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## Research Article

# Autonomic Innervation from the Aortic Root Ventricular Ganglionated Plexi to the Pulmonary Vein: A Novel Pathway

### Abstract

**Background:** Autonomic nerve innervation pathway from the ventricular GP to the pulmonary veins (PV) remains unclear.

**Aim:** This study investigates the autonomic innervations from aortic root ventricular GP to the PVs. Nissl's staining and fluorescent dual label staining were performed to determine the neuron structure in the aortic root GP in five dogs. Avidin Biotin Complex (ABC) staining were performed to study the efferent autonomic pathway from the aortic root GP to the PVs.

**Results:** Adrenergic and cholinergic neurons were both present in the aortic root GP, with the majorities were cholinergic. ABC positive nerve fibers that contained both cholinergic and adrenergic neurotransmitters penetrated directly from the aortic root GP to the left PVs.

**Conclusion:** Autonomic innervation of the Left PVs is partly originated from the aortic root ventricular GP.

## Introduction

There are at least 7 ganglionated plexi (GP) located in the canine epicardium including 4 atrial GP and 3 ventricular GP [1]. Several studies have demonstrated that most autonomic nerve fibers penetrated from the atrial GP to the pulmonary vein (PV) [2,3]. And the ventricular GP, has long been regarded only relative to the function of ventricle, eg. Blood pressure [4,5]. However, our previous study [6] demonstrated stimulation of the aortic root ventricular GP provoked AF in the absence of any extrinsic cardiac nerve activity. The aortic root ventricular GP was also an important element in the intrinsic neuronal loop that can increase the risk of AF in isolated heart models.

We hypothesized that some autonomic nerve fibers may directly project from the ventricular GP to the PVs except the atrial GP. And the goals of the present study were therefore two-fold. First, we determined the neuronal cell structure present in the aortic root GP by Nissl's staining and fluorescent staining to determine if existed neuronal cells that expressing dual neuronal markers. Second, we performed biotinylated dextran amine (BDA) tracing and the Avidin Biotin Complex (ABC) staining to study the specific efferent autonomic pathways from the aortic root GP to the PV.

## Methods

### Neuronal cell structure of the aortic root GP

**Tissue preparation:** Five adult mongrel dogs were anesthetized with sodium pentobarbital (30 mg/kg IV), then hearts were excised from each of the dog, and a cannula was infused into the aorta

ascends. This was followed by perfusion with 250ml of physiologic saline to clean the hearts. The tissues were perfused and fixed with 1L of 4% formalin, and then stored in 30% sucrose overnight after an additional post-fixation for 4h in 4% formalin. The tissues were then embedded in OCT (TissueTek) and cut into 20 mm sections at -25 °C with a cryostat (Kryostat 1720; Leitz, Mannheim, Germany).

**Histochemistry and immunohistochemistry:** The aortic root GP is embedded in adipose tissues surrounding the root of aorta and connected by the mesangial ligament as described in our previous study [6]. Nissl's staining was used first to determine the location of the GPs in the aortic root GP. Fluorescent dual-labeling with antityrosine hydroxylase (TH) and anticholine acetyltransferase (ChAT) antibodies was performed to determine the neuron cell types in the GPs and to determine whether both adrenergic and cholinergic nerves co-existed within the GPs. Mouse anti-TH (1:500 dilution; Abcam) antibody was used to label the adrenergic nerves, and goat anti-ChAT (1:100 dilution; Millipore) antibody was used to label the cholinergic nerves. Fluorescein-labeled anti-mouse IgG was used as the secondary antibody. Fluorescein isothiocyanate (FITC) (1:50 dilution; KPL) was conjugated to TH, and Rhodamine-Labeled Anti-Goat IgG (1:50 dilution; KPL) was conjugated to ChAT. The dual-labeled sections were then visualized with a laser-scanning confocal microscope.

### Autonomic innervation pathway from the aortic root GP to the PVs

**Tissue preparation:** 5 adult mongrel dogs were anesthetized with the same method in protocol 1. The chest was opened using the median sternotomy method, and the cardiac pericardium was cut to

expose the aortic root GP. Five microliters of 10% (w/v) biotinylated dextran amine (BDA) anterograde tracer (MW 10,000; lysine fixable; Molecular Probes, Invitrogen) dissolved in 0.05 M PBS was then injected into the aortic root GP with micropipettes. A total of 5 injection sites adjacent to the root of the right region of the aortic root GP were selected, and the micropipettes were used at each injection site for 5min. After BDA injection, the dogs were allowed to survive for 7 days, then were sacrificed, perfused, and fixed trans-aortically as described above.

**Histochemistry and immunohistochemistry:** The four PVs with injected BDA were cut into 10- $\mu$ m-thick slices on a cryostat. Avidin-Horseradish peroxidase (HRP) complex (1:100) was first used to label BDA. The series of slices were incubated at room temperature for 2-3h, and BDA positive nerve fibers was showed by the reaction solution (Tris-HCl (0.05mol/L,pH7.6)50ml,DAB50mg,twice distilled water 50ml, Nickel ammonium sulfate solution 200 $\mu$ l). After DAB was dissolved, the incubated slices were put into the reaction solution, and 5 $\mu$ l of 0.03% H<sub>2</sub>O<sub>2</sub> was add each time to allow better reaction. Finally, the slices were cleaned with PBS and were examined under a laser-scanning confocal microscope.

Then the series of slices were processed for BDA, TH, and ChAT triple immunofluorescence histochemistry. Avidin-conjugated CY<sub>5</sub> (1:50 dilution; Southern biotech) was used to label BDA, and both TH and ChAT were labeled as described above. Finally, the labeled slides were examined under a confocal laser-scanning microscope.

### Statistical analysis

All data are expressed as means $\pm$ SD. The LSD-t test was adopted to analyze the difference between two groups. Probability values  $\leq$ 0.05 were considered significant.

## Results

### Co-expression of adrenergic and cholinergic neurons in the aortic root GP

The aortic root GP were cut into three parts from right to left, and Nissl's staining was first performed to determine the location of the GPs. The results revealed that neurons are widely distributed in the GPs and mixed with fat cells, which present in small clusters of 3 to 15 cells, with most embedded in the right part of aortic root GP (Figure

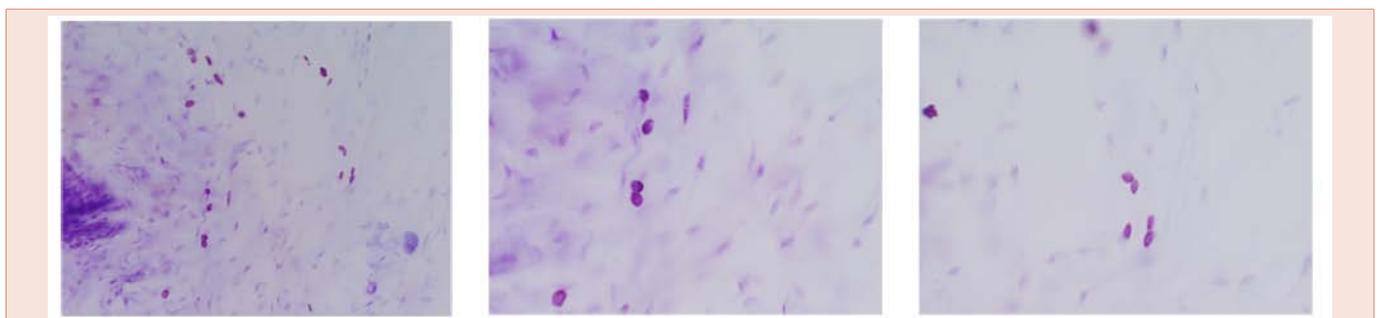
1). Fluorescent double staining showed adrenergic and cholinergic neurons were both observed, but the majority of neurons in the GP were of the cholinergic subtype (Figures 2A, 2B). Moreover, a proportion of neurons expressed dual adreno-cholinergic phenotypes (Figure 2C). This suggested that the ventricular GP had the same neuron structure with the atrial GP.

### Direct autonomic innervation from aortic root GP to the left PVs

ABC staining showed that neuron cells located in clusters among the fat cells in the aortic root GP (Figure 3). Autonomic nerve fibers projected directly from the aortic root GP to both the left PVs. The density of BDA-containing varicose fibers seemed thicker in the left superior PV (Figures 4A,4B) than that in the left inferior PV (Figures 4C,4D). A total 106 A total of 106 BDA-positive varicose fibers were counted in the left superior PV of five animals, and the counts were obtained for 20 sections (four sections in each animal).while the amount of BDA-positive varicose fibers was 44 in the left inferior PV. But the same BDA-containing varicose fibers were not observed in the right PVs as expected. These results demonstrated that autonomic innervation of the left PVs was partly originated from the aortic root ventricular GP.

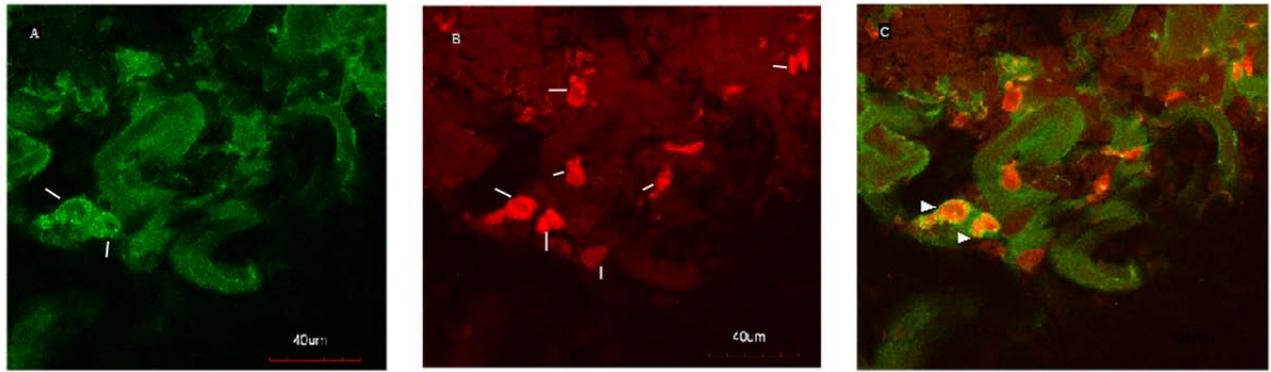
## Discussion

It is widely accepted that the atrial GP play an important role in both the initiation and the maintenance of AF [7-9]. Rapid repetitive activities arising from PVs especially from the Left PV may initiate AF. The basis of these rapid repetitive activities remains unclear, but recent evidence suggests that the autonomic nervous system plays an important role in their formation [10]. Noheria et al., claimed that post-cardiac transplant patients caused disruption of autonomic innervation and was shown to not have AF [11]. Therefore, how to decrease the activity of autonomic nervous system in the PVs appears to be a key point in AF control. Recently, researchers recognized that understanding the anatomy and autonomic modulation of PVs is very important in performing catheter ablation of AF [12]. The location and distribution of intrinsic cardiac nerves has been detailed presented in Vaitkevicius's study [2,13] that epicardial extension of mediastinal proceeded separately into the the PVs by seven atrial GPs. And then Pauza and colleagues [14] compared the findings between man and dog, and claimed that autonomic innervations of the canine PVs were also originated from some atrial GPs but different in

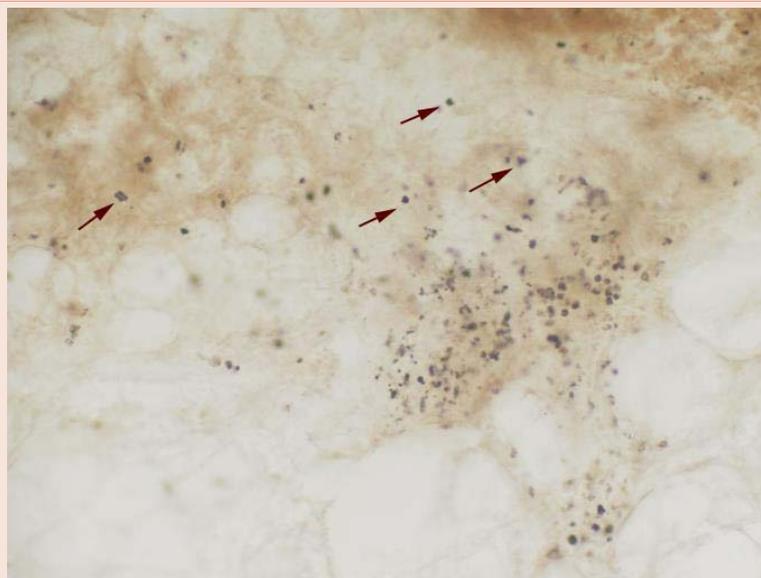


**Figure 1:** Location of the neurons in the aortic root GP.

Illustration: Figure 1A (1 $\times$ 20 eye lens) showed that neurons distributed widely in the aortic root GP, and were surrounded by fat cells. Figures 1B and 1C (1 $\times$ 40 eye lens) showed neurons presented in small clusters of 7 and 5 cells respectively. Location of the neurons in the aortic root GP.



**Figure 2:** Chemical properties of the neurons in the aortic root GP.  
**Illustration:** Fluorescence photomicrographs showed TH and ChAT are co-expressed in the aortic root GP. Figure 2A showed TH (green,white bar)-labeled adrenergic neurons; Figure 2B showed ChAT (red,white bar)-labeled cholinergic neurons; Figure 2C showed the co-expression of both TH and ChAT (white arrow).



**Figure 3:** BDA combined neuron cells in the aortic root GP.  
**Illustration:** BDA combined neuron cells scattered in the fat cells and were stained black-brown by ABC staining (red arrow).

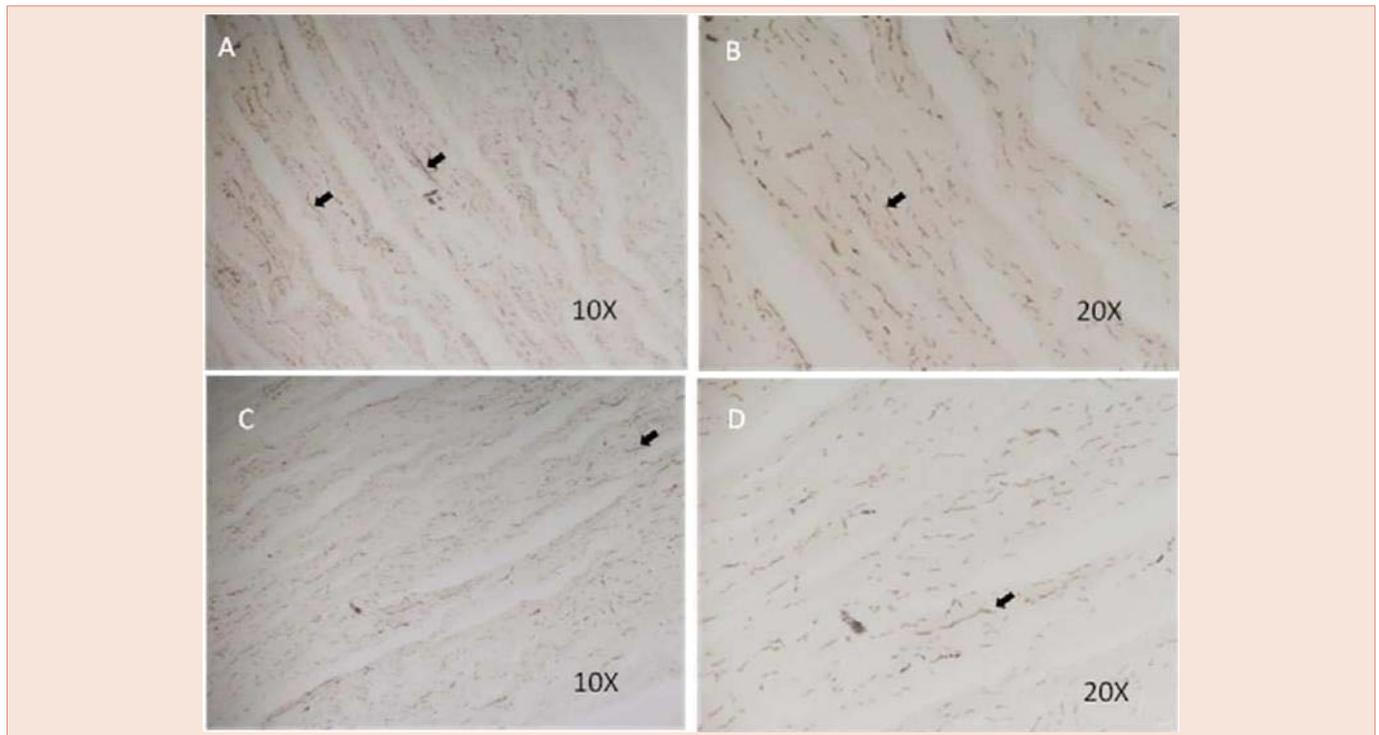
structural and quantitative.

However, the atrial GP may also have relationship with the function of the ventricle. He and coworkers demonstrated that stimulation of atrial GP may affect the electrophysiological properties in the ventricle including prolongation of ventricular ERP and delay of action potential duration [15]. Another study conducted by their group showed that atrial GP ablation increased the risk of ventricular arrhythmias under acute myocardial ischemia [16]. And the present study further demonstrated that some autonomic nerve fibers penetrated from the aortic root ventricular GP to left PVs. That may be the mechanism of why the aortic root GP stimulation/ablation was associated with AF.

**Clinical implications**

PV isolation has been widely used in clinic for managing AF; however, the reoccurrence rate was still high. DeSimone CV claimed modulation arrhythmogenic substrate was a safe and a few invasive way that may lead to improve treatment of AF in humans [17]. Zhou [18], demonstrated that a hyperactive state of either axons or ganglionated plexi in the intrinsic ANS may initiate AF from sites adjacent to the PV-LA junction. And this phenomenon may be the cause of AF reoccurrence after PV isolation. As a result, how to diminish the autonomic innervation of PVs was a key point to decrease the AF reoccurrence rate after PV isolation.

Previous ablation strategy usually focused on the atrial GP, because the autonomic innervation of the PVs was proved to be from the atrial GP. However, our study suggested that the ventricular GP was also an origin of the ANS that innervate the PV. Therefore,



**Figure 4:** Autonomic innervations from the aortic root GP to the left PVs.

**Illustration:** Expression of BDA in the left superior and left inferior PV. BDA positive nerve fibers were stained in black brown (black arrow) by Avidin-HRP complex. And expressed in left superior PV (4Aand 4B) and left inferior PV (4Cand 4D). The amount of BDA positive nerve fibers was 106 in the left superior PV and 44 in the left inferior PV. A and C=1×10 eye lens; B and D=1×20 eye lens.

additional modification of the aortic root ventricular GP may help to improve the success rate in AF control.

### Study limitation

Autonomic innervation between the aortic root GP and the atrial GPs were not determined in this study. In addition, further study should investigate whether ablation of the aortic root GP influenced the electrophysiology of the atrium and the PVs.

### Conclusion

The autonomic innervation of the left PVs is partly originated from the aortic root ventricular GP.

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### Ethics

This study complies with the Declaration of Helsinki, and the research protocol is approved by the locally appointed ethics committee, and the informed consent of the subjects (or their parents) has been obtained.

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