Chronic Electrical Neuronal Stimulation Increases Cardiac Parasympathetic Tone by Eliciting Neurotrophic Effects


Rationale: Recently, we provided a technique of chronic high-frequency electric stimulation (HFES) of the right inferior ganglionated plexus for ventricular rate control during atrial fibrillation in dogs and humans. In these experiments, we observed a decrease of the intrinsic ventricular rate during the first 4 to 5 months when HFES was intermittently shut off.

Objective: We thus hypothesized that HFES might elicit trophic effects on cardiac neurons, which in turn increase baseline parasympathetic tone of the atrioventricular node.

Methods and Results: In mongrel dogs atrial fibrillation was induced by rapid atrial pacing. Endocardial HFES of the right inferior ganglionated plexus, which contains abundant fibers to the atrioventricular node, was performed for 2 years. Sham-operated nonstimulated dogs served as control. In chronic neurostimulated dogs, we found an increased neuronal cell size accompanied by an increase of choline acetyltransferase and unchanged tyrosine hydroxylase protein expression as compared with unstimulated dogs. Moreover, β-nerve growth factor (NGF) and neurotrophin (NT)-3 were upregulated in chronically neurostimulated dogs. In vitro, HFES of cultured neurons of interatrial ganglionated plexus from adult rats increased neuronal growth accompanied by upregulation of NGF, NT-3, glial-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF) expression. NGF was identified as the main growth-inducing factor, whereas NT-3 did not affect HFES-induced growth. However, NT-3 could be identified as an important acetylcholine-upregulating factor.

Conclusions: HFES of cardiac neurons in vivo and in vitro causes neuronal cellular hypertrophy, which is mediated by NGF and boosters cellular function by NT-3-mediated acetylcholine upregulation. This knowledge may contribute to develop HFES techniques to augment cardiac parasympathetic tone. (Circ Res. 2011;108:1209-1219.)

Key Words: atrial fibrillation · tachyarrhythmias · acetylcholine · nervous system

During the last 10 years, techniques have been developed to directly and selectively interfere with the intrinsic cardiac neural system via electric stimulation catheters or leads in animals and patients.¹⁻⁵ The baseline observation for these applications was that depolarization of intracardiac neural fibers by high-frequency electric stimulation (HFES) delivers neurotransmitters that subsequently act locally on cardiac structures (eg, stimulation of sympathetic fibers along the coronary sinus selectively increases left ventricle inotropy).⁵ Furthermore, acute, short-term endocardial HFES of the right inferior ganglionated plexus (RIGP) elicits selective negative dromotropic effects in awake patients and slows atrioventricular (AV) conduction during atrial fibrillation.³ The RIGP is located between the inferior caval vein, the left atrium and the ostium of the coronary sinus. It contains abundant parasympathetic fibers that affect AV node conduction.⁶⁻⁷ After proof of concept in humans, we and others developed a technique for chronic endocardial⁸⁻⁹ and epicardial¹⁰ neurostimulation of the RIGP for ventricular rate control during atrial fibrillation (AF) in dogs. Of note, the negative dromotropic effect during endocardial HFES could be maintained throughout the entire neurostimulation period of 2 years.⁹ The effect of HFES on neural structures rapidly terminated after intermittent cessation of stimulation. This is mainly attributable to the short half-life time of the neurotransmitter acetylcholine (ACh), the main parasympathetic neurotransmitter. One interesting phenomenon during chronic HFES of the RIGP was a significant time-dependent decrease

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of the intrinsic ventricular rate during AF in the first 4 to 5 months when stimulation was intermittently switched off.9 The same observation was made by Zhang et al when epicardial HFES of the RIGP was delivered for 5 weeks.10 We thus hypothesized that, besides acute transmitter release, HFES might elicit neurotrophic effects on cardiac neurons, which in turn might increase baseline parasympathetic tone of the AV node. Therefore, the present study aimed to investigate the impact of HFES on neural growth and morphology of intrinsic cardiac neurons and to elucidate the underlying molecular mechanisms. For this purpose, we used an in vitro model of chronic parasympathetic neurostimulation in dogs and an in vitro model of cultured rat parasympathetic neurons under conditions of HFES.

Methods

Animal care, investigations, and euthanasia were performed according to the guidelines of the American Society of Physiology and with permission of the appropriate authorities.

Chronic Electrical Stimulation of the RIGP in Dogs

Six male mongrel dogs (19 to 27 kg) were anesthetized (propofol, 5 mg/kg body weight intravenously) and intubated, while breathing spontaneously. Analgosedation was maintained with fentanyl (0.5 to 1 mg/kg per hour) and pentobarbital (0.3 mg/kg per hour). The lead was identified by probatory HFES at the low interatrial septum.9 A chronic active fixation lead in the inferoseptal right atrium was then implanted at a site at which a negative dromotropic effect during induced AF was obtained in all 6 dogs. The lead was connected to a neurostimulator that was implanted subcutaneously. Chronic continuous neurostimulation with 20 Hz was performed for 2 years in 4 dogs to achieve a continuous 50% decrease of the ventricular rate during AF. Two dogs served as sham-operated nonstimulated control dogs.

Cell Culture of Neurons of Interatrial Ganglionated Plexus

Primary cultures of intrinsic neurons of interatrial ganglionated plexus (NIGP) were performed from adult Sprague–Dawley rats (Charles River, Germany). The interatrial ganglionated plexus (IAGP) was removed from the interatrial fat pad between the left and right cranial vein and the caudal vein11 and was dissociated enzymatically with collagenase (1.0 mg/mL) and trypsin (0.5 mg/mL) and then mechanically with fire-polished glass pipettes. NIGPs were grown in Neuronal Base Medium AD (PAA Laboratories GmbH, Austria) supplemented with 10% FBS, 2 mmol/L L-alanyl-L-glutamine (Gibco In Vitrogen Corp), B-27 Supplement (Gibco In Vitrogen Corp), and 10 μmol/L 5-bromo-2′-deoxyuridine (Sigma-Aldrich). Three days after cell isolation, serum-containing medium was changed to serum-free supplemented medium with 2 mmol/L L-alanyl-L-glutamine.

High-Frequency Electric Field Stimulation of NIGP

Three days after cell isolation and change of medium to serum-free, NIGPs were exposed to bipolar electric field stimulation with 1 to 3 V/cm, 0.4 ms impulse duration and different frequencies (2, 5, 20, and 50 Hz) for 48 hours with the C-PaceEP external pacing system (IonOptix, The Netherlands) as described previously.12 Because neurite outgrowth response of NIGP was maximum with 2 V/cm at 20 Hz, all experiments were performed with 2 V/cm. During the electric stimulation period cells were incubated at 37°C with 5% CO2.

RNA Preparation, First-Strand cDNA Synthesis, and Quantitative Real-Time RT-PCR

RNA extraction, first-strand cDNA synthesis and quantitative real-time PCR experiments were performed as described previously.13 PCR primers and fluorogenic probes for NGF, BDNF, CNTF, GDNF, NT-3, and the endogenous control were purchased from Applied Biosystems (Foster City, CA). The assay numbers were as follows: Rn01533872_m1 (Ngfb), Rn00560868_m1 (Bdnf), Rn00755092_m1 (Cntf), Rn00569510_m1 (Gdnf), Rn00579280_m1 (Ntf3), Rn00560865_m1 (β-2 microglobulin).

ELISA and Western Blot

At 0, 24, and 48 hours the conditioned medium was collected and assayed. NGF, BDNF, CNTF, GDNF, and NT-3 were assessed by ELISA kits from R&D Systems (Minneapolis, MN) according to the protocol of the manufacturer. Western blot experiments were performed as described previously.14

Measurement of Neurite Outgrowth and Cell Body Size

To measure the net increase of neurite outgrowth of NIGP, 10 light-optical microscopic images (×200 magnification) were taken before (0 hours) and after (48 hours) the application of HFES of each preparation. The mean length of all neurocyte cell extensions was measured with the help of the ImageJ 1.43 software (http://rsbweb.nih.gov/ij/download.html) as described previously.15 The net increase of neurite outgrowth was assessed by subtraction of the mean neurite outgrowth after 48 hours of HFES from the mean neurite outgrowth before HFES. Relative cell body size was also calculated with the help of the ImageJ software. The neurons were interactively contoured. Finally, all values were calculated as relative values and were statistically compared with each other.

Histo- and Cytofluorescence Experiments

Hearts were fixed in 4% formaldehyde. The right atrium was opened and the position of the neurostimulation lead inside the right atrium was inspected. For microscopic analysis a 1×1 cm tissue block around the lead tip was removed and the lead was carefully removed. The lead channel was marked with a blue dye to recognize the former lead position during further analysis. From the paraffinized tissue blocks 2 μm thick sections were taken, mounted on charged slides, and stained as described.

ACh Analysis

NIGPs were isolated as described above and incubated with 100 μmol/L eserine (Sigma–Aldrich, E8375) to inhibit acetylcholine

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er with different frequencies. HFES-induced increase of cell body size was accompanied by increased expression of growth associated protein 43 (GAP-43) and neurite outgrowth (Figure 2B and 2C). To analyze whether neuronal cell growth during HFES was dependant on depolarization of the neurocyte outer cell membrane, we applied lidocaine (100 μmol/L) during HFES. This concentration was specifically chosen, because it has been demonstrated that the half-maximum inhibitory concentration values for lidocaine to block tetrodotoxin-resistant (TTX-R) Na⁺ channels, and thereby inhibiting nerve conduction in rat sensory neurons, were 73 μmol/L and 128 μmol/L, respectively.17,18 Lidocaine completely reversed the HFES-induced increase of GAP-43 levels and neurite outgrowth (Figure 2B and 2C).

HFES of NIGPs Resulted in an Upregulation of NGF, GDNF, NT-3, CNTF, and BDNF Gene and Protein Expression
To characterize the mechanisms of increased neuronal growth during HFES, the gene and protein expression levels of the neurotrophins NGF, GDNF, NT-3, CNTF, and BDNF were assessed by real-time PCR and ELISA (Figure 3). There was an upregulation of all neurotrophins at all frequencies despite for NT-3 and HFES with 50 Hz. Upregulation of all neurotrophins was strongest during HFES with 20 Hz.

HFES-Induced Growth of NIGPs Is Mediated by NGF
To investigate by which neurotrophin the HFES-induced increase in neuronal growth is mediated, we performed loss-of-function experiments using neurotrophin neutralizing antibodies during HFES with 20 Hz. NGF neutralizing antibodies completely prevented the HFES-induced neurite outgrowth, whereas BDNF, NT-3, CNTF, and GDNF neutralization remained without a significant effect (Figure 4A). Likewise, incubation of control NIGPs with recombinant NGF without HFES induced significant neuronal growth, whereas BDNF, NT-3, CNTF, or GDNF did not elicit such an effect (Figure 4B).

HFES of NIGPs Increased ChAT and VACHT Expression and ACh Excretion
To evaluate whether the HFES-induced morphological changes of NIGP are paralleled by functional properties, the ChAT and VACHT gene and protein expression of NIGPs exposed to HFES with different frequencies were investigated (Figure 4C through 4E). ChAT gene and protein expression increased from stimulation with 5 Hz to 20 Hz but did not show further increase during stimulation with 50 Hz,
whereas stimulation with 2 Hz remained without significant effect. The same observation was achieved for VAChT.

To investigate whether the HFES-induced increase of ChAT and VAChT is paralleled by functional upregulation of neurotransmitter release, we measured ACh concentrations in the NIGP cell culture supernatant at different time points during HFES by HPLC. HFES induced a significant upregulation of ACh at all frequencies with maximal increase at 20 Hz followed by 50 and 5 Hz (Figure 4F).

HFES of NIGPs Did Not Alter TH Expression
Only 5% of resting NIGPs displayed markers of both cholinergic and noradrenergic transmission by expressing ChAT and TH as demonstrated above. To check the influence of HFES on TH expression, NIGPs were exposed to HFES with different frequencies and TH expression was measured by Western Blot. By HFES no increase or decrease of TH expression was observed (Online Figure II). Moreover, we found an unchanged number of ChAT-positive (100%) and TH-positive (6%) neurons after exposure of NIGPs to HFES with 20 Hz for 48 hours.

NT-3 Increased ChAT and VAChT Expression and Extracellular ACh Concentration of NIGPs Without HFES
NIGPs not exposed to HFES were incubated with recombinant NGF, BDNF, NT-3, CNTF, or GDNF, and the ChAT and VAChT expression was examined by Western blotting and the ACh concentration was measured in the cell culture supernatant by HPLC. The ChAT (Figure 5A) and VAChT (Figure 5B) protein expression and ACh concentration in the cell culture supernatant (Figure 5C) significantly increased when NT-3 was applied to cultured NIGPs, whereas NGF, CNTF, GDNF, and BDNF application to NIGPs had no significant effect.

NT-3 Neutralizing, but Not NGF Neutralizing, Significantly Inhibited the HFES-Induced ChAT, VAChT, and ACh Upregulation
To check whether neurotrophins and neurotrophins associated neurocyte growth are involved in the HFES-induced upregulation of ACh, NIGPs were exposed to HFES with 20 Hz for 48 hours in the presence of NT-3 and NGF neutralizing antibodies. NT-3 neutralization blunted but did not completely prevent the HFES-induced upregulation of ChAT, VAChT and ACh, whereas NGF blockade remained without effect (Figure 5D through 5F).

Part 2: In Vivo HFES
Chronic In Vivo HFES of the RIGP Induced Neuronal Growth Paralleled by Increased ChAT but Unchanged TH Expression
Figure 6A shows immunohistologic analysis of the site at which chronic HFES with 20 Hz was applied in vivo over 2 years. The lead position and surrounding nerve fascicles were located septally and were embedded in the fat-fibrous tissue surrounding the transitional zone between the inferior vena cava, the ostium of the coronary sinus and the low right atrium. Of each animal 30 slides of the lead implantation side...
were prepared and each slide was analyzed in total in a blinded fashion. Abundant NF-positive nerve fascicles containing neurons and fibers (see black arrows in Figure 6A) were identified in the vicinity of the implanted lead. In view of the profound negative dromotropic effect of electric stimulation over the lead, which was abolished by atropine, points to the presence of several parasympathetic nerve fascicles coursing to the AV node (Figure 6B). This is further supported by subsequent histological analysis shown in Figure 7A, where blue marking dye was used during explantation of the lead (see blue arrow) to later identify the stimulation site. Numerous nerve fascicles were retrieved in close proximity to the lead implantation site which stained positive for NF and ChAT, the latter being a specific marker for parasympathetic neurons and nerve fibers (Figure 7B and 7C). By double immunostaining of ChAT and TH we found an overall reduced TH expression compared with ChAT (Figure 7D and 7E). Although all resting neurons of the RIGP were ChAT only 27% coexpressed TH. We did not observe an increased number or percentage of TH/ChAT cells after chronic in vivo HFES (control dogs: 279 total cells; stimulation: 349 cells; control: 1.13±0.23, n=4, 642 cells; P<0.001; Figure 7H). Interestingly, we only found a 1.46±0.36-fold increase of cellular size of TH/ChAT cells after chronic HFES versus control (P<0.05). The growth response of TH/ChAT cells, however, was smaller than the increase in cell size of ChAT neurons. Thus, HFES seems to induce neurotrophic effects both in ChAT and to a lesser extent in TH/ChAT cells. The latter would not be surprising because NGF has already been known to be an important neurotrophic factor in TH sympathetic neurons. However, because the NGF mediated growth response in the present study was even larger in ChAT cells the increase of cellular size of the TH/ChAT neurons is unlikely to be dependent on the expression of markers of noradrenergic transmission.

Neuronal NGF and NT-3 Is Upregulated Because of Chronic HFES of the RIGP

Because NGF was identified as a neuronal growth-inducing factor and NT-3 as an important ACh-regulating factor in vitro, alterations of these neurotrophins were investigated in vivo chronic neurostimulated dogs. We found a significant upregulation of intracellular NGF (Figure 8A, 8B, and 8E) and NT-3 (Figure 8C, 8D, and 8F) after chronic HFES of the RIGP.

Discussion

The major findings of this study are (1) chronic HFES of neurons of the RIGP in vivo increases growth and upregulation of the neurotrophins NGF and NT-3; (2) HFES of NIGP
in vitro increases neuronal cellular growth that is mediated by NGF; (3) HFES of NIGP in vitro enhances neuronal cellular function by NT-3–mediated but growth-unrelated upregulation of ChAT, VAChT expression, and ACh excretion; and (4) HFES of NIGPs in vitro and HFES of neurons of the RIGP in vivo did not alter TH expression. Taken together, these findings provide strong evidence that HFES of intrinsic cardiac neurons not only stimulates neurotransmitter release by neuronal cell membrane depolarization but may augment parasympathetic tone by eliciting distinct and diverse neurotrophic effects. Thus, the observed decrease of the baseline ventricular rate observed during long term electric neural parasympathetic stimulation may at least in part be attributable to electrically induced neurotrophic effects.

NGF is an important growth-inducing factor for NIGP, and HFES increases ChAT and VAChT gene and protein expression. A, During HFES with 20 Hz, NIGPs were treated with neurotrophin neutralizing antibodies and neurite outgrowth was measured after 48 hours (n=5 preparations). B, Control NIGPs were stimulated with recombinant neurotrophins and neurite outgrowth was assessed after 48 hours (n=5 preparations). C, Gene expression of ChAT and VAChT in NIGPs after HFES with different frequencies (n=5 preparations). D, Relative CHAT and VAChT protein expression after exposure of HFES to NIGPs with different frequencies (n=5 preparations). E, ACh concentration by HPLC of cell culture supernatant of NIGPs after exposure to HFES with different frequencies (n=5 preparations). F, ACh concentration in nmol by HPLC.

NGF is a prototypic member of the neurotrophin family that is pivotal in the differentiation, survival, and synaptic activity of the cardiac sympathetic nervous system. NGF exerts his effects by binding to its high-affinity tyrosine kinase A (TrkA) receptor. Up to now, NGF has mainly been regarded as a neurotrophic factor which initiates pathological sympathetic neural growth, eg, after myocardial infarction and in heart failure, thereby increasing sympathetic tone aggravating the progression of the disease or contributing to cardiac arrhythmias. In the present study, we provide first evidence that NGF elicits growth-inducing properties in cardiac intrinsic ganglionated neurons. It is unclear whether this growth response may exert negative functional effects. For example, increased parasympathetic neural growth in the atria might decrease overall atrial refractory period and increase its heterogeneity thereby favoring atrial arrhythmias. On the other hand, increased parasympathetic tone has since long been advocated to be protective against lethal arrhythmias, ischemia, and deterioration of heart failure. Thus, it seems intriguing to speculate whether approaches, which increase the NGF expression in cardiac parasympathetic neurons, may be used to augment chronic baseline parasympathetic cardiac tone. HFES might be a target technology suitable to be developed to achieve this. A first clinical application might be chronic selective parasympathetic stimulation of the RIGP for ventricular rate control during AF. Neurotrophic effects in addition to the acute release of neurotransmitters by electric stimulation might increase neurostimulation efficacy and potentially decrease energy consumption and thereby device longevity.

In the present study, a frequency optimum of the achieved neurotrophic effects (both NGF- and NT-3–mediated effects) was found at 20 Hz. The observed frequency-neurotrophic response relationship was similar to the one reported for the acute electrophysiological effect of HFES (eg, acute prolon-
gation of AV conduction within the first second after onset of neural stimulation). Thus, both the immediate onset depolarization dependent liberation of neurotransmitters and the more latent upregulation of protein expression share similar frequency optima. This may be relevant for future neurostimulation device designs because efforts to induce mere electrophysiological effects by HFES without inducing neurotrophic effects by means of choosing differential HFES frequencies may not be successful (eg, choosing a distinct frequency for electrophysiological and another for neurotrophic effects). It is intriguing to speculate whether lower frequency neural stimulation (eg, as applied during clinical cervical vagal nerve stimulation) may also elicit neuronal growth effects. Our preliminary results on short-term HFES in vitro with lower frequencies (2 Hz) do not support such a hypothesis. In fact, there seems to be a lower frequency limit for neurotrophic effects as induced by short-term (48 hours) electric stimulation in vitro. However, this does not exclude the possibility that longer duration electric stimulation with lower frequencies in vivo may still induce trophic effects. This will have to be evaluated in further in vivo experiments on chronic neurostimulation with lower frequencies (eg, cervical vagal stimulation). Of note, De Ferrari et al reported the favorable effects of chronic cervical vagal stimulation in heart failure patients and observed a significantly increased heart rate variability (pNN50) after 3 to 6 months of neural stimulation. The measurement of heart rate variability was performed in continuous Holter recordings but neural stimulation was only performed for maximal 25% of the total recording time. Thus, it seems at least possible that other factors than an immediate, short-lived transmitter release as achieved by neural stimulation may have caused an increase of heart rate variability and associated baseline parasympathetic tone. Alternatively, the latter might completely be attributed to the improved hemodynamic state of the heart failure patients.

A second interesting finding of the present study was that the neurotrophin NT-3 seems to exert neurotrophic effects on cardiac neurons, which are beyond mere morphological growth effects. It has been demonstrated that NT-3 participates in the maturation of normal transmitter packets in developing neurons, and that NT-3 modulates muscarinic acetylcholine receptor signaling. Furthermore, it has been shown that NT-3 rapidly increases the frequency of spontaneous action potentials, and it synchronized excitatory synaptic activities in developing cortical neurons via binding to the tyrosine kinase C receptor. In the present study, we could demonstrate that NT-3 increases ACh availability in cardiac parasympathetic neurons by upregulation of ACh-synthesizing enzymes.
Up to now, there is a limited body of evidence about the expression of different neurotrophins by cardiac parasympathetic neurons and its potential physiological role. Hasan and Smith demonstrated that extracardial parasympathetic ganglionated neurons have the ability to express NGF.28 Furthermore, several studies have reported on differential expression of NGF by sympathetic cardiac neurons, eg, upregulation of NGF in cardiac ischemia and down regulation in congestive heart failure.13,15 We recently could demonstrate that sympathetic neurons of the superior cervical ganglia (SCG) have the ability to express and excrete NGF, CNTF, GDNF, and NT-3 in the extracellular space to mediate auto-/paracrine effects.15 However, we failed to detect BDNF on mRNA and protein level in sympathetic neurons of SCG.15

The present study extends these findings by providing first evidence that, besides NGF, intrinsic cardiac parasympathetic ganglionated neurons express BDNF, CNTF, GDNF, and NT-3 mRNA with NGF and NT-3 exerting measurable neuronal cell size compared with sham-operated nonstimulated dogs (control: 1 ± 0, n=2, 349 cells; stimulation: 1.86 ± 0.23, n=4, 642 cells).

Figure 6. The neurostimulation lead implantation site. A, Demonstrating the site where chronic neurostimulation was applied to the RIGP by neurofilament (NF) staining. Abundant NF-positive nerve fascicles containing neurons and fibers (see black arrows) were identified in the vicinity of the implanted lead. The section enlargement demonstrates a nerve fascicle with NF-positive neurons and nerve fibers. SS indicates stimulation site. B, Demonstrating the location of the RIGP in the right atrium and the relationship to the AV node (AVN). IVC indicates inferior vena cava; SVC, superior vena cava.

Figure 7. Chronic in vivo HFES of the RIGP increases neuronal cell size and ChAT protein expression. A, Hematoxylin/eosin-stained paraffin section showing the stimulation site where HFES was applied. SS indicates stimulation site. Blue arrow, Lead channel, which was marked with a blue marking dye to recognize the former lead position during analysis; black arrow, nerve fascicles. B and C, Representative sections of double immunostaining with ChAT and NF demonstrating neurons of the RIGP in a sham-operated nonstimulated dog and a chronic neurostimulated dog over 2 years. D and E, Representative sections demonstrating neurons of the RIGP double immunostained with ChAT and TH in a sham-operated nonstimulated dog and a chronic neurostimulated dog over 2 years. F, By chronic HFES of the RIGP for 2 years, no significant alterations of neuronal TH expression were noticed (control: 1 ± 0, n=2, 279 cells; stimulation: 1.13 ± 0.19, n=4, 602 cells). G, ChAT immunoreactivity, normalized to cell size, was significantly higher in chronic neurostimulated dogs compared with control dogs (control: 1 ± 0, n=2, 379 cells; stimulation: 1.76 ± 0.24, n=4, 631 cells). H, Chronic electrostimulation significantly increased
morphological and functional effects. Of note, all these neurotrophins were also detected in the cell culture supernatant suggesting that parasympathetic neurons possess the ability to excrete these neurotrophins into the extracellular space possibly affecting other neuronal and nonneuronal cell types. Although research about the exact role and regulation of these neurotrophins but also of CNTF, GDNF, and BDNF in the healthy and diseased heart is still in its infancy it certainly deserves future attention.

Finally, in line with previous studies, we observed that neurons of the RIGP and IAGP displayed markers of both cholinergic and noradrenergic transmission by expressing ChAT and TH. This phenomenon was found in 5% of resting NIGPs. Likewise, Hoard et al demonstrated dual cholinergic and noradrenergic properties with 21% TH expression in adult mice intrinsic cardiac neurons maintained in culture.29 Similarly, Hasan and Smith showed that surgical sympathetic denervation of the intrinsic cardiac ganglia did not affect VMAT expression (a sympathetic marker protein) but significantly reduced VACHT and NGF expression, thus pointing to a significant dependence of parasympathetic neuronal growth on sympathetic neuronal input.30 Of note, in our study HFES did not affect immunoreactivity of TH in cardiac ganglia but significantly increased NGF and VACHT expression. Thus, in view of these 2 studies, it seems possible that HFES in vivo concomitantly stimulated intracardiac sympathetic axons projecting to the RIGP, thereby augmenting NGF and VACHT expression. However, because these effects were also elicited in vitro, a direct cellular effect of HFES on RIGP neurons independent of sympathetic neuronal input seems to be operative.

Study Limitations
In vivo, HFES may also act on local cardiac myocytes near the tip of the neurostimulation lead. We have recently demonstrated that HFES of neonatal rat atrial cardiac myocytes increases NT-3 and decreases NGF expression.13 These changes of local neurotrophin levels may have affected function and growth of the adjacent neuronal structures in this study. We did not analyze the neurotrophin expression of cardiac myocytes close to the HFES site in vivo. The profound changes of neurotrophin expression during HFES in vitro, however, point to a significant role of neurons as a source for the observed functional and morphological changes.

The control dogs in the present study were investigated under conditions of sinus rhythm, whereas in the chronic

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Figure 8. Chronic in vivo HFES of the RIGP increases neuronal NGF and NT-3 protein expression. A and B, NGF and NF double immunostaining of RIGP neurons in control and stimulated dogs. Green indicates NGF (left); red, NF (middle); merged image (right). C and D, NT-3 and NF double immunostaining of RIGP neurons in control and stimulated dogs. Green, NT-3 (left); red, NF (middle); merged image (right). E and F, Relative NGF (control: n=2, 395 cells; stimulation: n=4, 612 cells) (E) and NT-3 immunofluorescence signal (control: n=2, 312 cells; stimulation: n=573 cells) (F) in neurons of the RIGP in control and stimulated dogs.
neurostimulated dogs, AF was induced by rapid atrial pacing until persistent AF occurred. The long-term effect of AF on parasympathetic neuronal structures and neurotrophin alterations has not been investigated in this study. However, the observed upregulations of neurotrophins in vitro are strong evidence for a substantial HFES associated effect.

Because of the predominance of ChAT, we classified the neurons of the RIGP and NIGP as “cholinergic,” although up to 29% of the neurons of the RIGP and 5% of the NIGP displayed markers of both cholinergic and noradrenergic transmission by expressing ChAT and TH. This was based on the fact that even in those cells showing TH immunoreactivity the expression of TH was lower than ChAT and because HFES elicited predominant parasympathetic effects on the AV node. However, it is conceivable that distinct other neuronal subpopulations like interneurons or afferent fibers possibly containing additional neuronal markers than ChAT may be located in the RIGP. Because no attempts were made to monitor remote or afferent neural excitation and subsequent physiological responses during HFES, the role of the ChAT/TH-positive neurons remains unclear at this stage of investigation.

The reduced increase of growth by 50 Hz compared with 20 Hz can be explained by a reduced increase of neurotrophin expression by 50 Hz compared with 20 Hz. A further explanation could be a cellular injury or apoptosis attributable to HFES with 50 Hz, which increases reactive oxygen species and thereby induction could be a cellular injury or apoptosis attributable to HFES. This knowledge may contribute to development of HFES techniques to augment cardiac parasympathetic tone. This was funded by the Deutsche Forschungsgemeinschaft.

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Disclosures
None.

References


Novelty and Significance

What Is Known?
• Chronic electric vagal stimulation improves ventricular function in heart failure and decreases the risk of ventricular arrhythmias.
• Nerve growth factor (NGF) promotes pathological sympathetic hyperinnervation which is known to accelerate the development of lethal arrhythmias after myocardial infarction and heart failure.
• Because there is evidence for an increase of baseline parasympathetic tone during chronic parasympathetic stimulation, neurotrophic effects might be operative.

What New Information Does This Article Contribute?
• In vivo, chronic electric stimulation of intracardiac parasympathetic ganglia induces neuronal growth, which is accompanied by an increase in the expression of NGF and neurotrophin (NT)-3.
• In vitro, electric stimulation of intrinsic cardiac parasympathetic neurons increases neuronal cellular growth, which is mediated by NGF.
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Supplemental Material

Materials and Methods

Chronic electrical stimulation of the right inferior ganglionic plexus (RIGP) in dogs

Chronic stimulation in all animals was well tolerated without signs of distress or pain. Throughout a 6-month monitoring period, all animals were kept under close surveillance. The animal care staff checked the animals for any obvious behavioural changes twice a day. A veterinarian was present during telemetric interrogations of the device to observe any signs of pain or distress. However, it has to be mentioned, that parasympathetic stimulation was performed to achieve a decrease of ventricular rate in order to achieve a decrease of the mean ventricular rate of 50%. This is far less stimulus energy than required to achieve more pronounced AV block which certainly would induce some pain reception in awake dogs and humans. In order to substantiate the tolerability of stimulation we also have recorded videos of the dogs with/without HFES.

Neurotrophins, Antibodies and Drugs

Neurotrophins: recombinant human beta-nerve growth factor (NGF, Biomol, #86423, 1ng/ml), recombinant human brain derived neurotrophic factor (BDNF, Calbiochem, #203702, 5ng/ml), recombinant rat ciliary neurotrophic factor (CNTF, Calbiochem, #231000, 5ng/ml), recombinant rat glial derived neurotrophic factor (GDNF, Calbiochem, #PF039, 5ng/ml), recombinant human neurotrophin-3 (NT-3, Calbiochem, #480875, 5ng/ml).

Primary antibodies: mouse anti-Neurofilament (NF, Santa Cruz, sc-25652), rabbit anti-choline acetyltransferase (ChAT, Chemicon, #AB5042), mouse anti-high-affinity choline transporter 1 (CHT-1, Santa Cruz, sc-33713), goat anti-vesicular acetylcholine transporter (VACHT, Santa Cruz, sc-7717), rabbit anti-tyrosine hydroxylase (TH, Santa Cruz, sc-14007), rabbit anti-growth associated protein 43 (GAP-43, Santa Cruz, sc-10786), rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Cell Signaling, #2118).

Neurotrophin neutralizing antibodies: anti-NGF (Sigma-Aldrich, #N6655, 1:500 dilution), anti-BDNF (PeproTech, #500-P84, 5µg/ml), anti-CNTF (R&D Systems, #MAB557, 5µg/ml), anti-GDNF (R&D Systems, #MAB212, 5µg/ml), anti-NT-3 (Promega, #G1651, 5µg/ml).

Drugs: Lidocaine (1x10⁻⁵mol/l, Sigma-Aldrich).

ELISA

NGF, BDNF, CNTF, GDNF and NT-3 were assessed by ELISA kits from R&D Systems (Minneapolis, USA). NGF was detected by primary anti-rat β-NGF (DY556), NT-3 by anti-human NT-3 (DY267), CNTF by anti-rat CNTF (DY557), BDNF by anti-human BDNF (DY248) and GDNF by anti-human GDNF antibodies. Biotinylated secondary antibodies were used according to the manufacturer’s protocol. The resultant reaction of the streptavidin-horseradish-peroxidase was read at a wavelength of 450 nm on a microplate reader (Spectrafluor Plus from Tecan, Switzerland).

Western Blot

Cells were homogenized in lysis buffer containing 128 mM Tris–HCl (pH 7.6), 4.6% SDS and 10% glycerol. Cell lysates were cleared by centrifugation at 17,000g for 20 min. Extracts (100-300 µg) were subjected to electrophoretic separation through a 10% SDS-polyacrylamide gel and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% bovine serum albumin in phosphate-buffered saline containing 0.1% Tween 20 (PBST). Primary antibodies for GAP-43, ChAT, VACHT and TH detection were incubated for 1 h. Blots were washed with PBST and incubated with secondary antibodies conjugated with horseradish peroxidase (HRP) for 1 h. Finally, the ECL AdvanceWestern Blotting Detection Kit (GE-Healthcare Life Sciences, Germany) was used to visualize the bands with the advanced luminescent image analyzer LAS-3000.
from Fujifilm. Relative densitometry analyses of the bands were performed with the Multi-Gauche V3.0 software from Fujifilm.

**Histo- and cytofluorescence experiments**

Tissue block sections were deparaffinized and rehydrated through a graded ethanol series. For immunofluorescence staining, sections were incubated at room temperature with primary antibodies for 2 h, then with a secondary antibody coupled to Alexa Fluor 488 and Alexa Fluor 647 (Invitrogen). For immunocytochemistry, NIGP were fixed in 4 % paraformaldehyde, permeabilized with TritonX-100 and blocked with 1 % BSA. Cells were incubated with the primary antibody overnight at 4 °C and then with a secondary antibody coupled to Alexa Fluor 488 and Alexa Fluor 647 (Invitrogen). Nuclei were stained with DAPI. To visualize fluorescence signals for the measurement of the relative fluorescence profiles the Axiovert-200 M microscope and the AxioVision Rel.4.5 software from Zeiss were used as described previously. Briefly, neurons were interactively contoured, and the colour values were exported as tabular data. Finally, all values were calculated as relative values and were statistically compared to each other in a blinded fashion by investigators.

**ACh analysis**

NIGP were isolated as described above and incubated with 100 µmol/L eserine (Sigma-Aldrich, E8375) to inhibit acetylcholine esterase activity (AChE). The ACh concentration was determined in cell supernatant by a combination of HPLC and enzyme reaction. A solution that consisted of 400 mg/L sodium decansulfonate, 50 mg/L EDTA-2Na and 5 g/L potassium hydrogen carbonate was delivered as the mobile phase at a rate of 150 µL/min. 100 µL of samples were injected into the HPLC system (BioRad BioLogic Duo Flow, BioRad BioLogic QuadTec UV–Vis Detector, BioRad BioFac Fraction Collector). After passage through the separation column (AC-GEL, Eicom, Japan), 100 µL fractions were collected. With a high rate of reproducibility, ACh was detected as the mean of fractions 28 to 30 (18.76 to 20.76 min) with the help of the Amplex Red Acetylcholine / Acetylcholinesterase Assay Kit (Invitrogen, A12217). Briefly, first, AChE converts the acetylcholine substrate to choline. Choline is in turn oxidized by choline oxidase to betaine and H₂O₂, the latter of which, in the presence of horseradish peroxidase, reacts with Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine). The reaction results in a magenta colour, which was quantified using a microplate reader set to 540 nm without wavelength correction.

**References**

Figure I

(A) All NIGP (100%) stained positively for NF, red=NF, green=nucleus (DAPI). (B) Double immunostaining for ChAT and TH. Only 5% of neurons stained positively for TH, while all neurons stained positively for ChAT (total cells: 489, ChAT: 489 (100%), TH: 24 (5%), green=ChAT, magenta=TH. To confirm the parasympathetic origin NIGP were stained for (C) the vesicular acetylcholine transporter (VACHT=green, nucleus=blue) and (D) the high-affinity choline transporter 1 (CHT-1=green, nucleus=blue).
Relative tyrosine hydroxylase (TH) protein expression remained unchanged in NIGP after exposure to HFES with different frequencies.