undergo any procedure were removed and fixed in 4% buffered formalin for 1 h and preserved in 70% ethanol. Tissues were obtained from multiple sites of the left ventricular free wall. The nerve markers tyrosine hydroxylase (TH), synaptophysin (SYN), and growth-associated protein-43 (GAP43) were stained on 5-μm transmural sections. The immunostaining methods and the sources of antibodies have been reported elsewhere (1).

NERVE DENSITY MEASUREMENT. For protocol 1, the nerve density, expressed as the total number of nerve fibers per unit area (number of nerves/mm²) or the total area of nerve fibers per unit area (μm²/mm²), was measured by a computer-assisted image analysis system (Image-Pro Plus 4.0, MediaCybernetics, Carlsbad, California), according to methods described previously (3,7).

For protocol 2, the nerve density (number of nerves/mm²) was measured manually, so that the results could be compared with those of a previous study (1) in which the nerve density was determined manually after NGF infusion to the LSG.
Table 1. Cardiac Nerve Density With 20-Hz Versus 5-Hz Stimulation to the Left Stellate Ganglion

<table>
<thead>
<tr>
<th></th>
<th>Left Ventricle</th>
<th>Right Ventricle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 Hz</td>
<td>5 Hz</td>
</tr>
<tr>
<td>GAP43</td>
<td>268 ± 250</td>
<td>209 ± 102</td>
</tr>
<tr>
<td>SYN</td>
<td>594 ± 251</td>
<td>759 ± 396</td>
</tr>
<tr>
<td>TH</td>
<td>86 ± 20</td>
<td>99 ± 62</td>
</tr>
</tbody>
</table>

Data are presented as the mean value ± SD (number of nerves per mm²). All nerves were counted with an imaging analysis system. There was no difference in cardiac nerve density between the 20-Hz and 5-Hz stimulation rates. GAP43 = growth-associated protein-43; SYN = synaptophysin; TH = tyrosine hydroxylase.

Statistical analysis. Data are presented as the mean value ± SD. The nonpaired t test was used to compare the mean values of nerve density. Analysis of variance with Dunn’s (Bonferroni) correction was used to compare the mean values of three or more groups. A p value ≤0.05 was considered statistically significant.

RESULTS

Protocol 1: Normal Dogs

The purpose of protocol 1 was to test the hypothesis that LSG subthreshold stimulation can induce nerve sprouting and sympathetic hyperinnervation in normal ventricles.

Cardiac nerve density. The ventricular nerve density with 20 Hz (n = 3) or 5 Hz (n = 3) subthreshold stimulation was similar (Table 1). Therefore, the data were pooled together for subsequent analyses. The nerve densities in the left ventricle were significantly higher (p < 0.01) in dogs with LSG subthreshold stimulation than in normal control dogs (Figs. 1 and 2). With GAP43 staining, the maximum amount of nerve density in the control group was always less than the minimum amount of nerve density in the LSG stimulation group, indicating that significant nerve sprouting occurred in all dogs studied. The nerve densities induced by subthreshold LSG stimulation were significantly higher than those induced by NGF infusion, as observed in a previous study (1).

Heart weight. The heart weight of dogs with LSG subthreshold electrical stimulation was 208 ± 33 g (8.4 ± 0.98 g/kg body weight), significantly heavier than that of the control dogs (172 ± 20 g [7.0 ± 0.42 g/kg body weight]; p < 0.02 and p < 0.05, respectively).

Stellate ganglia. Histologic evaluation of the left and right stellate ganglia was performed in four dogs. Neither the left nor right stellate ganglion had neuronal degenerative changes on Nissl staining or macrophage infiltration on immunostaining for macrophages (anti-CD68, alpha1 antitrypsin, and alpha1 anti-chemotrypsin). These histologic data indicate that the subthreshold stimulation did not cause neuronal injury in the LSG.

Protocol 2: Dogs With MI and CAVB

Protocol 2 was performed to further test our previous hypothesis that sympathetic nerve density is crucial for the development of ventricular arrhythmia in electrically remodeled ventricles.

Cardiac nerve density and heart weight. The densities of GAP43-positive, SYN-positive, and TH-positive nerves (measured manually as number of nerve fibers/mm²) in the left ventricle were 177.5 ± 37.4, 163 ± 31.6, and 72.3 ± 22.6, respectively. These nerve densities were significantly higher than or comparable with (p < 0.03, p < 0.03, and p = NS, respectively) the nerve densities induced by NGF infusion to the LSG in our previous study (1). Figure 3 shows examples. The average heart weight (289 ± 23 g) of these dogs with MI, CAVB, and LSG stimulation was also increased compared with normal unstimulated control dogs.

Ventricular arrhythmia and SCD. SUBTHRESHOLD ELECTRICAL STIMULATION OF THE LSG. Recordings from the DSI transmitter and ICD showed that there were very frequent spontaneous VT episodes. We observed 36 ± 60 VT episodes per day (range 1 to 188). Among them, 11 ± 17 VT
episodes per day (range 0 to 52) were longer than 20 beats. The period from the first surgery to either euthanasia or SCD was 41 ± 29 days (range 11 to 77). Four of six dogs died suddenly at days 77, 17, 11, and 37 days (Table 2). The longest VT event recorded in dog 1 started on day 73 at 3:00 PM and lasted for 41 days (range 11 to 77). Four of six dogs died suddenly (Fig. 5A) occurred during subthreshold stimulation of the LSG. In dog 5, the LSG stimulation was turned off on day 30, and ventricular fibrillation and sudden death (Fig. 5B) occurred a week later, when the LSG was not being stimulated.

INFUSION OF NGF TO THE LSG. Nerve growth factor was continuously infused to the LSG for 82 days in dog 7 and for 44 days in dog 8 (Table 2). Similar to our previous data, these two dogs had cardiac hypertrophy and an increase of VT. The hearts weighed 250 and 320 g, respectively. The DSI recordings showed an average of 4.7 ± 6.1 VT episodes per day (range 0 to 16), comparable to our previous study. Among them, 0.1 ± 0.33 VT episodes per day (range 0 to 1) were longer than 20 beats, and the nonsustained polymorphic VT episodes were 1.0 ± 1.2 per day (range 0 to 4).

DISCUSSION

This study showed that in normal dogs, subthreshold stimulation of the LSG induced cardiac nerve sprouting, sympathetic hyperinnervation, and ventricular hypertrophy. Subthreshold LSG stimulation in dogs with MI and CAVB resulted in frequent episodes of ventricular arrhythmias and a high incidence of SCD in addition to nerve sprouting and hypertrophy. The magnitude of nerve density and the frequency of VT induced by subthreshold electrical stimulation to the LSG were significantly higher than those elicited by NGF infusion to the LSG. Therefore, subthreshold LSG electrical stimulation induced anatomic remodeling (hypertrophy) and neural remodeling (nerve sprouting), which can be pro-arrhythmic in dogs with electrically remodeled ventricles. In this study, we also demonstrated that it is feasible to use a DSI transmitter to continuously monitor cardiac arrhythmia in a canine model of SCD.

Comparison with NGF infusion to the LSG. The magnitude of left ventricular nerve sprouting induced by LSG stimulation (protocol 2A) in the current study was much greater than that induced by NGF infusion to the LSG in dogs with both MI and CAVB in our previous study (1). There was also an obvious trend toward a higher density of TH-immunopositive nerve fibers, although not statistically significant, in the LSG stimulation group compared with the NGF infusion group (72.3 ± 22.6 vs. 33.2 ± 12.1 nerve fibers/mm²). Accompanied with the greater magnitude of nerve sprouting, there was a higher incidence of VT (36 ± 60/day) and SCD (4 of 6) in dogs with LSG subthreshold stimulation than in dogs with NGF infusion to the LSG (2.0 ± 2.0 VT/day; 4 of 9 dogs with SCD) (1). These findings further highlight the importance of LSG in arrhythmogenesis (8). Although increased sympathetic nerve density of left origin may be causally related to the development of VT and SCD in MI (1), we cannot exclude the possibility that a reflex withdrawal of vagal activity resulting from LSG subthreshold stimulation also contributed to arrhythmogenesis in the current model (9). A previous study by Schwartz et al. (10) showed that electrical stimulation to the LSG can induce ventricular arrhythmia even in normal dogs. Although the stimulus strength is subthreshold, we cannot completely rule out the direct effects of electrical current on arrhythmogenesis. We did
not monitor cardiac rhythm in normal dogs with LSG stimulation, but none of those six dogs with cardiac sympathetic hyperinnervation developed SCD. Dogs with MI and CAVB but no NGF infusion to the LSG had a low incidence of SCD (1). Putting these data together, we propose that the interaction between neural remodeling, resulting from sympathetic nerve sprouting, and electrical remodeling is the mechanism underlying ventricular arrhythmia after MI. Neural remodeling may serve as a trigger of inducing VT in electrically remodeled myocardium (substrate).

**Variations in nerve staining.** Similar to our previous studies (1,3,7,11), there is a large standard deviation of the nerve counts. Although the mechanisms by which large variations occur are not completely understood, we have found that variation of the magnitude of nerve sprouting among individual subjects to be a rule rather than an exception. It is possible that differential nerve sprouting in human subjects partially accounts for the differential propensity of cardiac arrhythmias after MI.

We also found a large difference between the densities of GAP43- and TH-immunolabeled nerves. The expression of TH often lagged behind and showed a much lower quantity initially (7). The implication was that these sprouting nerves (GAP43-positive) might not be the sympathetic nerves, or that they had not yet matured to express TH at the time of histologic study.

**Mechanisms of LSG stimulation-induced nerve sprouting.** Long-term potentiation (LTP) is a use-dependent form of long-lasting enhancement of synaptic efficacy (12). Brief tetanic stimulation of the preganglionic nerves to the superior cervical ganglion or ganglionic cells induces this LTP phenomenon (12,13). Electric currents delivered to the LSG can stimulate both the presynaptic ganglionic fibers and postsynaptic ganglionic cells in the LSG. Either mode of stimulation can elicit LTP in the LSG. In the central nervous system, LTP-like stimulation can elicit nerve sprouting (5). Stimulation that induces LTP can evoke oncogene c-fos expression in the hippocampus (14,15). Transcription c-fos has been associated with neurite outgrowth in PC12 pheochromocytoma cells (16). It is reasonable to postulate that cardiac sympathetic nerve sprouting, as shown in this study, was a direct result of long-term electrical stimulation to the LSG.

![Figure 3. Cardiac nerves immunolabeled with GAP43, SYN, and TH (brown twigs) in protocol 2. The nerve density of the myocardium in dogs with left stellate ganglion stimulation (LSGS) is higher than the nerve density in dogs with NGF infusion to the LSG. Magnification 40×. Abbreviations as in Figure 1.](image)

**Table 2. Summary of Protocol 2**

<table>
<thead>
<tr>
<th>Dog No.</th>
<th>LSGS or NGF</th>
<th>DSI or ICD Recording</th>
<th>Survival Period (days)*</th>
<th>SCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LSGS</td>
<td>DSI</td>
<td>77</td>
<td>Yes†</td>
</tr>
<tr>
<td>2</td>
<td>LSGS</td>
<td>DSI</td>
<td>25 (sacrificed due to sepsis)</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>LSGS</td>
<td>DSI</td>
<td>17</td>
<td>Yes‡</td>
</tr>
<tr>
<td>4</td>
<td>LSGS</td>
<td>DSI</td>
<td>11</td>
<td>Yes§</td>
</tr>
<tr>
<td>5</td>
<td>LSGS</td>
<td>ICD</td>
<td>37</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>LSGS</td>
<td>ICD</td>
<td>76</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
<td>NGF</td>
<td>DSI</td>
<td>82</td>
<td>No</td>
</tr>
<tr>
<td>8</td>
<td>NGF</td>
<td>DSI</td>
<td>44 (sacrificed due to sepsis)</td>
<td>No</td>
</tr>
</tbody>
</table>

*Period from the first surgery to the second surgery or to sudden cardiac death (SCD). †Sudden cardiac death occurred after the transmitter battery ran out. ‡Died suddenly while the transmitter pocket was being surgically revised. Dog was off monitor, and no rhythm was recorded. §Ventricular fibrillation documented.

DSI = Data Sciences International system; ICD = implantable cardioverter-defibrillator; LSGS = subthreshold electrical stimulation left stellate ganglion; NGF = nerve growth factor infusion to the left stellate ganglion.
Stimulation of the LSG and a model of SCD. In this study, we demonstrated that subthreshold electrical stimulation to the LSG is more potent than NGF infusion in inducing cardiac sympathetic hyperinnervation and ventricular arrhythmia. We included CAVB in this model to maximize the repolarization abnormalities (17). Although combined neural and electrical remodeling is needed for the development of SCD, a weakness of this model is that most patients who have SCD do not have CAVB. Other investigators (18) have demonstrated that it is possible to develop a model of SCD without CAVB. In that model, coronary artery occlusion and submaximal exercise were used to induce ventricular arrhythmia. We also demonstrated the feasibility of long-term implantation of a DSI transmitter for continuous monitoring of heart rhythm. Therefore, this study demonstrates an effective canine model for studying the mechanism of, and testing therapeutic modalities for, SCD.

Conclusions. Subthreshold electrical stimulation of the LSG is a potent method for inducing nerve sprouting in the heart. Applying this method to dogs with CAVB and MI resulted in a high incidence of VT and SCD. The data further support the hypothesis that sympathetic nerve sprouting is an important arrhythmogenic mechanism in electrically remodeled myocardium. This method might be useful in developing a high-yield animal model of SCD.

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