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Sympathetic Reinnervation is Required for Mammalian Cardiac Regeneration

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Abstract

Rationale—Although mammalian cardiac regeneration can occur in the neonatal period, the factors involved in this process remain to be established. As tissue and limb regeneration require concurrent reinnervation by the peripheral nervous system, we hypothesized that cardiac regeneration also requires reinnervation.

Objective—To test the hypothesis that reinnervation is required for innate neonatal cardiac regeneration.

Methods and Results—We crossed a Wnt1-Cre transgenic mouse with a double-tandem (td) Tomato reporter strain to identify neural crest-derived cell lineages including the peripheral autonomic nerves in the heart. This approach facilitated the precise visualization of subepicardial autonomic nerves in the ventricles using wholemount epifluorescence microscopy. Following resection of the left ventricular apex in 2-day-old neonatal mice, sympathetic nerve structures, which envelop the heart under normal conditions, exhibited robust re-growth into the regenerating myocardium. Chemical sympathectomy inhibited sympathetic regrowth and subsequent cardiac regeneration following apical resection, significantly (scar size as cross-sectional percentage of viable LV myocardium: n=9, 0.87±1.4% vs. n=6, 14.05±4.4%; $p<0.01$).

Conclusions—These findings demonstrate that the profound regenerative capacity of the neonatal mammalian heart requires sympathetic innervation. As such, these data offer significant insights into an underlying basis for inadequate adult regeneration following myocardial infarction, a situation where nerve growth is hindered by age-related influences and scar tissue.

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DISCLOSURES

Dr. Hare reported having a patent for cardiac cell-based therapy. He holds equity in Vestion and maintains a professional relationship with Vestion as a consultant and member of the Board of Directors and Scientific Advisory Board. Vestion Inc. did not play a role in the design and conduct of the study. The other authors report no conflicts.

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Keywords

Cardiac regeneration; neonatal repair; cardiac innervation; sympathetic denervation; neonatal mouse cardiac myocyte; sympathic nervous system

INTRODUCTION

It has recently been established that unlike the limited cardiac regeneration demonstrated by the injured adult mammalian heart, the neonatal heart remains permissive for near-complete cardiac regeneration during a finite developmental period[1, 2]. In a similar fashion to other regeneration-competent species including the salamander[3] and zebrafish[4, 5], the dominant mechanism underlying cardiac regeneration in the neonatal mouse appears to be de-differentiation and proliferation of resident cardiac myocytes[2]. However, the underlying basis for regeneration and revascularization of the neonatal tissue are not fully understood. Other vertebrate[6–9] and invertebrate[10] models of tissue regeneration exhibit a complete dependence on reinnervation of the regenerating tissue by nerves of the peripheral nervous system (PNS). Despite the clinical importance of cardiac autonomic innervation, the neuroanatomy of the sympathetic nerve plexus innervating the ventricular myocardium remains incompletely characterized[11].

To address these issues, we used a combination of genetic and pharmacologic tools to map the cardiac PNS and to test the role of peripheral nerve innervation in mammalian cardiac regeneration. Post-ganglionic, subepicardial sympathetic axons make up the bulk of nerve fibers in the ventricles[11] and we demonstrate, for the first time, that these nerve fibers undergo robust re-growth and reinnervation during the regeneration of resected ventricular tissue. Furthermore, sympathectomy abrogates cardiac regeneration and promotes collagenous scar formation, demonstrating that innate mammalian cardiac regeneration in neonates is dependent on sympathetic innervation. Together these findings suggest that concurrent reinnervation of injured adult cardiac tissue is essential for functional and complete cardiac regeneration.

METHODS

A detailed description of the experimental procedure and statistical analysis is provided in the online data supplement. Briefly, Wnt1-Cre mice were crossed with tdTomato reporter mice (The Jackson Laboratory). Cre/$^{+}$; tdTomato/$^{+}$ animals were used in experiments where direct observation of nerves was required. BALB/cJ animals (The Jackson Laboratory) were used in apical resection experiments. Studies were performed on 2-day-old neonates and tissue was collected at either day 14- or day 21-post injury.

RESULTS

The proto-oncogene *Wnt1* is only expressed during the development of the central nervous system and demarcates lineages derived from the neural crest[12]. The Wnt1-Cre transgenic mouse strain is a widely used and well-validated model for neural crest lineage tracing studies[13, 14]. Wnt1-Cre transgenic mice were mated with mice expressing the tdTomato

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reporter[15] so that neural crest-derived cells, and their progeny, are permanently labeled with the red fluorescent tdTomato reporter. Using this system, together with whole-mount, broad focal plane, epifluorescent stereomicroscopy, we visualized the subepicardial neural network in unprecedented detail (Figure 1A, B).

The structures observed in whole-mount were confirmed to be nerves by staining for β-tubulin III (Figure 1C) and the presynaptic marker synapsin1 (Figure 1D). In order to determine from which autonomic branch these nerves derive, we performed immunofluorescence histology against tyrosine hydroxylase (TH), and choline acetyltransferase (ChAT). Co-localization of TH^+ fibers only with Wnt1-Cre^+ fibers was observed at the base of the heart where nerve density is greatest (Figure 1E–G), which agrees with the published distribution of the sympathetic branch primarily to the subepicardium[11, 13].

In contrast to the adult neonatal sympathetic neurons, sympathetic ganglia and dorsal root ganglia exhibit enhanced plasticity, both in vitro[16] and in vivo[17]. Therefore, we assessed the ability of neonatal subepicardial sympathetic nerves to reinnervate ventricular myocardium following injury. We resected the apex of the left ventricle of 2-day-old Wnt1-Cre:tdTomato mice, as described[18]. Under normal conditions the neonate heart is capable of a robust regenerative response following apical resection within 21 days. At 14 days post-resection, we observed an area of heavy dendrite hyperinnervation at the injury border (Figure 2A), which is consistent with that described in studies of acute myocardial infarction[19] and varicose fibers emerging from the border into the site of active regeneration (Figure 2B). By day 21 post-injury the entire apex had regenerated and had become reinnervated by organized, arborized and anastomosed fibers (Figure 2C–F).

Denervation of tissue following injury in other animal models results in a block of the innate regenerative response with subsequent scar formation at the expense of functional tissue[6, 8]. Considering the vast abundance of sympathetic nerves associated with the ventricular myocardium and their ability to regrow in the model of neonatal cardiac regeneration, we sought to determine whether these nerves were a necessary component of the cardiac regenerative response. Beginning 48 hours after apical resection, chemical sympathectomy of adrenergic nerves[20, 21] was induced by treatment of neonatal mice with 3 doses of 6-Hydroxydopamine hydrobromide (6-OHDA, 250 mg/kg, intraperitoneal injection) 48 hours apart. In response to 6-OHDA, the subepicardial sympathetic nerves demonstrated classical features of Wallerian degeneration[22] throughout the surface of the heart (Figure 3A). Treatment resulted in robust denervation (n=9, $1.74 \times 10^5 \pm 7447$pxl; n=9, $3.11 \times 10^4 \pm 3863$pxl; $p<0.01$) of the subepicardial nerves in the heart, as quantified by densitometry (Figure 3B).

In the absence of 6-OHDA neonatal mouse hearts underwent robust reinnervation and regeneration of the ventricular apex following resection with little or no signs of injury (Figure 3C) (n=9, scar as a percentage of cross-sectional myocardial area = 0.87±1.4%,). In contrast, the hearts of sympathectomized mice consistently exhibited extensive scarring, lack of regeneration and failure to replace the ventricular myocardium following apical resection (Figure 3C) and denervation (n=6, 14.05±4.4%, $p<0.01$) (Figure 3D).

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In summary, we have demonstrated that sympathetic denervation completely inhibits the ability of the neonatal heart to regenerate following injury. These findings correlate with those from other animal models of tissue regeneration, which describe a critical dependence on peripheral nerves in order to regenerate injured tissue[6–10].

DISCUSSION

Despite the clinical importance of the cardiac autonomic system, there is still much to learn regarding the neuroanatomy of the mammalian heart. Here we combine a strong fluorescence lineage tracing reporter system with broad focal plane stereomicroscopy to visualize the vast cardiac sympathetic neural network in unprecedented detail. We demonstrate large nerve bundles entering the heart from the dorsal aspect, which then arborize and anastomose throughout all four chambers of the heart. The resulting dense network of nerve fibers makes extensive contact, via “en-passant” synapses, with subepicardial myocytes in the ventricles and with vessels of the cardiovasculature.

Direct visualization of these nerves facilitated investigation into the ability of the nerves to regenerate in vivo following injury and identified that cardiac regeneration is dependent on a nerve supply. Our data support the hypothesis that innervation is critical for innate cardiac regeneration exhibited by neonatal mice, as inhibition of sympathetic nerves completely blocks myocardial repair. Several other vertebrate and invertebrate models of tissue regeneration support this concept, including a recent report demonstrating that ablation of the parasympathetic branch of the autonomic system of neonatal mice by surgical vagotomy inhibits cardiac regeneration, despite an assumed presence of sympathetic nerves[9]. Together, these results suggest that contributions from both branches of autonomic nerves are needed to support full cardiac regeneration. Further studies are required to tease apart the relationship between the branches of the autonomic nervous system, the myocardium and the cardiovasculature and to address the structural changes in cardiac neuroanatomy that result from injury.

Substantial evidence is accumulating that implicates peripheral autonomic nerves in several aspects of tissue regeneration and homeostasis, including stem cell niche maintenance[23], vasculogenesis and patterning[24], and tissue hyperplasia[6, 7]. Cardiac nerves clearly function as more than just simple impulse conduits. A recent report demonstrated that uninjured rats treated with 6-OHDA to ablate the sympathetic while sparing the parasympathetic nerves, exhibit myocardial injury and fibrosis[21]. This injury was prevented when a neuroprotectant was co-administered, demonstrating that myocardial injury was a secondary response to sympathetic nerve loss.

Taken together, these data support the idea that peripheral cardiac autonomic nerves play a role not only in repair, but also in chronic disease. Peripheral neuropathies due to diabetes or heart transplant, are typically associated with idiopathic vasculopathy and heart failure[25, 26]. Further work is needed to investigate whether the loss of cardiac nerves in these patients results in a pathological loss of trophic support for the myocardium, vasculature or cardiac stem cell niches. Considering the physiological differences between fetal/embryonic and

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adult post-ganglionic nerves, these findings have important implications for improving adult cardiac regeneration, which has, as of yet, remained elusive.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

WNT1	Wingless-Type MMTV Integration Site Family, Member 1
CRE	Cre recombinase
LV	Left Ventricle
PNS	Peripheral Nervous System
TH	Tyrosine Hydroxylase
ChAT	Choline Acetyltransferase
6-OHDA	6-hydroxydopamine

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Novelty and Significance

What Is Known?

- Whereas adult mammals, including humans, exhibit regenerative capacity inadequate to repair injury due to heart attack or other damage, the neonatal mouse is capable of regenerating injured myocardium during a finite developmental window.
- The mammalian heart is extensively innervated by peripheral nerves.
- Peripheral nerves are essential for tissue repair in newts and zebrafish.

What New Information Does This Article Contribute?

- We used a Wnt1 transgenic reporter mouse, which allowed for high resolution visualization of cardiac sympathetic nerves.
- Using this transgenic mouse we demonstrated concurrent nerve and myocardial tissue regrowth and repair at the site of cardiac regeneration in the neonatal mouse.
- Cardiac regeneration in the neonatal mouse is critically dependent on sympathetic nerves, as denervation blocks cardiac tissue regeneration.

Our understanding of cardiac regeneration has advanced significantly in recent years. However, large-scale regeneration or full recovery remains elusive, suggesting that an important component of the regenerative process is being overlooked. Unlike the adult, the neonatal mouse heart retains an innate ability to regenerate following injury, providing a compelling model to study the mechanisms of mammalian cardiac regeneration. Several animal species exhibit a robust capacity to regenerate tissue and limbs and this process is critically dependent upon intact innervation. Here we demonstrate that neonatal cardiac regeneration is critically dependent on sympathetic nerves and identify a potentially novel strategic target for improving cardiac repair. This finding has important implications for adult regeneration following myocardial infarction where nerve growth is hindered by age related influences, disease processes and scar tissue.

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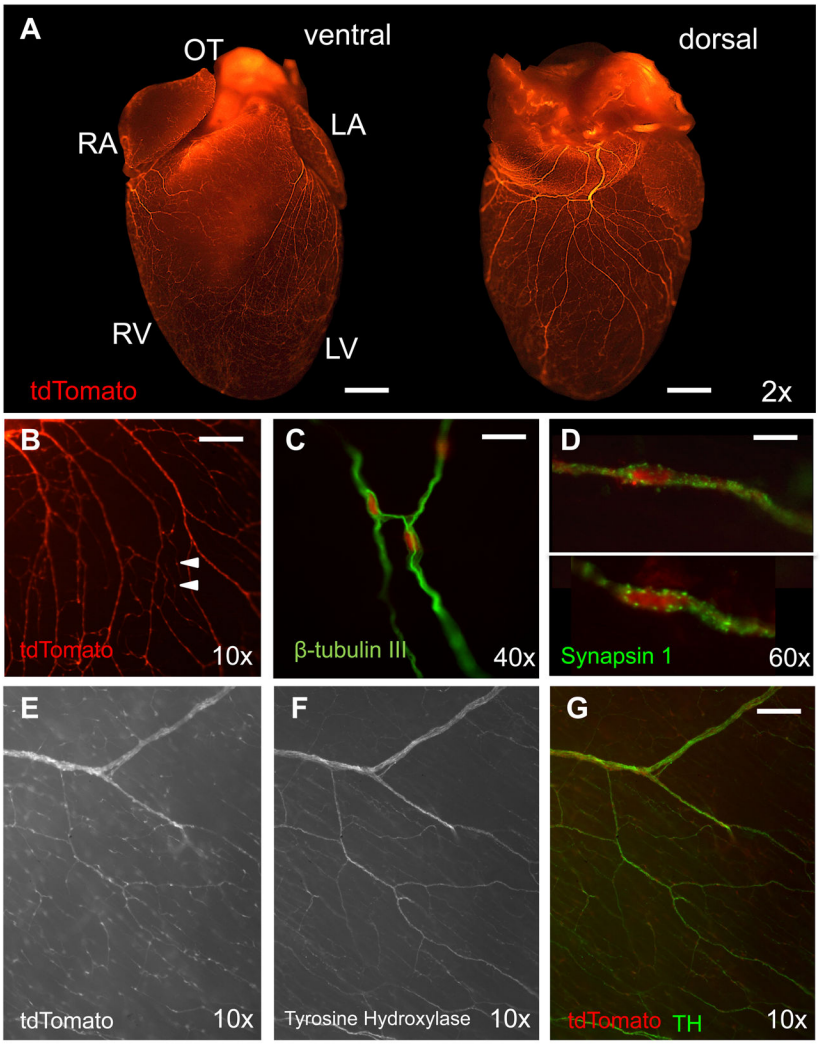

Figure 1. Subepicardial distribution of post-ganglionic sympathetic nerve fibers in the mouse heart

A, Crossing Wnt1-Cre transgenic mice with tdTomato reporter mice identifies nerve fibers throughout the entire subepicardium from the base of the heart to the apex of the ventricles (bar, 1 mm). **B**, The fibers are heavily varicosed (arrows) (bar, 200 μm). **C,** They stain positive for the neurofilament marker β-tubulin III (bar, 50 μm), the presynaptic marker synapsin 1 (**D**) (bar, 20 μm) and the sympathetic nerve fiber marker TH (**E–G**) (bar, 200 μm).

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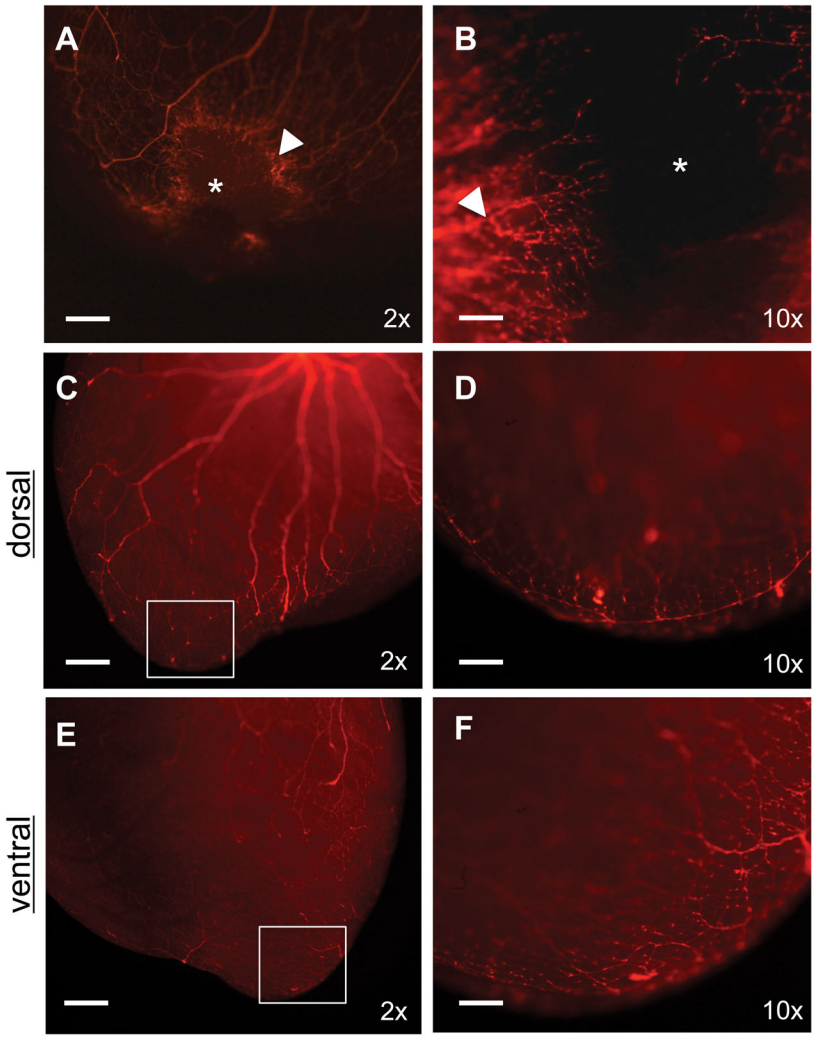

Figure 2. Concurrent reinnervation and cardiac regeneration

A, B, At day 14 post-resection the apex is in the process of regenerating (*****) as nerves regrow into the injury (arrow marks site of hyperinnervation at injury border) (bars, 500 μm and 100 μm, respectively). **C–F.** After 21 days the apex has regenerated and fully reinnervated (bar, 500 μm). Boxes from **C** and **E** are expanded with greater magnification in **D** and **F** (bar, 100 μm). All images are of Wnt1-Cre;tdTomato hearts.

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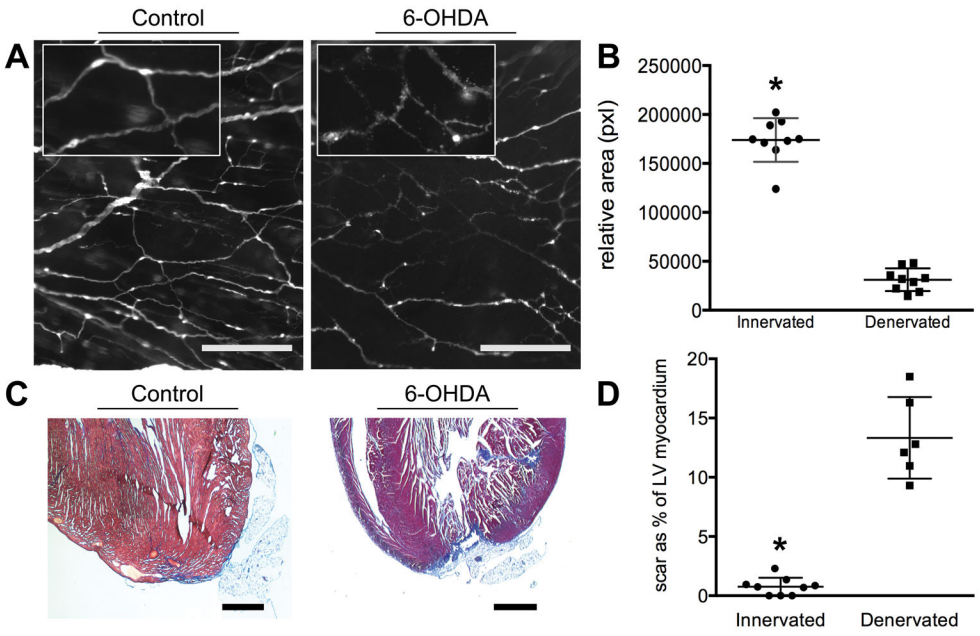

Figure 3. Cardiac innervation is required for myocardial regeneration

A, Administering 6-OHDA to neonates induces Wallerian degeneration of sympathetic nerve fibers. Inset demonstrates degeneratio of nerve structure (epifluorescence, Wnt1-Cre;tdTomato. bar, 200 μm). **B**, Densitometry of Wnt1-Cre;tdTomato nerves from 21-day-old mouse hearts that are innervated compared to denervated (n=9, 1.74×105±7447pixels; n=9, 3.11×104±3863pixels; p<0.01). **C**, In the presence of sympathetic nerves the apex can regenerate with minimal fibrosis; visualized histologically with Masson's Trichrome stain (blue). Small amounts of adipose are adherent to the apex on occasion. Following denervation with 6-OHDA innate regeneration is blocked and extensive scar and adipocyte deposition can be observed (bar, 1 mm). **D**, Scar area as a percentage of left ventricular (LV) myocardium in cross-section (n=9, 0.87±1.4% vs. n=6, 14.05±4.4%; p<0.01).