Bilateral distribution of vagal motor and sensory nerve fibers in the rat’s lungs and airways

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Received 15 January 2000; accepted in final form 14 March 2000

Pérez Fontán, J. Julio, Catherine T. Diec, and Christine R. Velloff. Bilateral distribution of vagal motor and sensory nerve fibers in the rat’s lungs and airways. Am J Physiol Regulatory Integrative Comp Physiol 279: R713–R728, 2000.—This study combined single and transneuronal labeling to define the origin of midline-crossing vagal fibers projecting to the rat’s lungs. Injections of the β-subunit of cholera toxin (CT-β) into the lungs labeled similar numbers of neuronal somata in the nucleus ambiguus and dorsal motor nucleus of the vagus on both sides of the medulla, even though vagal stimulation increased lung resistance 50% less in the contralateral than in the ipsilateral lung. Unilateral cervical vagotomy prevented CT-β labeling of ipsilateral neuronal somata and sensory fibers, indicating that lung-bound vagal fibers undergo decussation only inside the thorax. Injections of CT-β and FluoroGold into opposite main stem bronchi double labeled 30% and 11% of all neuronal somata immunoreactive for CT-β and FluoroGold, respectively, showing that one single vagal motoneuron can innervate airways on both sides. Injections of pseudorabies virus into the right lung revealed a bilateral network of infected neurons, even after unilateral vagotomy. The latter did not prevent infection of the ipsilateral vagal nuclei. These findings demonstrate that vagal motoneurons that project to the lungs receive contralateral inputs from the airway premotor network and vagal bronchomotor centers.

For such a system to be mechanically efficient, however, preganglionic inputs must reach distant regions of both lungs at approximately the same time and with similar intensity. Vagal stimulation and neuroanatomic tracing studies suggest that these requisites are met in part by a dual-innervation arrangement in which each lung receives fibers from both vagus nerves. The existence of such an arrangement has been suspected since the early neuroanatomic studies of Larsell and Mason in rabbits (16) and Honjin in mice (13) revealed that a substantial number of lung vagal preganglionic and sensory fibers do not undergo degeneration after ipsilateral cervical vagotomy. Olsen et al. (23) provided functional evidence for the presence of midline-crossing vagal fibers by showing that unilateral electrical stimulation of the vagus nerve increases the airflow resistance of both lungs in dogs and cats. More recently, using horseradish peroxidase as a retrograde neuronal tracer, Kalia and Mesulam (15) demonstrated that the right main stem bronchus of the cat receives a bilateral supply of motor fibers that originate from the nucleus ambiguus and dorsal motor nucleus of the vagus. Interestingly, the same investigators reported that the apical lobe of the right lung receives bilateral innervation only from the dorsal motor nucleus of the vagus; innervation from the nucleus ambiguus was unilateral.

The present study was designed to uncover some unexplored features of this dual-innervation arrangement in the rat. First, we assessed the size and trajectory of the contingent of vagal motor and sensory fibers that cross the midline to innervate the contralateral lung by analyzing the effects of cervical and thoracic vagotomies on the topography of neuronal labeling by the β-subunit of cholera toxin (CT-β) injected into the lungs. Second, we investigated whether individual vagal motor fibers are committed to the innervation of unilateral targets or divide to reach bilateral targets by determining the rate of double labeling of medullary neurons after injections of CT-β and FluoroGold (FG) into opposite main stem bronchi. Finally, we elucidated the existence of intramedullary connections between vagal preganglionic centers by examining the effects of...
unilateral vagotomy on the retrograde transsynaptic labeling of brain stem neurons by pulmonary injections of pseudorabies virus.

**METHODS**

All experiments were performed in male Sprague-Dawley rats (300–400 g body wt, 10–12 wk old; Charles River, Wilmington, MA) following protocols approved by the Washington University Animal Studies Committee. The rats were housed at 23°C in a climate-controlled room with access to standard rat chow and water.

**Midline Crossing by Vagal Motor and Sensory Fibers**

The experiments described here ascertained the trajectory and size of the contingent of vagal fibers that cross the midline to innervate the contralateral lung. Our experimental approach included two complementary strategies. The first, aimed at defining whether vagal motor and sensory fibers undergo decussation in the brain stem (Fig. 1A), compared the topographical patterns of neuronal labeling produced in the medulla oblongata by the injection of CT-β into the lungs of rats that had intact vagi (n = 16, divided into 2 groups of 8 rats, depending on the side of the injection) or had undergone unilateral cervical vagotomies (n = 32, divided into 4 subgroups of 8 rats, each representing a different combination of lung injection and vagotomy sides). The second strategy was designed to elucidate whether midline-crossing vagal fibers exit the vagal trunk above or at the origin of the recurrent laryngeal nerve or follow the path of smaller parahilar branches that emerge from the nerve near the tracheal bifurcation. To this end, we analyzed the effects of a low-thoracic vagotomy on the response of each individual lung to unilateral vagal stimulation (Fig. 1D; n = 18) and the retrograde labeling of vagal medullary neurons by CT-β injected into the ipsilateral lung (n = 6).

**Pulmonary injections of CT-β**. The rats were anesthetized with halothane (0.5–3%), which was piped initially into an induction box or, after the institution of mechanical ventilation, blended into the inspiratory limb of the ventilator’s circuit. The trachea was intubated through the mouth with the help of a pediatric otoscope, and the endotracheal cannula was connected to a rodent ventilator (Harvard Apparatus, South Natick, CA). The lung, right or left, was exposed under a dissecting microscope through a lateral thoracotomy at the fourth or fifth intercostal space. A 50-μl volume of 0.1% CT-β suspension (List Biological Laboratories, Campbell, CA) was then injected into the lung parenchyma with use of a Hamilton microsyringe (Reno, NV). The injectate volume was divided into 5–10 injections, each performed with the needle tip ~1–3 mm below the pleural surface while lung volume was held constant to minimize disruption of the lung tissue. After every injection, the visceral pleura was blotted with a cotton-tipped probe to minimize nonspecific neuronal labeling by leaked CT-β suspension. After completion of the injections, the lung surface was washed thoroughly with normal saline and allowed to dry for 3–5 min before the thoracotomy was closed by layers. A small silicone rubber tube was left inside the chest for removal of any air remaining in the pleural space at the end of the surgery. On recovery from anesthesia, the rat was returned to the holding facility, where it remained for 9–11 days.

**Cervical and thoracic vagotomies**. Cervical vagotomies were carried out through a paramedial neck incision. The vagus nerve was isolated from the carotid artery, and a 0.5-cm segment of the nerve distal to the origin of the superolaryngeal nerve was removed. The incision was closed by layers. Thoracic vagotomies were performed only on the right side to minimize animal usage. The vagus nerve was visualized through a lateral thoracotomy by retracting the lung anteriorly. The entire trajectory of the nerve caudal to the origin of the recurrent laryngeal nerve was dissected from the surrounding tissues and removed. The thoracotomy was closed as described above.

**Effect of unilateral vagal stimulation on lung resistance**. Rats were anesthetized with pentobarbital sodium (50 mg/kg ip). The trachea and the vagus nerves were exposed through a midline neck incision, and a tracheostomy was performed.

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![Fig. 1. Schematic illustration of experimental strategies. A: retrograde labeling of vagal motoneurons by injection of β-subunit of cholera toxin (CT-β) into right lung after right cervical vagotomy. If, as hypothesized, motor nerve fibers cross the midline caudal to the vagotomy, then only neurons on the left side of the medulla would be labeled. B: retrograde double labeling of medullary vagal motoneurons by injections of CT-β (black) and FluoroGold (FG; gray) into right and left main stem bronchi, respectively. Vagal neurons innervating exclusively one bronchus would be labeled only by the corresponding marker (solid black or gray circles); in contrast, neurons that innervate both bronchi may be immunoreactive for both markers (split black and gray circles). C: input pathways between vagal nuclei by pseudorabies virus injected into the right lung after right cervical vagotomy. Because the vagotony severs all nerve fibers originating on the right side of the medulla (A), viral infection (dark circles) of neurons in right nucleus ambiguous indicates retrograde transneuronal labeling via vagal motoneurons on the opposite side. D: right thoracic ambiguous vagotomy caudal to the origin of the recurrent laryngeal nerve (RLN). Preservation of right or left lung responses to right cervical vagus stimulation (Stim) would indicate that fibers bound to the right and left lungs, respectively, exit the nerve trunk above or with this nerve. VN, vagus nerve; NG nodose ganglion.](image-url)
at the second intercartilaginous space. A cannula made from a 6-cm section of PE-150 tubing (0.12 cm ID) tapered at the end was inserted into the trachea and cannula was connected to the rodent ventilator through an ensemble consisting of a small tube with a lateral port for measurement of airway pressure (model MAP45, ±56 cmH₂O, Validyne Engineering, Northridge, CA) and a Fleisch no. 000 pneumotachograph attached to a differential pressure transducer (model MAP45, ±2.3 cmH₂O) for measurement of airway gas flow. The length of the tubing connections was adjusted to ensure that the transducer outputs were well matched dynamically and had an amplitude-frequency response appropriate for the conditions of the experiments. The transducer outputs were amplified and recorded digitally at an acquisition rate of 500 Hz.

To determine the effects of vagal stimulation on lung resistance, we first exposed the lungs through a widened median sternotomy. After cutting the vagus nerves caudal to the origin of the superior laryngeal nerves, we denuded the distal stump of each nerve and placed it on a bipolar electrode attached to a nerve stimulator (model 50-5008, Harvard Apparatus), with the nerve and electrode covered with mineral oil to prevent dehydration. Next, we defined the supramaximal stimulus for each rat by varying stimulation frequency between 5 and 40 Hz and potential between 2 and 30 V while monitoring airway pressure as described previously (24). Pulse width was maintained constant at 0.5 ms. We then loosened the tie around the trachea and advanced the tracheal cannula into a main stem bronchus, right or left in random order, until the cannula’s tapered end was wedged inside the bronchial lumen without obstructing segmental bronchial branches, as demonstrated by the selective and complete inflation of the corresponding lung. Before performing any measurements, we inflated the lung to an airway pressure of 30 cmH₂O for 1–2 s to provide a consistent volume history. We then applied the supramaximal stimulus to the distal stump of the vagus nerve while the lung was ventilated with a peak pressure of 15 cmH₂O at a rate of 80 breaths/min. The stimulus was discontinued as soon as its effect on airway pressure reached a maximum, usually within 30 s of stimulation. Finally, we pulled the cannula tip back into the trachea and allowed the rat to recover for 3–5 min before repeating the measurements in the contralateral lung. Total lung resistance was calculated as the ratio of the airway pressures and flows measured at midvolume points during inspiration and expiration (20). The increase in lung resistance produced by vagal stimulation was computed as the difference between the maximum resistance and the resistance determined before the initiation of the stimulus.

**Bilateral Innervation of Airways by Vagal Motoneurons**

These experiments tested the hypothesis that a single vagal motoneuron can provide innervation to airways in both lungs. Our experimental approach was based on a simple double-labeling scheme in which CT-β and FG were used as retrograde neuronal markers (Fig. 1B). To ensure consistency between injections and reduce the potential for contamination of other organs within the chest, we injected both markers into the main stem bronchi at the lung hilum. At this location, the bronchi are large enough to allow visual inspection of the site as the injection progresses. They are also separated from each other by the mediastinum and distant enough from the esophagus and the large vessels and heart to minimize the potential for nonspecific labeling of vagal neurons innervating these structures when small volumes of tracer are injected.

The rats (n = 24) were prepared as described above for the pulmonary injections. The main stem bronchus, right or left, was visualized through a lateral thoracotomy by retracting the lung anteriorly. CT-β (0.1%) or FG (15% solution, Fluorochrome, Denver, CO), dissolved in a 1- to 1.5-μl volume, was injected through a glass micropipette mounted on an X-Y-Z micromanipulator (Stoelting, Wood Dale, IL) in two to five individual injections distributed over the external surface of the bronchus. The bronchial serosa was lifted slightly with the tip of the pipette before the injections to facilitate diffusion of the injectate within the subserosal space and minimize backpressure-induced leakage. Once again, the surface of the bronchus was blotted with a cotton-tipped probe after each injection, and the pleural space was washed thoroughly with normal saline before the thoracotomy was closed. The contralateral bronchus was injected with the alternative neuronal marker 24 h later after a similar procedure. The survival period before removal of the brain and spinal cord was 11–12 days.

**Interconnections Between Vagal Medullary Centers: Pseudorabies Virus Injections**

This final group of experiments was designed to determine whether vagal motoneurons projecting to the lungs receive inputs from contralateral vagal premotor or motor neurons. We took advantage of the retrograde transteun neuronal labeling properties of the Bartha strain of pseudorabies virus (29). We also relied on the demonstration by the experiments described above that no pulmonary-bound vagal motor fibers cross the midline above the neck. We reasoned that a unilateral cervical vagotomy would preclude labeling of ipsilateral medullary neurons unless this labeling occurred transsynaptically through neurons located in the contralateral side of the medulla (Fig. 1C).

Pseudorabies virus was injected into the right lung of 28 rats, which were divided into 2 groups of 14 rats each, depending on whether the cervical vagotomy was on the right or left side. (Once again, no injections were performed into the left lung to minimize animal usage.) The procedure was analogous to that described above for the CT-β injections. The viral suspension containing 3–4 × 10⁷ plaque-forming units of the Bartha strain of pseudorabies virus was injected in 0.5-μl aliquots to a total injection volume of 1–1.5 μl. The survival period after the injections was 5 days.

**Selectivity of Neuronal Tracer Injections**

The selectivity of neuronal labeling by the pulmonary injections of the various neuronal tracers used in the study relied on the restriction of the injectate to the injected lung. In designing the experiments, we were especially concerned that leakage from the lung CT-β injection sites into the pleural cavity and diffusion of CT-β across the visceral pleura into thoracic organs such as the esophagus or the heart, which receive a rich supply of motor and sensory nerve fibers from the vagus (1, 5, 7, 9, 28, 30, 31), could result in spurious labeling of medullary neurons (8). We performed four types of control experiments to address this concern.

The first experiment studied the distribution over time of a sample of 125I-CT-β injected into the lungs of nine rats. Radioiodination of CT-β was performed as described previously (17). A standard 0.25% suspension of CT-β (List Biological) was diluted 1:1 to a final volume of 200 μl with 0.1 M NaPO₄-buffered saline and incubated for 10 min with 20 μl of a solution containing 2 mCi of Na¹²⁵I (New England Nuclear,
Boston, MA) in the presence of two Iodo-Beads (Pierce Chemical, Rockford, IL). After addition of 30 μl of cytochrome c (20 mg/ml; Sigma-Aldrich, St. Louis, MO) as a color label, the reaction volume was fractionated in a Sephadex G-10 column (Sigma-Aldrich). A 20- to 40-μl volume of the radiolabeled CT-β fraction (specific activity ~4,600 cpm/ng, concentration 0.08%) was then injected in 10-μl aliquots into the right or the left lung following the procedure described above. After survival periods of 8 h (n = 2), 24 h (n = 3), and 7 days (n = 4), the thoracic viscera were removed. The lungs were cut in coronal slices, which were then mounted flat on a film plate along with the trachea and esophagus to obtain an autoradiographic image. Lastly, the lung tissue was homogenized, and the number of radioactive counts in representative samples from each lung was determined in a scintillation counter.

The second control experiment was aimed at establishing whether CT-β suspension leaked from the injection sites may cause unintended labeling of medullary neurons innervating other organs in the thoracic cavity. We performed a small

Table 1. Primary antisera used for immunohistochemical analysis

<table>
<thead>
<tr>
<th>Immunizing Protein/Selectivity</th>
<th>Raised in</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT-β</td>
<td>Goat</td>
<td>1:10,000</td>
<td>List Biological Laboratories (Campbell, CA)</td>
</tr>
<tr>
<td>FG</td>
<td>Rabbit</td>
<td>1:500</td>
<td>Chemicon International (Temecula, CA)</td>
</tr>
<tr>
<td>Pseudorabies virus</td>
<td>Pig</td>
<td>1:60,000</td>
<td>Loewy*</td>
</tr>
<tr>
<td>Human choline acetyltransferase</td>
<td>Rabbit</td>
<td>1:500</td>
<td>Biogenesis (Poole, UK)</td>
</tr>
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CT-β, β-subunit of cholera toxin; FG, Fluoro Gold. *See Ref. 29.

Fig. 2. Effect of right or left cervical vagotomy on topographical distribution of vagal motoneurons labeled retrogradely by injections of CT-β into the left or right lung of rats. Counts of neuronal somata labeled by CT-β in the left (LNA) or right (RNA) nucleus ambiguous and left (LDMV) or right (RDMV) dorsal motor nucleus of the vagus are plotted at 0.5-mm intervals from 14.5 to 11.5 mm caudal to bregma (B = 14.5 and B = 11.5). Cervical vagotomies precluded labeling of ipsilateral vagal motoneurons but had no discernible effect on the number of labeled contralateral neurons. When the vagi were left intact, a greater number of neuronal somata was labeled by the left than by the right lung injections (P = 0.002). Shading of squares and connecting lines are used to identify each rat at various levels of the medulla.
right lateral thoracotomy in six rats and instilled a 30-μl volume of a 0.1% suspension of CT-β on the lung surface. The thoracotomy was then closed by layers. The survival period after the injections was 11 days.

The third control experiment compared the topographic distribution of medullary neurons labeled by concomitant injections of CT-β into the lung and FG into the esophagus. This comparison was intended to identify double-labeled neurons, which would alert us of the presence of CT-β leakage from lung injection sites. The injections were performed through a right thoracotomy in 13 rats. CT-β (50 μl of 0.1% suspension) was injected into the right rostral and medial lobes as described above. FG (0.8 μl of a 4% solution) was injected into the right aspect of the wall of the midthoracic portion of the esophagus with a glass micropipette. The survival period was 11–13 days.

Finally, the last control experiment tested our ability to discern between neurons innervating adjacent airway targets by comparing the distribution of medullary neurons labeled by CT-β and FG, each injected alternately into the extrathoracic and intrathoracic trachea of six rats. Because innervation of the trachea appears to follow a segmental pattern (15), we predicted that these injections would label two distinct populations of parasympathetic preganglionic neurons. CT-β (1 μl of 0.1% suspension) and FG (1 μl of 15% solution) were injected into the anterior or lateral wall of the trachea, which was exposed through a midline neck incision for the extrathoracic injections and through a lateral thoracotomy for the intrathoracic injections. The survival period was 11–13 days.

**Preparation and Staining of Brain Tissue**

At the end of the preestablished survival period after the neuronal tracer injections, anesthesia was induced again, this time with pentobarbital sodium (50 mg/kg ip). The heart and lungs were exposed through an extended median sternotomy, and heparin (1,000 U/kg) was injected into the right ventricle. The rat’s systemic circulation was perfused with 0.1 M sodium phosphate-buffered saline and then with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.40) through a cannula inserted into the aortic root through the left ventricular wall. The spinal cord and brain were removed, submerged in 4% paraformaldehyde solution for 2 days, and stored in buffered 30% sucrose until they were sectioned.

The brain stems were cut into 50-μm-thick coronal sections on a freezing microtome. A 1-in-5 series of the sections was immersed in a blocking solution containing 5% donkey serum (Sigma-Aldrich) in 0.3% Triton X-100 and 0.02 M potassium phosphate buffer at room temperature for 30 min and incubated overnight, also at room temperature, with the appropriate solution of primary antiserum (Table 1). After they were rinsed thoroughly in buffered saline, the sections were placed for 3–4 h in a 1:100 dilution of biotinylated anti-IgG antiserum (double-labeling studies) or 1:50 dilutions of fluorescent-labeled (FITC or FITC and tetramethylrhodamine isothiocyanate) anti-IgG antisera (single-labeling studies) or 1:50 dilutions of 0.1% suspension of CT-β leakage. Finally, the last control experiment tested our ability to discern between neurons innervating adjacent airway targets by comparing the distribution of medullary neurons labeled by CT-β and FG, each injected alternately into the extrathoracic and intrathoracic trachea of six rats. Because innervation of the trachea appears to follow a segmental pattern (15), we predicted that these injections would label two distinct populations of parasympathetic preganglionic neurons. CT-β (1 μl of 0.1% suspension) and FG (1 μl of 15% solution) were injected into the anterior or lateral wall of the trachea, which was exposed through a midline neck incision for the extrathoracic injections and through a lateral thoracotomy for the intrathoracic injections. The survival period was 11–13 days.

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p-phenylenediamine for fluorescent-stained sections to prevent fading), and protected with coverslips.

**Data Analysis**

Diaminobenzidine- and fluorescent-stained sections were examined with bright-field and fluorescence microscopy, respectively. Labeled neuronal somata and fibers were placed on a computerized map of the rat’s brain stem (23a); a color code was used to differentiate single- and double-labeled neuronal somata. Labeled neurons were counted in medullary sections obtained at 0.5-mm intervals starting at the medullary-spinal junction, and the results were analyzed for the effects of injection site, vagotomy, and section level with an ANOVA with replication. Fisher’s protected least significant difference was used for a posteriori comparisons. Increases in total lung resistance during vagal stimulation were compared for differences between lungs or the side of the stimulated vagus also with an ANOVA. All the photomicrographs used in the illustrations were scanned digitally into Adobe Photoshop format for mounting and labeling. Only color and contrast were adjusted to reproduce the conditions viewed under the microscope.

**RESULTS**

**Midline Crossing by Vagal Motor and Sensory Fibers**

Characteristics of neuronal and sensory fiber labeling by lung injections of CT-β. Neuronal somata and sensory fibers were labeled in the medulla of all but 3 of...
the 32 rats that survived the 9- to 11-day period allowed for transport of CT-β from the lung injection site to the central nervous system. Deaths usually occurred within 12 h of the surgery, bearing no apparent relationship in their frequency to the side of the injection or to whether the rat had undergone a cervical vagotomy.

When both vagus nerves were intact, injection of CT-β into one lung caused labeling of surprisingly similar numbers of neuronal somata on both sides of the medulla (Fig. 2). Although labeling density varied substantially from rat to rat, the total number of CT-β-labeled neurons was consistently greater after left than after right lung injections. Labeled neurons were grouped in two distinctive nuclear divisions. The main or ventral division consisted of large, often multipolar, neurons, which were clustered in the rostralmost portion of the nucleus ambiguous proper, corresponding to the compact formation of the nucleus ambiguous (5), or distributed more loosely in the region of the lateral tegmental field immediately ventral to the nucleus (Fig. 3), in the subdivision known as the external formation in the same nomenclature. Although isolated neurons were labeled caudally in the area often referred to as nucleus retroambigualis, the majority of the neurons labeled in the nucleus ambiguous complex were aligned rostrocaudally in a 1.5-mm-long column rostral to the obex (from 13 to 11.5 mm caudal to the bregma). The second or dorsal division of retrogradely labeled neurons was considerably sparser and included smaller, frequently bipolar neuronal somata located in the dorsal region of the dorsal motor nucleus of the vagus.

Sensory fibers were labeled bilaterally in the area of the nucleus of the tractus solitarius. Fiber density was usually greater on the side of the CT-β injection, but in some sections, differences between sides were not apparent. Labeled sensory terminals were more abundant below the obex (Fig. 4), concentrating in the commissural subnucleus, in the caudalmost sections, and in the medial and ventrolateral subnuclei of the nucleus of the tractus solitarius, more rostrally.

Effect of unilateral vagotomy on neuronal and sensory fiber labeling. Cervical vagotomies prevented labeling of neuronal somata and sensory fibers on the ipsilateral medulla but did not affect labeling in the contralateral medulla (Figs. 2–4). The vagotomies had no apparent effect on the proportions of neuronal somata labeled in the nucleus ambiguous complex and dorsal motor nucleus of the vagus or, within the nucleus ambiguous, on the partition of labeled neurons between the compact and external formations.

Effect of thoracic vagotomy on response to vagal stimulation and CT-β labeling after lung injections of CT-β. Electrical stimulation of the distal stump of the cervical vagus increased the resistances opposed by both lungs to airflow (Fig. 5). The increases were maximal at stimulation frequencies of 30–40 Hz and were more pronounced in the ipsilateral than in the contralateral lung (2.1 ± 0.2 and 2.9 ± 0.7 times greater, depending on whether the left or the right vagus was stimulated) and during left than during right vagal stimulation.

Interruption of the right vagus nerve caudal to the origin of the recurrent laryngeal nerve reduced but did not eliminate responses of both lungs to stimulation of the right cervical vagal trunk, suggesting that lung-bound vagal motor fibers emerge from the vagal trunk at the origin of the recurrent laryngeal nerve or rostral to this nerve.
Bilateral Innervation of Airways by Vagal Motoneurons

Labeling with CT-\(\beta\) and FG was found to coexist in 13 of the 18 rats that survived the appointed period of 11 days after the injections (6 injected with FG into the left bronchus and CT-\(\beta\) into the right bronchus and 7 injected with the alternative scheme). Although there were no differences in the anatomic organization of the neurons labeled by the two retrograde neuronal markers (Fig. 6), considerably fewer neurons were labeled by CT-\(\beta\) than by FG. The topographic distribution of the neuronal soma labeled by the bronchial injections was undistinguishable from that produced by the injection of CT-\(\beta\) into the lungs.

Double labeling (Fig. 7), indicating uptake of both markers by the same neuron, was observed in one or more sections in all the rats. Of all the neurons labeled by CT-\(\beta\) in the nucleus ambiguus, 30% were also labeled by FG; conversely, of all the nucleus ambiguus neurons labeled by FG, 11% were also labeled by CT-\(\beta\).

Interconnections Between Vagal Medullary Centers

We found extensive neuronal labeling indicating transsynaptic infection with pseudorabies virus on both sides of the brain stem in 13 of the 24 rats that survived 5 days after injection of the virus (Fig. 8). In addition to areas known to contain vagal preganglionic neurons (nucleus ambiguus and dorsal motor nucleus of the vagus), virus-infected neurons were present in the nucleus of the tractus solitarius (including the subnuclei found to contain labeled sensory fiber terminals after lung injections of CT-\(\beta\)), midline reticular nuclei (raphe pallidus, raphe obscurus, parapyramidal, and raphe magnus nuclei), ventrolateral medulla (lateral paragigantocellular, gigantocellular, and retroganglionic nuclei), and noradrenergic nuclei of the medulla and pons (A5, locus ceruleus, and locus subceruleus). There were no labeled neurons in the gracilis or cuneate nucleus, confirming previous re-

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**Fig. 6.** Retrograde labeling of vagal motoneurons in the nucleus ambiguus by separate injections of CT-\(\beta\) and FG into the main stem bronchi. Counts of single (CT-\(\beta\) or FG)- and double-labeled (CT-\(\beta\) + FG) neurons in the left (LNA) and right (RNA) nucleus ambiguus are plotted at 0.5-mm intervals from 14.5 (B – 14.5) to 11.5 mm (B – 11.5) caudal to bregma for each of the 2 labeling schemes used in the experiment.
ports that, at the viral doses used in the experiments, the virus does not infect sensory pathways (26).

Unilateral cervical vagotomies reduced but did not eliminate the presence of virus-infected neurons in the nucleus ambiguus and dorsal motor nucleus of the vagus ipsilateral to the cut vagus nerve (Fig. 9). Simultaneous staining with antibodies against choline acetyltransferase confirmed the cholinergic identity of these neurons (Fig. 10).

**Controls for Selectivity of Tracer Injections**

*Distribution of $^{125}$I-CT-β injected into the lung.* Autoradiographic analysis confirmed that, although the injected $^{125}$I-CT-β was almost entirely removed from the lung after 7 days, within the lungs, the tracer did not migrate beyond the immediate vicinity of the injection sites (Fig. 11). No accumulation of radioactivity was found in the contralateral lung, the trachea, or the esophagus. The ratio of total lung count measured in homogenates of injected and contralateral lungs removed at 8 h to that removed at 24 h was <0.02.

*Labeling of vagal medullary neurons by intrapleural CT-β.* None of the six rats injected with CT-β suspension into the pleural space exhibited labeling of neuronal somata or sensory fiber terminals in the medulla (data not shown).

*Distinction between pulmonary and esophageal motoneurons.* Injections of CT-β into the rostral and medial lobes of the right lung and FG into the wall of the midthoracic esophagus produced concurrent labeling in the nucleus ambiguus and dorsal nucleus of the vagus in 7 of the 12 rats that survived the procedure. No double labeling was detected in any of these rats (Fig. 12). The somata of the majority of the neurons labeled by the injections of CT-β into the lungs were once again distributed bilaterally between the compact and the external formations of the nucleus ambiguus in the sections located between 13.5 and 11.5 mm caudal from the bregma. The somata of the neurons labeled by the injections of FG into the esophageal wall were also found bilaterally, but they were circumscribed to the compact formation, where they were interspersed with the CT-β-labeled neurons. Esophageal neurons adopted a more rostral distribution than the pulmonary neurons; they were found rarely in medullary sections >12.5 mm caudal to the bregma.

*Distinction between motoneurons innervating extra- and intrathoracic trachea.* Labeling by CT-β and FG injected into extra- and intrathoracic segments of the trachea coexisted in all six rats included in this control experiment. We found no double-labeled neuronal somata in the nucleus ambiguus or dorsal motor nucleus of the vagus in any of these rats (Fig. 13).

**DISCUSSION**

The autonomic innervation of bilateral organs that are functionally interdependent must contain mechanisms to prevent asymmetries in neural input from interfering with mechanical efficiency. In the case of the lungs, the mechanism appears to be based in part
Fig. 8. Composite chart showing distribution of virus-infected neurons (red stars) in rats that had undergone a left \((n = 6)\) or right \((n = 7)\) cervical vagotomy before being injected with pseudorabies virus into the right lung (incubation period after injections = 5 days). Unilateral vagotomy reduced, but did not eliminate, retrograde viral labeling in the ipsilateral nucleus ambiguus and dorsal motor nucleus of the vagus. Considered in conjunction with the information shown in Fig. 3, this finding suggests that vagal motoneurons projecting to the lungs receive inputs from the contralateral vagal centers. These inputs may help coordinate vagal outflows from both sides of the medulla. Vagotomy also had no apparent effect on distribution of second- or higher-order premotor neurons, but some of these neurons may have been infected via the sympathetic nerve supply of the lung, which was not interrupted. A5, A5 noradrenergic group; Cu, cuneate nucleus; DC, dorsal cochlear nucleus; DMV, dorsal motor nucleus of the vagus; Gi, gigantocellular reticular nucleus; GiV, ventral division of gigantocellular reticular nucleus; Gr, nucleus gracilis; KF, Kölliker-Fuse nucleus; LC, locus ceruleus; LPGi, lateral paragigantocellular nucleus; LVe, lateral vestibular nucleus; MoV, motor trigeminal nucleus; Mve, medial vestibular nucleus; NA, nucleus ambiguus; NRA, nucleus retroambiguus; NTS, nucleus of the tractus solitarius; Pr, prepositus nucleus; PT, pyramidal tract; RMg, raphe magnus nucleus; ROb, raphe obscurus nucleus; Rpa, raphe pallidus nucleus; SpV, spinal trigeminal nucleus; SC, locus subceruleus; SpVe, spinal vestibular nucleus; VII, facial nucleus; XII, hypoglossal nucleus.
on a system of double innervation, whereby each lung receives supplies of motor and sensory nerve fibers from both vagus nerves. In the present study we demonstrate that 1) as many as one-half of the vagal motor fibers and a smaller, but still substantial, proportion of the sensory fibers destined for the rat’s lungs cross the midline, and they do so exclusively inside the thorax; 2) at least for the main stem bronchi, a single medullary motoneuron can innervate airways on both sides of the midline; and 3) medullary vagal motoneurons that project to the lungs receive inputs from an extensive bilateral neuronal network that includes sensory relay areas in the nucleus tractus solitarius and the contralateral nucleus ambiguus and dorsal motor nucleus of the vagus.

**Midline Crossing by Vagal Motor Fibers**

The counts of neurons labeled by lung injections of CT-β suggest that each lung receives a unexpectedly symmetrical supply of vagal motor fibers from both sides of the medulla. This symmetry contrasts with the ipsilateral preponderance of lung innervation reported by Kalia and Mesulam (15), who used injections of horseradish peroxidase to analyze the topography of the medullary neurons that innervate the cat’s lungs and airways. These investigators found that, when the tracer was injected into the right main stem bronchus, over one-third of the neuronal somata labeled in the nucleus ambiguus and dorsal motor nucleus of the vagus and a substantial portion of the central sensory fiber terminals were on the left side of the medulla. When the injections were made into the apical lobe of the right lung, however, only dorsal motor nucleus neurons and sensory terminals were labeled in the left side of the medulla. No labeled neurons were present in the left nucleus ambiguus. Differences in the distributions of lung nerves between cats and rats provide the most obvious explanation for the discrepancy between these findings and ours. There are, however, other potential explanations worthy of consideration. First, labeling of the contralateral nucleus ambiguus in our rats could have resulted from transbronchial migration of the tracer to the opposite lung. The distribution of the radiolabeled CT-β injected into the lung, however, makes this explanation unlikely. Bilateral labeling of vagal neurons in the rats’ medulla could have been also the unsuspected consequence of contamination of the esophagus or the heart by leaked CT-β. The results of
the control studies also render this explanation improbable. Finally, it is possible that the injections of Kalia and Mesulam were inefficient at labeling contralateral neurons. This interpretation derives some support from the results of the vagal stimulation experiments. Particularly when considered in contrast to the symmetry with which both lungs are represented in the vagal nuclei, the weak response of the contralateral lung to unilateral vagal stimulation suggests that the midline-crossing motor fibers have a more limited arborization than their ipsilateral counterparts. If this is so, horseradish peroxidase, a less-sensitive retrograde tracer than CT-β (33), may not have been taken up by these fibers in amounts sufficient for the identification of neuronal somata in the contralateral medulla. The consequences of the tracer’s reduced sensitivity may have been compounded by the relatively large size of the cat’s lungs. A wider separation between the pleural surface and the lung hilum in this

Fig. 10. Infected cholinergic neurons in the rostralmost portion of the right nucleus ambiguus compact formation in a rat that underwent a right cervical vagotomy before injection of pseudorabies virus into the right lung. Left photomicrograph shows choline acetyltransferase immunoreactivity (green fluorescence with FITC-conjugated antiserum) in right ventrolateral medulla (bregma − 12.5 mm). Right photomicrograph is an enlarged double exposure of the nucleus ambiguus, where virus-infected neurons appear yellow (double fluorescent staining) or red (arrows, TRITC-conjugated antiserum) depending on whether they are cholinergic or not (scale bar 100 μm). Gi, gigantocellular nucleus; LPGi, lateral paragigantocellular nucleus; NA, nucleus ambiguus; VII, facial nucleus. Line drawing of brain stem section is modified from Paxinos and Watson (23a).

Fig. 11. Local permanence of radioactive tracer 8 h, 24 h, and 7 days after injection of 125I-CT-β into 1 lung. Each rat’s lungs were sectioned in the coronal plane. Lung sections (top at each time) and esophagus and trachea (small squares below lung sections) were laid out flat on a glass surface and covered with photographic film. After a 90-min exposure, the film was removed and the sections were photographed. Autoradiograph and photographic images were scanned digitally, scaled, and superimposed. The contour of the tissue was then traced over its corresponding location on the autoradiograph before the tissue image was deleted.
species may have placed the lung injections at a
greater distance from central airways (where intrapul-
monary parasympathetic ganglia concentrate) than in
the rat.

Two additional observations of our study deserve
mention in connection with the lateral distribution of
vagal fibers in the lungs. First, left lung injections
labeled a larger number of neurons in the nucleus
ambiguus (right and left) than right lung injections.
Although we did not compare the diffusion of CT-β in
the lungs quantitatively, we believe that this disparity
is likely to have resulted from a greater spread of the
neuronal tracer in the unilobar left lung than in the
multilobar right lung. Second, the increases in airflow
resistance elicited by unilateral vagal stimulation were
greater in the ipsilateral than in the contralateral
lung. To interpret this observation (or any other quan-
titative results obtained from vagal stimulation), it is
important to consider that lung resistance increases
during vagal stimulation are influenced by factors such
as the geometry of the airways (24) and the effective-
ness with which the electrical stimulus mimics physi-
ological conduction in the nerve. The latter, in partic-
ular, is influenced by the proportion of myelinated and
unmyelinated fibers present in each vagus. There is
convincing evidence that, in the guinea pig, capsaicin-
responsive fibers remain committed to the ipsilat-
eral vagus nerve (32). Thus, at least in this species, unilat-
eral vagal stimulation at the frequencies and pulse
duration used in our experiments (32) is likely to elicit
greater contraction of ipsilateral than contralateral
airways. Tachykinins released antidromically by ipsi-
lateral unmyelinated sensory fibers in response to the
stimulus can be expected to sensitize local ganglia to
the effects of other depolarizing stimuli (4, 21) and may
cause bronchoconstriction directly (18). A similar effect
would be observed if vagal motor fibers were segre-
gated to provide preferential innervation to ipsilateral
airway smooth muscle targets (as opposed to glands or
blood vessels) or established a larger number of gan-
glionic synapses in the ipsilateral than in the con-
tralateral airways. Such arrangements would have the

Fig. 12. Photomicrographs (top) show vagal motoneurons labeled
retrogradely in the right nucleus ambiguus complex by concurrent
injections of CT-β into the rostral and medial lobes of the right lung
(stained red with a TRITC-conjugated antiserum) and FG into the
wall of the midthoracic esophagus (stained green with FITC-conju-
gated antiserum). Esophageal motoneurons were circumscribed to
the compact formation of the nucleus and present only in the ro-
stralmost sections; pulmonary motoneurons were distributed between
the compact and external formation of the nucleus and had a wider
rostrocaudal range. No double-labeled neuronal somata were found
in any of the 7 rats that exhibited uptake of both neuronal tracers by
medullary neurons. Bar graph (bottom) shows counts of motoneurons
(horizonal line indicates median numbers, and bars indicate 10th–
90th percentile ranges) labeled by pulmonary injections of CT-β (red)
and esophageal injections of FG (green) in the compact (CF) and
external (EF) formations of the nucleus ambiguus (n = 7 rats). Line
drawings of brain stem sections are modified from Paxinos and
Watson (23a); distance from bregma is shown at top of each drawing.
Gi, gigantocellular reticular nucleus; LPGi, lateral paragigantocel-
lar nucleus; L Ret, lateral reticular nucleus; NA, nucleus am-
biguus.
advantage of allowing the vagal centers to retain individual control of the cholinergic outflow to each lung without losing the benefits of a bilateral distribution in the prevention of large asymmetries in airway tone.

Location of Lung Vagal Motoneurons and Central Afferents in the Medulla Oblongata

The organization of the lower airway motoneurons and central afferents has received surprisingly little attention in the neuroanatomic literature compared with other thoracic visceras, such as the esophagus (1–3, 5) or the heart (1, 5, 7, 9, 28, 30, 31). The precise origin of the airways' motor nerve supply was not examined with modern neuroanatomic methods until Kalia and Mesulam (15) demonstrated that the cat's trachea, bronchi, and lung parenchyma receive variable proportions of preganglionic fibers from neurons located in the compact and external formations of the nucleus ambiguus and in the dorsal motor nucleus of the vagus. Subsequent studies by Haxhiu et al. (11), Haxhiu and Loewy (12), Hadziefendic and Haxhiu (10), and our own laboratory (25) have confirmed the participation of the same two subdivisions of the nucleus ambiguus and, to a lesser extent than reported originally by Kalia and Mesulam, of the dorsal motor nucleus of the vagus in the innervation of the trachea and lungs in the rat, dog, ferret, and sheep.

The participation of the compact formation of the nucleus ambiguus in the innervation of the airway and lung tissues challenges some of the views expressed by Bieger and Hopkins (5) in their authoritative analysis of the representation of the upper alimentary tract in the rat's medulla oblongata. These investigators asserted that this subdivision of the nucleus ambiguus is dedicated exclusively to the efferent innervation of striated esophageal muscle. The results of our control experiments, especially the absence of double labeling of neuronal somata by the concurrent injections of CT-β into the lung and FG into the esophagus, argue against any concerns that the visualization of compact formation neurons after lung tracing experiments was an artifact caused by spread of the tracer to the adventitial surface of the esophagus. Far from negating that the nucleus ambiguus is organized in a viscerotopic fashion, the coexistence of esophageal and lung neurons in the same nuclear subdivision is, in our opinion, consistent with the common embryologic origin of the two organs and with the observation that a subpopulation of esophageal parasympathetic ganglia participates directly in the control of airway tone (6).

Kalia and Mesulam (15) were also the first to report that afferent nerve fibers from the cat's right lung end bilaterally in the nucleus of the tractus solitarius, concentrating in the ventrolateral, dorsolateral, and commissural subnuclei of the nucleus of the tractus solitarius. More recently, Haxhiu and Loewy (12), using CT-β as an anterograde tracer, described a slightly different topographical termination pattern for the trachea's sensory nerves of rats, ferrets, and dogs, whereby the majority of the sensory fibers ended in the medial, ventrolateral, and commissural subnuclei of the same nucleus. These findings, which are similar to those presented here for the rat's lungs, verify that the airways and the esophagus have anatomically separate

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Fig. 13. Fluorescence-labeled neurons in the left nucleus ambiguus of a rat injected with FG (stained in green with FITC-conjugated antiserum) into the intrathoracic trachea and CT-β (stained in red with TRITC-conjugated antiserum) into the extrathoracic trachea. No double-labeled neurons are present. NA, nucleus ambiguus; LPGi, lateral paragigantocellular nucleus. Section is located 13 mm caudal to bregma.
sensory relays in the nucleus of the tractus solitarius, with most of the esophageal fibers ending in the central subnucleus (1). Evidence of this separation, which had been noticed by Altschuler et al. (1) in their detailed description of the viscerotopic sensory representation of the upper alimentary tract in the rat, adds further support to the contention that CT-β injected into the lung did not spread to the esophagus in our experiments.

**Bilateral Innervation of Airways by Vagal Motoneurons**

The presence of double-labeled neurons after the injections of CT-β and FG into the main stem bronchi demonstrates that a single vagal motoneuron can innervate airway structures located on both sides of the midline. Although this finding reveals a previously unsuspected characteristic of the vagal innervation of the airways, its validity is restricted to the main stem bronchi. Our present results offer no proof that a similar arrangement is in place for more distal airways.

Clearly, a considerable number of vagal motoneurons project to both main stem bronchi. However, the observed discrepancy in the degree of neuronal labeling by CT-β and FG (most likely the result of the greater tissue spread of the latter) limits our ability to draw a more quantitative estimate of the number of cells with bilateral projections. This discrepancy may reflect differences in the uptake, transport, and immunoreactive characteristics of the two markers rather than to nonspecific labeling by either of them. Except for the number of labeled neurons, the distributions of the CT-β- and FG-labeled neurons were undistinguishable. Furthermore, the labeling disparity occurred, despite the use of similar injectate volumes of CT-β and FG and the alternation of injection side. Finally, previous studies have shown that neither CT-β nor FG is incorporated by undamaged fibers of passage (19, 27), which reduces the possibility that FG simply labeled a greater number of fibers destined for distal airways than CT-β.

**Interconnections Between Vagal Medullary Centers**

Injection of pseudorabies virus into the right lung revealed an extensive bilateral network of infected brain stem neurons, even when all the efferent fibers from one side of the medulla were severed by unilateral cervical vagotomies. This network comprised first-order neurons (presumably preganglionic parasympathetic neurons) contralateral to the vagotomy and second- or higher-order premotor neurons in a variety of locations throughout the brain stem, including the nucleus of the tractus solitarius, the nucleus ambiguus, and the dorsal motor nucleus of the vagus ipsilateral to the vagotomy. The topographic distribution of the infected neurons was akin to those described after similar injections into rat and sheep tracheae and lungs (10, 11, 25). The pattern of medullary infection was also similar to that produced by injection of pseudorabies virus into the esophagus (3), denoting a considerable overlap in the regulatory networks of the respiratory and alimentary derivatives of the primitive foregut.

A note of caution is pertinent in interpreting these results. Unlike some of the earlier experiments (10, 11, 25), our design did not include the interruption of the sympathetic pathways from the lung (11, 25). The obvious conclusion that preganglionic neurons receive regulatory inputs from both sides of the medulla must therefore be tempered by the possibility that some of the extensive bilateral labeling by the virus occurred through sympathetic nerves.

Nevertheless, retrograde passage via the sympathetic system is unlikely to explain the presence of the virus in the vagal nuclei. Injections of pseudorabies virus into structures such as the stellate ganglion or the adrenal gland, which are innervated by medullary sympathetic neurons, do not infect neurons in the ambiguous complex or in the dorsal motor nucleus of the vagus (14). This leaves no plausible route for the infection of the vagal nuclei ipsilateral to the vagotomy other than the contralateral lung-projecting vagal motoneurons. The specific occurrence of interconnections between the nuclei ambiguus is well documented by direct injections of retrograde markers into various portions of the ambiguous complex in the rat (22). Here we show that at least some of these interconnections may be involved in the coordination of vagal output to the lungs from both sides of the medulla. Our present data do not allow us to establish, however, whether the coordinating inputs are channeled through medullary interneurons, respiratory vagal motoneurons, or other vagal premotor neurons.

**Perspectives**

Our results demonstrate that each lung receives a bilateral supply of vagal motor and sensory fibers. This system of double innervation may serve to coordinate parasympathetic outflow to the lungs by 1) relaying unilateral lung stimuli to sensory neurons in both nuclei of the tractus solitarius, 2) coordinating preganglionic responses to these stimuli via interconnections between bilateral vagal motor centers, and 3) ensuring that each lung receives a balanced apportionment of the outflow from both vagal motor centers.

The authors thank Prof. Arthur D. Loewy for advice and technical assistance.

This work was supported by National Heart, Lung, and Blood Institute Grant HL-57998.

**REFERENCES**


