New Aspects for the Treatment of Cardiac Diseases Based on the Diversity of Functional Controls on Cardiac Muscles: The Regulatory Mechanisms of Cardiac Innervation and Their Critical Roles in Cardiac Performance

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Abstract. The heart is abundantly innervated, and the nervous system precisely controls the function of this organ. The density of cardiac innervation is altered in diseased hearts, which can lead to unbalanced neural activation and lethal arrhythmia. For example, diabetic sensory neuropathy causes silent myocardial ischemia, characterized by loss of pain perception during myocardial ischemia, and it is a major cause of sudden cardiac death in diabetes mellitus. Despite the clinical importance of cardiac innervation, the mechanisms underlying the control of this process remain poorly understood. We demonstrate that cardiac innervation is determined by the balance between neural chemoattractants and chemorepellents within the heart. Nerve growth factor (NGF), a potent chemoattractant, is synthesized abundantly by cardiomyocytes, and is induced by the upregulation of endothelin-1 during development. By comparison, the neural chemorepellent Sema3a is expressed at high levels in the subendocardium in the early stage of embryogenesis and is downregulated as development progresses, leading to epicardial-to-endocardial transmural sympathetic innervation patterning. We also show that the downregulation of cardiac NGF is a cause of diabetic neuropathy and that NGF supplementation prevents silent myocardial ischemia in diabetes mellitus. Both Sema3a-targeted and Sema3a-overexpressing mice display sudden cardiac death or lethal arrhythmias due to disruption of innervation patterning. The present review focuses on the regulatory mechanisms controlling cardiac innervation and the critical roles of these processes in cardiac performance.

Keywords: cardiac nerve, nerve growth factor, Sema3a, arrhythmia, sudden cardiac death, cardiac disease

Introduction

Cardiac tissues are extensively innervated by autonomic nerves. The sympathetic nervous system produces norepinephrine and increases the heart rate, conduction velocity, as well as myocardial contraction and relaxation. It is well known that sympathetic innervation density, which is high in the subepicardium and the central conduction, is stringently regulated in the heart (1). Regional differences in sympathetic innervation correspond to different areas of influence over cardiac function to effectively control heart rate and myocardial contraction and relaxation. Despite the clinical importance of cardiac innervation, little is known about the developmental and regulatory mechanisms underlying cardiac sympathetic innervation patterning. Moreover, to date there has been no experimental demonstration of the consequences of disrupting this patterning.

The density of cardiac innervation is altered in diseased hearts, as in cases of congestive heart failure and myocardial infarction. Following myocardial injury, cardiac nerves undergo Wallerian degeneration, which may be followed by neurilemmal cell proliferation and axonal regeneration, resulting in heterogeneous innerva-
calcitonin gene-related peptide (CGRP), a sensory marker; protein gene product 9.5 (PGP 9.5), a general peripheral nerve marker; and growth-associated protein 43 (GAP43), a nerve sprouting marker. As discussed below, we and others have used these specific neural markers to demonstrate that the organization of cardiac innervation is strictly controlled in the heart during development, whereas in diseased hearts, innervation density and organization are dramatically altered.

Nerve sprouting and SCD

Sympathetic stimulation is important in the generation of SCD in diseased hearts. There is circadian variation in the frequency of SCD that parallels sympathetic nerve activity. β-Blocker therapy prevents SCD secondary to ventricular tachyarrhythmia in ischemic heart disease or congestive heart failure. Immunohistochemical analysis of cardiac nerves in explanted hearts of transplant recipients reveals a positive correlation between nerve density and clinical history of ventricular tachyarrhythmia (4). Zhou et al. showed that nerve growth factor (NGF), which is critical for sympathetic nerve sprouting, is upregulated after myocardial infarction (MI) in animal models, resulting in the regeneration of cardiac sympathetic nerves and heterogeneous innervation (5). In other experiments, augmented myocardial nerve sprouting through NGF infusion after MI results in a dramatic increase in SCD and a high incidence of ventricular tachyarrhythmia, compared with animals not receiving NGF infusion (6). These results demonstrate that NGF upregulation and nerve sprouting in diseased hearts may cause lethal arrhythmia and SCD. However, the molecular mechanisms that regulate NGF expression and sympathetic innervation in the heart are poorly understood.

The endothelin-1 (ET-1) / NGF pathway is critical for cardiac sympathetic innervation

In general, the growth-cone behavior of nerves is modulated by coincident signaling modulated by neural chemoattractants and chemorepellents synthesized in the innervated tissue. NGF, a potent neural chemoattractant, is a prototypic member of the neurotrophin family that plays critical roles in the differentiation, survival, and synaptic activity of the peripheral sympathetic and sensory nervous systems (7). The level of NGF expression within innervated tissue corresponds approximately to innervation density. NGF expression increases during development and is altered in diseased hearts.

ET-1 is a critical factor in the pathogenesis of cardiac hypertrophy, hypertension, and atherosclerosis. Gene targeting of ET-1 and its receptor ETₐ results in un-
expected craniofacial and cardiovascular abnormalities not observed in other hypertrophic factor–deficient mice (8). Although these phenotypes are consistent with interference of neural crest differentiation, the role of ET-1 in neural crest development remains underdetermined. We hypothesized that ET-1 affects the induction of neurotrophic factors and that the disruption of ET-1 contributes to the immature development of neural crest–derived cells.

We found that ET-1, but not angiotensin II, phenylephrine, leukemia inhibitory factor, or IGF-1, upregulates NGF expression in primary cultured cardiomyocytes (9). ET-1–induced NGF augmentation is mediated via the ETα receptor, Giβγ, PKC, the Src family, EGFR, extra-cellular signal-regulated kinase, p38MAPK, activator protein-1, and the CCAAT/enhancer-binding protein δ element. To study the role of the ET-1/NGF pathway in the development of the cardiac sympathetic nervous system, we analyzed various mouse models of modified genes. NGF expression, cardiac sympathetic innervation, and norepinephrine concentration are not reduced in ET-1–deficient mouse (Edn1−/−) hearts, but not in the hearts of angiotensinogen-deficient mice (Atg−/−). In Edn1−/− mice, the sympathetic stellate ganglia exhibited excessive apoptosis and display loss of neurons at the late embryonic stage. Moreover, we demonstrate that cardiac-specific overexpression of NGF in Edn1−/− mice rescues the heart from sympathetic nerve retardation. These findings indicate that ET-1 is a key regulator of NGF expression in cardiomyocytes and that the ET-1/NGF pathway is critical for sympathetic innervation in the heart. Given that ET-1 is strongly induced in pathological conditions, the ET-1/NGF pathway may also be involved in NGF upregulation and nerve regeneration after myocardial infarction.

NGF is critical for cardiac sensory innervation and rescues the diabetic heart from neuropathy

The cardiac autonomic nervous system is composed of efferent and afferent nerves. The cardiac sensory nervous system is responsible for pain perception and for initiating a protective cardiovascular response during myocardial ischemia. Cardiac sensory nerve impairment causes silent myocardial ischemia, which is a major cause of sudden death in DM patients. Despite the severity of this complication, the alterations in cardiac sensory innervation in diabetic sensory neuropathy and the molecular mechanism underlying this process are poorly understood. Moreover, little is known about the anatomical distribution of cardiac sensory nerves and the molecular mechanism controlling innervation during development.

Unlike somatic tissues, visceral organs, such as the heart, are believed to be rich in autonomic efferent innervation but poor in nociceptive afferent nerves. In fact, Zahner et al. report that vanilloid receptor-1–immunopositive sensory nerves are enriched in the epicardium but scarce in the myocardium (10). We show that cardiac sensory innervation is rich not only at epicardial sites but also in the ventricular myocardium and that sensory innervation increases with development (11). In a screen of several neurotrophic factors, we showed that development of cardiac sensory nerves coincides with synthesis of NGF in the heart. Cardiac nociceptive sensory nerves that are immunopositive for CGRP, including the dorsal root ganglia and the dorsal horn, are markedly retarded in NGF-deficient mice, while cardiac-specific overexpression of NGF rescues the heart from these deficits. Thus, NGF synthesis in the heart is critical for the development of the cardiac sensory nervous system.

To investigate whether NGF is involved in diabetic neuropathy, DM was induced with streptozotocin in wild-type (WT) and transgenic mice overexpressing NGF in the heart. DM-induced WT mice show down-regulation of NGF, CGRP-immunopositive cardiac sensory denervation, and atrophic changes in dorsal root ganglia, whereas these defects are prevented in DM-induced NGF-transgenic mice. Cardiac sensory function, as measured by myocardial ischemia-induced c-Fos expression in dorsal root ganglia, is also downregulated by DM in WT mice, but not by DM in NGF-transgenic mice. Direct gene transfer of NGF into diabetic rat hearts improves the impaired cardiac sensory innervation and function, as determined by the electrophysiological activity of cardiac afferent nerves during myocardial ischemia. These findings demonstrate that development of the cardiac sensory nervous system depends on the synthesis of NGF in the heart, and that DM-induced suppression of NGF expression may lead to cardiac sensory neuropathy.

Phase I and phase II clinical trials showed that systemic administration of recombinant NGF is safe and has potential efficacy in diabetic polyneuropathy, but a phase III trial did not show any beneficial effects, perhaps because the dosage and route of administration were suboptimal (12, 13). The dosage of NGF was restricted by side-effects in the phase III clinical trial, and the development of anti-NGF antibodies may have contributed to the lack of beneficial effects. We examined the possibility of avoiding these complications by direct administration of the NGF gene to the cells that require the factor. We showed that NGF expression
and CGRP-immunopositive nerves are proportionally reduced in diabetic hearts and thus demonstrated the successful treatment of cardiac sensory neuropathy by direct NGF gene transfer. Consistent with our findings, the efficacy of NGF gene therapy has been reported in diabetic cystopathy and neuropathy of the footpad (14). Further studies on the reliability and efficacy of NGF gene therapy are required before clinical trials can proceed.

Sema3a is critical for cardiac sympathetic innervation patterning

As discussed above, NGF plays critical roles in cardiac nerve development. In contrast, the neural chemorepellent that induces growth-cone collapse and repels nerve axons has not been identified in the heart. The Class 3 secreted semaphorin, Sema3a, has been cloned and identified as a potent neural chemorepellent and a directional guidance molecule for nerve fibers (15–17). However, it is not known whether cardiomyocytes produce Sema3a, and if so, whether this protein affects sympathetic neural patterning and cardiac performance.

We analyzed the kinetics and distribution of cardiac sympathetic innervation in developing murine ventricles (1). TH-immunopositive sympathetic nerve endings appear on the epicardial surface at embryonic day (E)15 and gradually increase in number in the myocardium after postnatal day (P)7 and P42. In the ventricular myocardium, sympathetic nerves are more abundant in the subepicardium than in the subendocardium, suggesting an epicardial-to-endocardial gradient. We analyzed heterozygous Sema3a knocked-in lacZ mice (Sema3a<sup>lacZ/+</sup>) to identify the Sema3a expression pattern and its relationship to innervation patterning in the heart. At E12, lacZ expression was detected strongly in the heart, especially in the trabecular components of the ventricles. In E15 hearts, lacZ expression was observed in the subendocardium but not in the subepicardium of the atria and ventricles (Fig. 1). At P1 and P42, lacZ expression was reduced in certain regions and highlighted the Purkinje fiber network along the ventricular free wall. Quantitative RT-PCR of Sema3a in developing hearts demonstrated strong Sema3a expression only in the subendocardium. Scale bar = 100 µm.

**Fig. 1.** Sema3a expression in murine hearts. X-gal staining (green) of Sema3a<sup>lacZ/+</sup> hearts at E15 demonstrates strong Sema3a expression only in the subendocardium. Scale bar = 100 µm.

**Fig. 2.** Regulation of cardiac innervation patterning. a: Cardiac sympathetic innervation shows an epicardial-to-endocardial transmural gradient. This patterning is established by the balance between ET-1/NGF and Sema3a expression in the heart. Note that NGF is expressed abundantly in the working myocardium, whereas Sema3a is expressed specifically in the subendocardium. b: Appropriate Sema3a-mediated sympathetic innervation patterning is critical for the maintenance of an arrhythmia-free heart. Sema3a<sup>−/−</sup> mice exhibit sinus bradycardia, and SemaTG mice are highly susceptible to ventricular tachyarrhythmias.
hearts confirmed the presence of Sema3a from E12 and the subsequent linear decrease in expression. The spatial and temporal expression pattern of Sema3a contrasts directly with the patterning of sympathetic innervation in developing hearts. These results indicate that Sema3a is a negative regulator of cardiac innervation. We analyzed Sema3a-deficient mice (Sema3a−/−) to investigate whether Sema3a is critical for cardiac sympathetic nerve development. The WT hearts show a clear epicardial-to-endocardial gradient of sympathetic innervation. In contrast, the sympathetic nerve density is lower in the subepicardium and higher in the sub-endocardium of Sema3a−/− mice, resulting in disruption of the innervation gradient in Sema3a−/− ventricles. The Sema3a−/− mice also exhibit malformation of the stellate ganglia that extend sympathetic nerves to the heart. To investigate whether the abnormal sympathetic innervation patterning in Sema3a−/− hearts is a secondary effect of stellate ganglia malformation, we generated transgenic mice overexpressing Sema3a specifically in the heart (SemaTG). SemaTG mice are associated with reduced sympathetic innervation and attenuation of the epicardial-to-endocardial innervation gradient. These results indicate that cardiomyocyte-derived Sema3a plays critical roles in cardiac sympathetic innervation by inhibiting neural growth. Since cardiomyocyte-derived NGF acts as a chemoattractant, it is possible that the balance between NGF and Sema3a synthesized in the heart determines cardiac sympathetic innervation patterning.

The growth-cone behavior of somatic sensory axons is also modulated by the coincident signaling of NGF and Sema3a (18, 19). During development, NGF and Sema3a are expressed within the spinal cord and influence the guidance pathway of sensory axons. Sema3a is specifically expressed in the ventral half of the spinal cord and mediates the termination of NGF-responsive sensory axons at the dorsal part of the spinal cord. The targeted inactivation of Sema3a disrupts neural patterning and projections in the spinal cord, thereby highlighting the critical role of Sema3a signaling in the directional guidance of nerve fibers (17, 20).

**Sema3a maintains arrhythmia-free hearts through sympathetic innervation patterning**

Most Sema3a−/− mice die within the first postnatal week, with only 20% surviving until weaning. We performed telemetric electrocardiography and heart-rate variability analysis to identify the cause of death and the effects of abnormal sympathetic neural distribution in Sema3a−/− hearts (1). In addition to multiple premature ventricular contractions, Sema3a−/− mice develop sinus bradycardia and abrupt sinus arrest due to sympathetic neural dysfunction.

By comparison, the SemaTG mice die suddenly at 10 months of age without symptoms. Sustained ventricular tachyarrhythmia is induced in SemaTG mice, but not in WT mice, after epinephrine administration, and programmed electrical stimulation reveals that SemaTG mice are highly susceptible to ventricular tachyarrhythmia. The β1-adrenergic receptor density is upregulated and the cAMP response after catecholamine injection is exaggerated in SemaTG ventricles. Action potential duration is significantly prolonged in hypoinnervated SemaTG ventricles, presumably via ion channel modulation. These results suggest that the higher susceptibility of SemaTG mice to ventricular arrhythmia is due, at least in part, to catecholamine supersensitivity and prolonged action potential duration, both of which can augment triggered activity in cardiomyocytes. Thus, Sema3a-mediated sympathetic innervation patterning is critical for the maintenance of arrhythmia-free hearts.

Sympathetic nerves modulate the function of ion channels and trigger various arrhythmias in diseased hearts (21, 22). Various studies highlight the importance of regulatory factors in sympathetic innervation patterning. For example, Sema3a−/− mice exhibit sinus bradycardia, abrupt sinus slowing, and stellate ganglia defects. Consistent with our data, right stellectomy induces sinus bradycardia and sudden, asystolic death in dogs (23). In addition, Stramba-Badiale et al. report that developmental abnormalities in cardiac innervation may play a role in the genesis of some cases of sudden infant death syndrome (24). The SemaTG hearts are also highly susceptible to ventricular arrhythmias, albeit without contractile dysfunction or structural defects. Given that catecholamine augments systolic function, it is surprising that SemaTG mice show normal cardiac function. Patients with denervated hearts who undergo heart transplantation do not develop heart failure but approximately 10% of the patients develop SCD (25). Together, these studies highlight the significance of cardiac nerve regulation as a new paradigm for the management of SCD.

**Conclusions**

Cardiac nerves are highly plastic, and innervation patterning is strictly controlled by the balance between NGF and Sema3a synthesized in the heart (Fig. 2a). ET-1 regulates NGF expression in cardiomyocytes, and the ET-1/NGF pathway modulates nerve sprouting and plays critical roles in sympathetic nerve development. NGF is also important in sensory nerve development, and NGF downregulation may result in sensory neuropathy in diabetic hearts. By comparison, Sema3a inhibits...
neural growth and establishes appropriate innervation patterning in the heart. The disruption of sympathetic innervation patterning may lead to SCD, in both diseased and developing hearts (Fig. 2b). An understanding of the mechanisms regulating cardiac innervation patterning in hearts represents an important step towards the development of therapies for SCD.

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