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Running Header: Dual Vagal Cardiac Efferent Pathways

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Abstract

In previous single-labeling experiments, we showed that neurons in the nucleus ambiguus (NA) and the dorsal motor nucleus of the vagus (DmnX) project to intrinsic cardiac ganglia. Neurons in these two motor nuclei differ significantly in the size of their projection fields, axon caliber, and endings in cardiac ganglia. These differences in NA and DmnX axon cardiac projections raise the question as to whether they target the same, distinct, or overlapping populations of cardiac principal neurons. To address this issue, we examined vagal terminals in cardiac ganglia and tracer injection sites in the brainstem using two different anterograde tracers (DiI and DiA) and confocal microscopy in male Sprague Dawley rats. We found that (i) NA and DmnX neurons innervate the same cardiac ganglia, but these axons target separate subpopulations of principal neurons; (ii) axons arising from neurons in the NA and DmnX in the contralateral sides of the brainstem enter cardiac the ganglionic plexus through separate bundles and preferentially innervate principal neurons near their entry regions, providing topographic mapping of vagal motor neurons in left and right brainstem vagal nuclei. Since the NA and DmnX project to distinct populations of cardiac principal neurons, we propose that they may play different roles in controlling cardiac function.

Key Words: brainstem, parasympathetic, anterograde tracing, heart, baroreflex
Introduction.

Vagal efferent nerve activation induces negative chronotropic, dromotropic, and inotropic changes in the heart (15, 16). Our knowledge, however, of how the brainstem circuitry is organized to control the heart via the vagus is limited because of the complexity of brain-heart connections and limitations of previous anatomical techniques (5).

Vagal efferent preganglionic axons arise mainly from neurons in two brainstem nuclei: the nucleus ambiguus (NA) and the dorsal motor nucleus of the vagus (DmnX), while a small number of vagal efferent axons arise from neurons in the intermediate zone between DmnX and NA. The functional roles of cardiac neurons in these nuclei are not well understood (5, 6, 11, 15, 16, 25). Furthermore, neurons in different cardiac ganglia control heart rate, A-V conduction, and myocardial contractility (17, 18, 20, 21, 22). In contrast to the conventional concept that cardiac ganglia are simple relay stations for central nervous system input, these structures are more likely operating as complex integration centers (19, 23). They consist of sensory neurons, motor neurons, and interneurons that employ complex chemical coding (12, 26), and are involved in local, as well as central, cardiac reflexes (1, 19, 23, 27). Thus, a major question is how cardiac ganglia are innervated and controlled by neurons in the NA and DmnX in the brainstem.

Traditionally, it has been assumed that the NA is the major vagal motor neuron pool controlling the heart whereas neurons in the DmnX plays only a minor role in cardiac control (15, 16). Using anterograde tracing and confocal microscopic techniques, however, we recently demonstrated that neurons in both NA and DmnX project to each cardiac ganglion where their...
axons form extensive basket endings around ganglionic principal neurons (5, 6). Therefore, this anatomical evidence suggests that both nuclei could be important for cardiac function.

Consistent with this morphological evidence, physiological data also support the functional significance of the two nuclei: 1) electrical stimulation of vagal efferent B fibers (presumably from NA) and vagal C fibers (presumably from DmnX) selectively evokes bradycardia, AV block, and reduction of cardiac contractility (9, 13, 14, 29); 2) stimulation of the DmnX elicits bradycardia and reduces myocardial contractility (8, 10, 24); 3) activation of the NA induces negative chronotropic, dromotropic, and inotropic effects (2, 3, 17, 28); and 4) both DmnX and NA neurons are barosensitive (30). However, whether the two vagal motor nuclei perform different functional roles remains unclear.

To study the difference between the NA and DmnX, we first compared cardiac projections of the two nuclei (5, 6). DmnX and NA motor neurons projections to the heart differ significantly: (a) efferent axons originating from NA neurons project to the heart and diverge and innervate principal neurons three times as much as the efferent fibers arising from DmnX neurons, (b) axons arising from NA neurons are larger in caliber than those arising from DmnX neurons, and (c) fibers from DmnX neurons also project to small intensely fluorescent cells (SIF; presumably interneurons) as well as to cardiac principal neurons, whereas axons from NA neurons innervate only principal neurons. Furthermore, we used domoic acid, an excitatory neurotoxin which acts on glutamate receptors, to selectively lesion the NA and DmnX. Lesions of the NA almost completely abolished the baroreflex control of the heart rate, whereas lesions of the DmnX did not, suggesting that the NA plays a more important role in baroreflex control of
the heart rate than the DmnX (7, 31)

Morphological and physiological differences between the NA and DmnX raise the question of whether the NA and DmnX control the heart through different pathways, and whether axons arising from neurons in these two nuclei target different populations of intrinsic cardiac principal neurons. We hypothesized that vagal fibers originating from neurons in the NA and DmnX project separately to cardiac ganglia, so that they innervate different populations of cardiac neurons and control different aspects of cardiac function. Alternatively, axons arising from NA and DmnX neurons might converge to innervate the same cardiac neurons because recruitment of C-fibers from the DmnX does not increase the degree of cardiac slowing produced by stimulation of B-fibers arising from NA (cf. 15).

To elucidate these issues, we used a double labeling strategy and confocal microscopy to examine dual projections of the NA and DmnX neurons to the heart. Our results show that neurons in the NA and DmnX project to neurons throughout individual cardiac ganglia. Rather than innervating the same principal neurons, however, these two vagal nuclei project to separate subpopulations of principal neurons within each cardiac ganglion.

Methods.

Male Sprague Dawley rats (300 - 350 g; Harlan Industries, Indianapolis, IN) were used for all experiments and were divided into 4 groups. The experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Louisville and are in agreement with the National Institutes of Health guidelines for the care and use of laboratory
In the first tracing group, each animal received a series of unilateral DmnX injections of the tracer DiI (1,1’-dioleyl-3,3’,3’,3’-tetramethylindocarbocyanine methanesulfonate, Rhodamine red, catalog 3886, Molecular Probes, Eugene, Oregon) paired with injections of DiA (4-(4-(dihexadecylamino)-styryl)-N-methylpyridinium iodide, fluorescein yellow-green; catalog 3883; Molecular Probes) into the contralateral NA (left DmnX: right NA, n = 4; right DmnX: left NA; n=4). In the second tracing group, each rat received a series of unilateral DmnX injections of DiI paired with injections of DiA into the ipsilateral NA (left DmnX: n=4; right DmnX: n=4). In the third tracing group, the tracers DiI and DiA were switched at each injection site. Each rat received a series of unilateral DmnX injections of the tracer DiA paired with injections of DiI into the ipsilateral (n=4) or contralateral (n=4) NA to test tracer effect. In the fourth group, each rat (n=2) was injected with DiI into the left DmnX and the left cervical vagal trunk was transected immediately after the DiI injection to assess the extent of DiI spread in the contralateral DmnX. Animals in these groups were also injected with Fluoro-Gold (FG: i.p., 2 ml of 2mg/ml) to counterstain cardiac ganglia. In a reference group, rats (n=10) were injected with FG (3 mg/ml, 5µl) into the cervical vagal trunk to identify DmnX and NA motor neurons. This group was used to ascertain the precision of DiI injections into DmnX, and of DiA injections into the NA, i.e., the registration of DiI in the brainstem with FG labeled DmnX motor neurons, and the registration of DiA with NA motor neurons.

Reference animals from the fourth group were sacrificed 5 days after injections to confirm tracer injection sites within the brainstem. In contrast, the other three experimental
groups were sacrificed three weeks after injections, and the brainstem tissue, nodose ganglia, and atrial specimens of these animals were harvested for analysis of NA and DmnX fibers and endings in cardiac tissues. In the event that tracer injection sites were off center from the target nuclei, data were discarded from further analysis.

**Tracer injection into the DmnX and NA**

Each animal in the tracing groups was anesthetized with sodium pentobarbital (60 mg/kg i.p.), treated with atropine (1 mg/kg, s.c.), and placed in a stereotaxic instrument equipped with a head holder adapted to permit the neck to be sharply flexed. A dorsal incision was made over the neck muscles which were retracted to expose the atlanto-occipital membrane. The membrane was opened with an incision, exposing the cisterna magna and the dorsal medulla. The occipital bone was trimmed with the bit of a dental drill until the caudal cerebellum was visible. The caudal end of the area postrema was used as a reference for stereotaxic coordinates. A glass micropipette, filled with DiI or DiA and connected to a picospritzer, was then advanced to the DmnX or NA. DiI or DiA was injected in small aliquots (2.5 - 12.5 nl each) either at 7 different sites for DmnX (-1200 to + 1200 µm; total volume 17.5-87.5 nl) or at 9 different sites (-1600 to + 1600 µm; total volume 22.5 - 112.5 nl) for NA, separated 400 µm longitudinally. All analyzed animals had well placed injections, as confirmed by post-mortem examination; medullary injection sites tended to fuse longitudinally. Injections were considered to be well placed only if (a) the injections--and their central cores--were centered in the DmnX and NA at all frontal levels, and (b) the injection spheres extended minimally both ventral to the DmnX and dorsal to
the NA (see injection sites in Figure 1; also Figure 1 in reference 5 and Figure 1 of reference 6). Diameters of DiI and DiA injections in NA are approximately 300 µm and 150 µm, respectively, (c) the series of injections primarily covered the bulk of the longitudinal extent of the DmnX and the NA, respectively.

Tissue preparation

Three weeks were allowed for the tracers to be transported to the heart. Animals were anesthetized with an overdose of pentobarbital sodium (100mg/kg) and perfused through the heart with 0.9% saline (300 ml) and 10% phosphate-buffered (pH = 7.4, 600 ml) formalin. Nodose ganglia and brainstems were removed. Each brainstem containing the entire DmnX and NA was stored in 10% sucrose formalin overnight and sectioned transversely at 100 µm on the second day. Thoracic vagal trunks, with the cardiopulmonary branches attached from the level of recurrent laryngeal nerves to a level 3 mm caudal to the junction of the inferior vena cava (IVC) and the right atrium, were also dissected. Atria were then separated and cut open as previously described (4). Briefly, the left atrium tissue block included the region of the junction with pulmonary veins; the right atrium had the superior vena cava, inferior vena cava, and the left pre-cava vein attached. The interatrial septum was separated from atria. Tissue surrounding the heart was gently removed, using extreme care to avoid peeling off the ganglionated plexuses(5) on the dorsal surface of the atria. Tissue was then dehydrated through graded concentrations (70%, 90%, and 2X100%) of glycerin for 4 hours. Finally, the tissue was mounted and coverslipped in 100% glycerin and n-propyl gallate (5%) to prevent fading.
**Examination of DiI and DiA injections sites.**

Brainstem slices were initially examined at 100X magnification using an epifluorescence microscope equipped with filter cubes appropriate for DiI, DiA, and FG, and were later scanned using a confocal microscope (Zeiss LSM 510) at 250X magnification. To present the registrations of DiI and DiA injections sites in vagal motor nuclei in a complete fashion, a montage of individual confocal projection images was assembled using Adobe Photoshop 5.5. DiI injection sites were evident by labeling with orange-red to red colors; DiA injection sites were in the yellow-green or green part of the spectrum; and FG labeled neurons appeared blue in color.

**Data acquisition and analysis of dual vagal projections in intrinsic cardiac ganglia**

A detailed description of the data acquisition strategy has been previously published (5, 6). Briefly, cardiac and nodose ganglia specimens were screened with a conventional epifluorescence microscope equipped with filter cubes appropriate for DiI (Rhodamine), DiA (FITC), and FG (UV), respectively. When DiI and DiA nerve fibers and endings were both found in the same region of cardiac ganglia at 200X magnification, their locations were recorded for subsequent detailed confocal microscopic analysis at 400X magnification. The digitized confocal images were stored on compact disks.

Consistent with earlier observations (5, 6), three major ganglionated plexuses labeled by FG were identified in the epicardium of the atria under UV light. Within each plexus, multiple ganglia were found (5). Using stacks of optical sections collected with the confocal microscope
through filter cubes appropriate to DiI and DiA, we examined the dual innervation of DmnX and NA cardiac axons and their terminal endings in each of the three ganglionated plexuses. Dual vagal axonal innervation of the three plexuses was examined systematically and completely. Stacks of confocal optical sections that contained both DiI and DiA fibers and endings were analyzed sequentially, and each cell apposed by labeled varicosities or fiber swellings was considered as being innervated. The presence and close proximity of varicosities were the criteria for judging a labeled fiber to have contact with a cell (5, 6).

Results.

1. Histological verification of DiI injection in the DmnX and DiA injection in the NA.

   We used a triple labeling technique to verify the DiI and DiA injection sites. Figure 1 is a montage of confocal photomicrographs obtained from a cross section of the brainstem at the level of the area postrema. Figure 1 shows that DiI injections into the left DmnX covered the whole left DmnX as well as some neurons in the medial portion of the right DmnX, whereas the DiA injections covered most of the NA. Therefore, both tracer injections were in precise registration with their targeted sites.

   Figure 1 goes about here.

2. DmnX and NA efferent fibers innervate different populations of intrinsic cardiac
ganglionic neurons.

Both DmnX and NA efferent fibers entered the epicardium of the atria and innervated the ganglia in all identified cardiac ganglionated plexuses (5). Frequently, DmnX and NA fibers and endings were found in the same cardiac ganglion with axons forming basket endings around cardiac principal neurons. As previously observed, DmnX neurons innervated SIF cells as well. In contrast, NA neurons did not. Figure 2 illustrates three examples showing that the DmnX and NA project to non-overlapping, different subpopulations of neurons within the same ganglion. We have carefully examined such doubly-labeled DiI and DiA terminals in 605 frames of confocal images randomly sampled from three ganglionated plexuses (which included 3,025 DiI and 1,815 DiA basket endings) and found that DmnX and NA neurons project to completely distinct populations of cardiac principal neurons in these plexuses.

3. Ipsilateral DmnX and NA efferent fibers distribute and innervate the same subregion of cardiac ganglia, but different populations of principal cardiac neurons.

Ipsilateral DmnX and NA axons entered cardiac ganglia in the same bundle and innervated different populations of cardiac principal neurons. Figure 3A is a montage of 26 frames of confocal projections and shows that DiI and DiA labeled fibers are intermingled and form basket endings very close to each other. The DiI and DiA basket endings are interdigitated
within the plexus. Examination of the DiI and DiA labeling in each frame of this large montage
did not reveal converging projections to the same principal neurons. It should be pointed out
that DiA is a weaker tracer than DiI in the sense that it labels fewer axons and terminals.
Therefore, the DiA labeled axons and endings projecting from the NA in this figure do not
appear as strongly labeled as those of DiI-labeled axons and endings. When tracers were
switched, i.e., the DmnX was injected with DiA and the NA was injected by DiI, the axons and
endings arising from the NA appeared much denser and stronger, as shown in Figure 4 D & E.
Therefore, it was necessary to switch tracers to investigate fluorescence properties of these
tracers.

Figs. 3 goes about here.

4. Contralateral DmnX and NA efferent fibers innervate neurons in separate subregions of
intrinsic cardiac ganglionic plexuses

Axons arising from neurons in contralateral DmnX and NA entered a cardiac
ganglionated plexus via separate routes or connectives at different entry points (a connective is a
mixture of extrinsic and intrinsic nerves connecting two separated cardiac ganglia in a
ganglionated plexus). Each plexus contains a number of ganglia. Figure 4 includes 3 montages
that show the right NA and left DmnX project to a cardiac ganglionated plexus (see also Figure 5
of Reference 5 for a FG labeled cardiac ganglionated plexus and different connectives at low
magnification). Figure 4A shows that a bundle of DiA-labeled NA fibers enter the plexus from a connective at one entry region, tending to innervate principal neurons situated near the entry region. Figure 4B shows that a bundle of DiI-labeled DmnX axons enters the same plexus through another entry point, to innervate principal neurons at the immediate entry region. Between these two opposite entry points, the DiA and DiI-labeled basket endings intermingle and innervate principal neurons interdigitately, as shown in Figure 4C. Therefore, neurons in the left and right sides of the brainstem appear to innervate topographically separated subdivisions of neurons in cardiac ganglionated plexuses.

Figs. 4 goes about here.

5. Projection of DmnX and NA efferent fibers to cardiac ganglia: Counterbalancing DiA and DiI.

To examine whether our results were influenced by the differential fluorescence properties of DiI and DiA, we switched the tracers used to inject the DmnX and NA. In 8 animals, we injected DiA unilaterally into the DmnX and DiI into the NA (either ipsilateral or contralateral). Comparable to previous labeling using alternate tracers in the brainstem nuclei, DiA labeled baskets are among numerous DiI baskets. Individually, DiA and DiI-labeled basket formations were evident. Labeled axons arising from the DmnX and NA innervated different
populations of principal neurons in the same cardiac ganglion (Figure 4D-E). Therefore, our results did not depend upon tracer fluorescence properties.

In contrast to the dense DiI-labeled axons and terminals projecting from the DmnX in Figure 3, the DiA labeled axons and basket endings projecting from the DmnX in Figure 4D were much more sparse than the DiI labeled axons and terminals originating from the NA, indicating that DiA is a weaker tracer.

6. Specificity of DiI and DiA labeling

We injected tracers to the DmnX and NA to label the cardiac motor axons and terminals in the heart. Three types of potential secondary labeling could occur. First, a tracer injection into the DmnX could also label ipsilateral NA neurons and *vice versa*. Second, a tracer injection into the DmnX or NA could also label the contralateral DmnX or NA. Third, a tracer injection into the DmnX or NA could label the nucleus of the solitary tract (NTS) region, and the vagal afferent terminals might pick up the tracer and transport it to the heart. For example, DiI injection into the DmnX may label NA, contralateral DmnX, and NTS; and DiA injection into the NA may label contralateral NA, DmnX, and NTS. Obviously, such potential problems could affect the interpretation of our findings.

According to our previous studies and after thorough examination of the brainstem, nodose ganglia, and atrial tissues used in the present study, we did not observe any evidence suggestive of such technical problems. Indeed, previously we have shown that DiI injections into the DmnX or NA did not label the other nucleus (5, 6). The present study confirms these
findings. In addition, after examination of the atrial tissue whole mounts, no flower-spray sensory terminals were found. Since flower-spray terminals are the major type of afferent terminals (4), we conclude that tracer injections did not label any vagal afferents in the heart. Examination of brainstem serial sections did not show any labeled contralateral NA neurons. In contrast, as shown in Figure 1, tracer injections into the DmnX could label the contralateral DmnX, especially in the caudomedial portion of the nucleus since the left and right DmnX are close to the midline and to each other at this level. Such contralateral labeling may account for the occasional DiI axons in the DiA bundle. However, this relatively limited technical issue concerning the two sides of the caudal DmnX has no bearing on our major observation that the DmnX and NA project to different populations of cardiac neurons.

It should be pointed out that it was important to make a relatively large injection of DiI into the DmnX as shown in Figure 1 because a major aim of the present study was to test the hypothesis that the NA and DmnX project to the same cardiac ganglia, but different populations of principal neurons therein. Therefore, we aimed to label as many DmnX and NA cardiac motor neurons as possible such that more DiI and DiA labeled terminals co-localizing at the same region within a cardiac ganglion would be available for analysis.

Such strategy, however, could lead to a potential leak of the tracer to the contralateral DmnX, as shown in Figure 1, and might affect our finding that neurons in the right and left brainstem topographically project to the different subdivisions of the plexus. In order to eliminate such possibility, we quantitatively estimated the maximal number of fibers being labeled from the contralateral DmnX and the innervation pattern of these axons. DiI was injected
into the left DmnX. The left cervical vagal trunk was transected in 2 rats to evaluate the extent of spread in the contralateral DmnX. In these animals, 5-7 axons and 20-28 basket endings were found, corresponding to 7.4% of the total axons and 3.9% of the total baskets which were estimated previously as originating from the left DmnX projection (5). Thus, only a minority of fibers on the contralateral DmnX were labeled by injections we employed in our experiments. In contrast to the well-labeled DiI axons from the ipsilateral DmnX that entered the heart in large bundles (Figure 4 A and 4B; also see 5, 6), these sparse secondarily labeled axons were weakly labeled and randomly distributed in the cardiac ganglionated plexuses. Since in the majority of other animals the large DiI and DiA bundles were separated and entered ganglionated plexuses through different pathways, and since the secondarily labeled fibers from the contralateral DmnX appeared weakly labeled, and sparsely and randomly distributed, the secondary labeling of the contralateral DmnX neurons should not detract from our findings.

Discussion.

Our results indicate that neurons in the NA and DmnX send axons which converge to the same cardiac ganglia and innervate distinctive principal neurons in these ganglia. Furthermore, we propose that neurons in the left and right sides of the brainstem vagal nuclei, project axons topographically to different neuronal subdivisions within the cardiac ganglionic plexuses. Our previous findings have shown that the NA and DmnX project axons to neurons in cardiac ganglia differently with respect to the size of projection fields, axon caliber, and terminal targets (5, 6). We have also found that lesions of the NA almost completely abolish the baroreflex control of
the heart rate (31) and that lesions of the DmnX do not change the baroreflex sensitivity (7, 31). Collectively, our data support the hypothesis that the NA and DmnX play different roles in the regulation of cardiac function.

**Topographical representation of left and right vagus in cardiac ganglionated plexuses.**

As mentioned above, the left and right vagal cardiac axons enter the cardiac ganglionated plexus through different routes. This observation raises the possibility that the left and right brainstem may have a well-defined topographical representation of the heart. Furthermore, this may indicate that cardiac ganglionic plexuses are not homogenous structures, and that they may be organized hierarchically to receive functionally-related inputs from the vagal brainstem nuclei in a topographical pattern. Whether our findings provide the anatomical basis for certain physiological observations indicative of left and right vagus lateralization is worthy of future investigation.

Regarding the topographic projections of the vagal motor nuclei to cardiac ganglionated plexuses, an additional intriguing issue is whether the different regions of the DmnX and NA project to different areas of cardiac ganglionated plexuses. In dogs and cats, Massari and his colleagues (3, 17) found that different locations of the NA projected to different cardiac ganglia in fat pads and had differential control over the heart rate, AV conduction and left ventricle contractility. Such data in the rat are not currently available. Our present dual labeling strategy, i.e., injection of different areas of the NA or DmnX with two tracers simultaneously and subsequent examination of cardiac ganglionated plexuses with the confocal microscope, may
provide a novel method for future investigation of this issue.

**NA and DmnX cardiac motor neurons: Functional significance.**

Conventionally, the NA was considered the major vagal motor nucleus which controls the heart. Recently, we demonstrated that neurons in the DmnX also project substantially to the heart, implying that the DmnX might control cardiac function, as well. The present finding that NA and DmnX project to the same cardiac ganglia suggests that they could both modulate the heart rate, A-V conduction, and myocardial contractility (6) because the adjacent cardiac principal neurons are thought to be have similar cardiac functions. From these data, we reasoned that NA and DmnX might be both involved in baroreflex control of heart rate.

In order to study the functional role of the NA and DmnX, we injected domoic acid to disrupt the integrity of the NA and DmnX, respectively, and studied their respective roles in baroreflex effect on the heart rate. To our surprise, lesions of the DmnX did not affect baroreflex sensitivity (7). In contrast, lesions of the NA almost completely eliminated baroreflex control of heart rate, indicating that the NA is critical for baroreflex control (31), whereas the DmnX is not.

Whether the DmnX may control the heart through some other cardiac reflexes is an interesting issue. Since the NA and DmnX innervate non-overlapping populations of cardiac principal neurons and play different roles in baroreflex, we hypothesize that the NA and DmnX may play distinct roles in different cardiopulmonary reflexes. Actually, it has been postulated that the DmnX may mediate pulmonary C-fiber-evoked bardycardia (14, 28).
In summary, neurons in two spatially separated medullary nuclei, the NA and DmnX, project axons to separate and non-overlapping populations of interspersed cardiac neurons. These findings provide the anatomical framework to account for the possibility that the NA and DmnX may have different functional roles in controlling the heart. The exact nature of such potential functional differences of the two nuclei as well as the property of the two populations of cardiac principal neurons await further studies.
Acknowledgments

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Figure Legends.

Figure 1. Confocal photomicrographs of a brainstem section at the level of the area postrema after DiA injection into the right NA and DiI injection into the left DmnX. (A) DiA and DiI injections in the brainstem selectively labeled NA and DmnX vagal cardiac motor neurons. In this brainstem section near the obex a DiA injection (yellowish-green or green) was positioned at the center of the right NA, where a group of FG-labeled NA neurons (blue) are located. A DiI injection (orange-red or red) effectively infiltrated the left DmnX area and the middle portion (pinkish-red) of the right DmnX (blue neurons). (B) The right DmnX in panel A at a higher magnification shows more clearly that the DiI injection into the left DmnX also partially covered the right DmnX. The pinkish neurons in the right DmnX were doubly labeled by DiI and FG. DiI labeling of these neurons became weaker as the distance increases to the right. (C) The FG labeled neurons in the right DmnX neurons in panel B were imaged in the UV channel. (D) The DiA injection site in panel A at a higher magnification. (E) The FG labeled NA motor neurons in panel D were imaged in the UV channel. DmnX: dorsal motor nucleus of the vagus. NA: nucleus ambiguus. NTS: Nucleus of the solitary tract. AP: area postrema. cc: central canal. Scale bar in A: 540 µm. Scale bar in C is 270 µm for B-C.

Of note, the FG labeled vagal motor neurons scanned using the UV laser were pseudocolored in blue arbitrarily to make them stand out of the multiple colored background.
Naturally, the FG labeled neurons under UV light look yellow-gold. Also, the center of the injection site of the DiI (Rhodamine red) had a yellow-orange helix or ring. This is because the image was scanned through three channels (rhodamine, FITC and UV) and emission light of DiI could not only be picked up by the rhodamine channel, but also picked by the FITC channel. Therefore, the DiI injection in the color merged image appears as yellow-orange-red. Similarly, the strong DiA (FITC) signal at the injection site bled through the rhodamine channel and hence appears yellow-green.

**Figure 2.** Confocal photomicrographs of DmnX and NA efferent axon innervation of cardiac ganglia principal neurons. In cardiac ganglia, DiI-labeled fibers and endings are red or orange-red and DiA-labeled NA fibers and endings appear green or yellowish-green. The soma of the principal neurons labeled by Fluoro-Gold (FG) are yellowish-brown. (A) A projection of a series of confocal optical sections shows that a DiI axon (arrow) forms a basket ending on a principal neuron at the center of the image (solid triangle) and a DiA fiber (arrow) innervates a principal neuron (open triangle) at the lower-left of the image. In addition, other green and red fibers are seen. A FG labeled principal neuron next to the DiI basket ending is designated by a *. (B) An optical section of the image in (A) illustrates the close contacts of a DiI fiber with the soma of the principal neuron, pointed by a solid triangle. It is apparent that DiA and DiI fibers innervate separate principal neurons in (A). The somata and nuclei of the ganglion cells are clearly visible. The same principal neuron as shown panel A is designated by *. (C) A projection showing that the DiI (red) and DiA (green) labeled basket endings occurred in close proximity to each other,
as shown in the upper part of the image. (D) A cluster of SIF cells (an arrow) in the cardiac ganglion are innervated by DiI fibers. The morphological characteristics of SIF cells has been fully described in (4) and 5 SIF cells are pointed by a solid triangle in Figure 4 C”. A DiA fiber innervates two principal neurons below the SIF cells. (E-H) From a 3-D perspective, NA and DmnX fibers and endings may be tightly packed or interdigitated in the cardiac ganglion. In (E), the projection of optically sectioned images shows several DiA and DiI baskets, which are intermingled and closely packed. In this projection image, some neurons appear to be dually innervated by DiI and DiA terminals, for example, the one indicated by a triangle. However, panels (F-H), which are the three optical sections of panel E, reveal that NA and DmnX neurons actually project axons to different populations of principal neurons. Note that panels E-F are such arranged that all four panels are spatially registered. No converging projections to the same principal neuron were detected. Scale bar in B is 20 µm for A, B, C, D, E, F, G, and H.

**Figure 3.** A montage of confocal photomicrographs shows that the ipsilateral NA (DiA) and DmnX (DiI) project to the same cardiac ganglionated plexuses but to different populations of principal neurons. (A) A bundle of mixed DiA- and DiI-labeled fibers entered a ganglionated plexus from the bottom right (two arrows), traversed the space between the ganglia, and generated basket endings around many principal neurons along its path. One arrow points to a DiI labeled basket ending and another arrow indicates a DiA labeled basket ending (see Figures 2B & 4C”” for optical sections of the basket endings; also Reference 5 and 6). (B) is the upper portion of panel A at a higher magnification. The separation line of the upper and lower portions
in panel A is indicated by * . Scale bars in A and B: 60 µm.

**Figure 4.** (A-C) Montages of confocal photomicrographs shows that neurons in left DmnX (DiI labeled) and right NA (DiA labeled) innervate topographically distinct regions within an intrinsic cardiac ganglionated plexus. In this plexus, the DiA-labeled NA fibers (green or yellowish-green) and DiI-labeled DmnX fibers (red or orange-red) enter the plexus through separate entry points. The NA and DmnX fibers tend to innervate principal neurons situated near their respective entries. (A) The NA fibers enter the plexus from the top-right and produce dense basket endings around principal neurons located in the vicinity of the entrance site. Only a couple of DiI endings are seen. (B) The DmnX fibers enter the same plexus from the bottom-left and densely innervate principal neurons at this entry. (C) In the middle of this plexus, abundant NA and DmnX fibers and endings are seen, and they together form a complex neural network. C’, C”, C’’’ expose “one” neuron pointed by an arrow in panel C which appears to be innervated by both DiI and DiA endings. Through examination of consecutive optical sections, 2 neurons were visualized instead of one, and these 2 neurons are separately innervated by DiI and DiA, as indicated by the arrows in C” and C’’’. In addition, a solid triangle points to 5 SIF cells in C” (note smaller size compared to principal neurons). As in Figure 3, the NA and DmnX project to separate populations of principal neurons. Scale bar: 30 µm for A, 50 µm for B and C, and 40 µm for C’, C’’, C’’’. (D) Confocal photomicrographs showing projections from NA (labeled with DiI) and DmnX (labeled with DiA) neurons to a cardiac ganglion. (E) An optically sectioned image of the field in panel (D) indicating that DiA-labeled (DmnX)and DiI-labeled (NA) endings innervate different principal neurons. Scale bar: 40 µm for A and B.
Figure 1
Figure 2
Figure 4