

## **Retrograde Neuronal Tracer Injected into the Diaphragm Labeled Neurons in the Brainstem: A Possible Direct Brain Connection for the Diaphragm**

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The diaphragm is under both voluntary and involuntary controls. It has a multi-functional role, serving primarily in respiration but also in coughing, sneezing, defecating, yawning, swallowing, and vocalization. This means that the diaphragm is intricately controlled, which should be reflected in the complexity of its neuronal innervations. To determine whether the neuronal innervations of the diaphragm reflect its multifunctional role, true blue (TB) was injected into the diaphragm of rats with intact nerves or with unilateral transected phrenic or vagal nerves. Horseradish peroxidase (HRP) was also injected into the crushed vagus nerve. Following the injection of TB into the diaphragm, neurons were bilaterally labeled in the phrenic motor nucleus (PMN) as well as in the nucleus ambiguus (NA), in the dorsal motor nucleus of the vagus nerve (DMNV), in the area postrema (AP), and in the caudal regions of the mesencephalic trigeminal nucleus (MTN). Transection of the phrenic nerve did not affect the labeling in the brain, which shows that the tracer was not transneurally transported via the PMN. Tissues prepared from rats with transected vagus nerves showed labeled neurons in the contralateral NA, DMNV, and AP and in the bilateral PMN and MTN to the transected vagus nerve. HRP microinjected into the crushed vagus nerve labeled nerve endings in the diaphragm. Choline acetyl

transferase (ChAT)-like immunoreactivity was observed in blood vessels. As controls, the injection of TB into the thoracic or abdominal cavity did not result in labeling the brain neurons. The findings suggest that direct projections from the brain to the diaphragm exist. Axons originating in the NA, DMNV, and AP traverse the vagus nerve to the diaphragm. The NA-DMNV connections may corroborate the finding of blood vessel-associated cholinergic fibers in the diaphragm (1) and may explain the ChAT-like activity seen in blood vessels. It indicates that neurons in the NA may regulate respiratory functions not only by modulating breathing but logically also by modulating vascular perfusion mechanisms. Based on the known functions of the NA, AP, and MTN, the labeled neurons in these areas may be involved in neuronal circuitry that control the reflex involvement of the diaphragm in functions like yawning, coughing, talking, vomiting, and gasping.

## I. Introduction

The diaphragm is the principal muscle of respiration. During contraction, its dome descends, the base of the thorax expands, intrathoracic pressure decreases, and abdominal pressure increases. These activities facilitate the expansion of the lung and cause inspiration. Besides respiration, the diaphragm also serves other functions. It contracts strongly during expulsive efforts, probably participating in all efforts requiring the rigidity of the thoracoabdominal system (2,3). Thus the diaphragm serves and maintains functional relationship with the systems that control coughing, sneezing, laughing, vocalization, defecation, yawning, and swallowing. During swallowing, for example, inspirational neurons instantly stop their discharge (4); vomiting is preceded by rhythmic spasmodic respiratory movement; and the expulsion phase of vomiting is brought about by the squeezing action of the diaphragm as well as the abdominal muscles. Most of the diaphragm contracts during both retching and expulsion, but the crural region around the esophagus is virtually inactive (5). This crural relaxation is necessary to augment the relaxed intrinsic smooth muscle sphincter mechanism at the gastroesophageal junction (6). Since the coastal region continues to contract when the crural region relaxes (6), it suggests that these two regions may be under different neural control mechanisms. Furthermore, the fact that the diaphragm participates in these various functions means that there may be still unresolved neuronal control mechanisms for the diaphragm in addition to that directly involving the phrenic motor nucleus (PMN). The normal neural mechanism that influences diaphragmatic functions is generally accepted to involve brainstem respiratory neuronal groups, notably the ventral respiratory group, with which the nucleus ambiguus (NA) is associated. The brain neurons, in turn, influence the PMN, which innervates the diaphragm via the phrenic nerve. In spite of this well-known pathway for the control of breathing,

other salient but direct projections from the brain to the diaphragm may exist, especially in light of the findings (7) that the dorsal and ventral respiratory neuronal groups do not have the appropriate firing pattern to activate phrenic motor neurons during vomiting. Neuronal connections from the brain to the diaphragm may make up the circuitry through which the diaphragm is modulated during its participation in expulsion and elimination functions—for example, vomiting, coughing, sneezing, defecation, and vocalization. Such circuitry will not be spontaneously active, neither will the activity of the circuitry be evoked during normal breathing. It means that the connections may elude identification using procedures that will normally identify active respiratory neuronal circuitry. Interestingly, respiratory electrical activity in the diaphragm of cats, dogs, monkeys, and rabbits following bilateral phrenicectomy has been reported (8). Such observation, however, was not supported (9) when it was shown that sectioning of the phrenic nerve roots in rats caused the respiratory electrical activity in the diaphragm to disappear. In his review, Felix (10) had maintained that there is some extraphrenic motor innervation of the diaphragm, but this too was not supported (11–14). More recently, however, documentation of the autonomic innervation of the diaphragm has been reported (1), using neuronal tracing, cholinesterase activity, and fluorescence microscopy for catecholamine. It was suggested that fibers enter the diaphragm from the gut wall. In the present study, neuronal projections to the diaphragm were determined by injecting retrograde neuronal tracers into the diaphragm and looking for labeled cell bodies in the spinal cord and brain.

## II. Materials and Methods

Sprague-Dawley male rats were kept in an animal room maintained at a 12-h light/12-h dark cycle; they were given free access to food and water. For the injection of neuronal tracers into the diaphragm, at least seven rats were anesthetized with chloral hydrate (400 mg/kg IP). Each rat was placed on an operating board and a longitudinal incision was made into the abdomen. The intestine was gently placed on the external abdomen, lateral to the incision, and was kept covered and moistened. The liver was gently pushed aside with cotton-tipped applicators to expose the diaphragm, and the diaphragm was injected at about 4 to 5 adjacent sites with about 8  $\mu$ L of 2% True Blue (TB) (Sigma Chemical Co., St. Louis, MO) using a 10  $\mu$ L Hamilton syringe. The sites of injection were blotted to remove traces of TB at the surface of the needle tract. The intestines were then repositioned, the wound closed, and the animals allowed to recover in a warmed cage. To determine whether the transport of TB from the diaphragm occurred via the phrenic nerve and/or the vagus nerve, these nerves were transected in two groups of rats prior to the injection of TB into the diaphragm. In another group of rats, the right vagus nerve was exposed

and crushed with a plastic-tipped forceps. Horseradish peroxidase (HRP) coupled to cholera toxin B-chain was prepared according to McIlhinney et al. (15) and about 3.0  $\mu$ L injected into the crushed vagus nerve. As controls, the injection of TB into the thoracic or abdominal cavity was done in two groups of rats to determine whether the labeled brain neurons were due to spillage and uptake of the marker by vagal fibers innervating the esophagus, lungs, and gut. The diaphragms of rats not injected with HRP were used as the HRP control.

For the TB experiments, the rats were reanesthetized with 400 mg/kg chloral hydrate at 4 to 5 days postinjection. They were then perfused transcardially with cold phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PF) in PBS. The spinal cord and brain were removed, postfixed for about 1 h in cold 4% PF, and placed in 15% sucrose-PBS for 1 day. The tissues were frozen in powdered dry ice and stored at  $-78^{\circ}\text{C}$ . Sections 30  $\mu\text{m}$  thick were prepared using a cryostat microtome and mounted on gelatin chrome-alum coated slides. In some experiments, the diaphragm was removed, processed, and examined to verify the injection sites. The sections were viewed with a Zeiss microscope equipped with epifluorescent illumination and with a filter-set G365 for TB visualization. Photomicrographs of the labeled neurons were prepared.

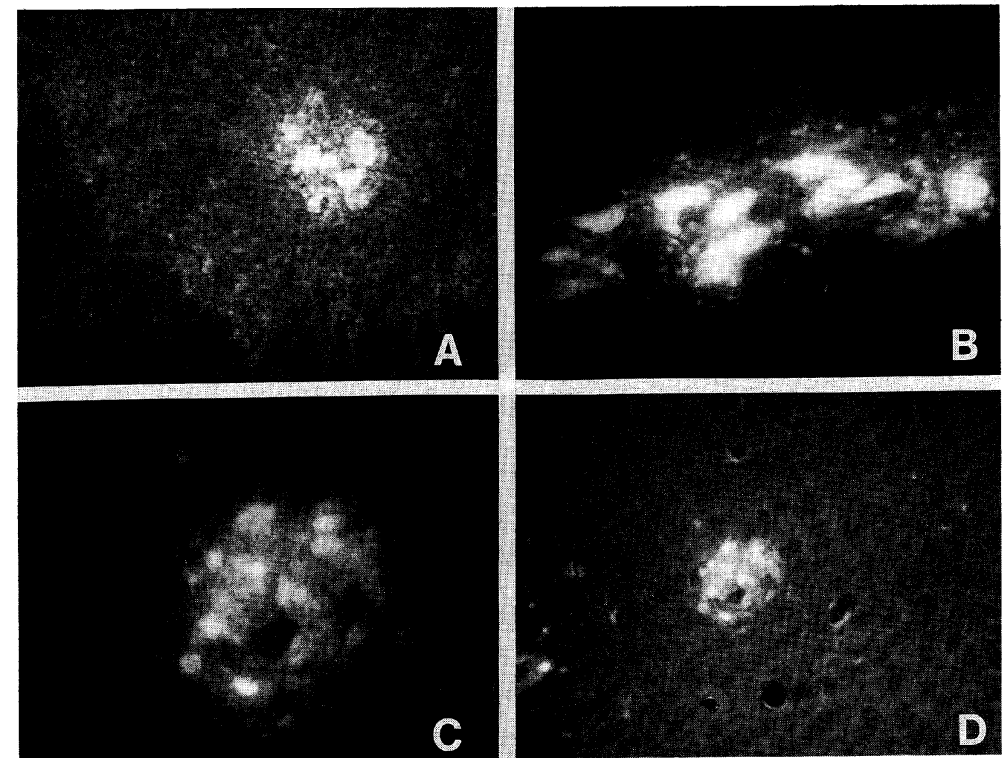
For the HRP experiments, the rats were reanesthetized after 3 days and perfused with 2% glutaraldehyde and 1% PF in PBS, pH 7.4. The diaphragm was removed and sections prepared. The sections were treated for the determination of HRP according to the modification of the methods of Graham and Kornovsky (16), Mesulam (17), and McIlhinney et al. (15). Sections were soaked in 0.1 M phosphate buffer, pH 7.4, then preincubated in a solution of 25  $\mu\text{g}/\text{mL}$  of tetramethylbenzidine (TMB) and 1 mg/mL of sodium nitroprusside in 0.05 M sodium acetate buffer, pH 3.3, for 30 min. This was followed by incubation, three consecutive changes, in a similar solution containing 0.006% hydrogen peroxide for 10 min each. The slices were then stabilized for 5 min in 0.9% sodium nitroprusside made up in 50% ethanol and 0.05 M sodium acetate buffer. They were then rinsed in buffer and lightly stained in 0.04% neutral red in 50% ethanol, rinsed, dried, and cover-slipped, using permount, and examined under a bright field microscope for TMB reaction products.

Immunohistochemical localization of ChAT was done in diaphragm slices. The procedure for tissue preparation was similar to that used for the detection of TB, in addition to a modification of the indirect immunohistochemical procedure of Coons (18). The slide-mounted slices were preincubated in 0.3% Triton X-100 in PBS, pH 7.4, for three 5-min periods, then incubated in a similar buffer containing ChAT antiserum (developed in rabbit or normal rabbit serum) as controls for 1 h at room temperature and about 16 h at  $4^{\circ}\text{C}$ . The slices were washed in 0.2% Triton X-100 buffer for three 5-min periods and incubated in a humidity box in the dark for about 30 min in the buffer containing the fluorescent-labeled goat antiserum, developed against rabbit IgG. The slides were then washed for 5 min in buffer and for two consecutive 5-min

periods in PBS. The sections were drained and cover-slipped using Fluoromount (Fisher Scientific Co.), and viewed with an epifluorescent microscope.

### III. Results

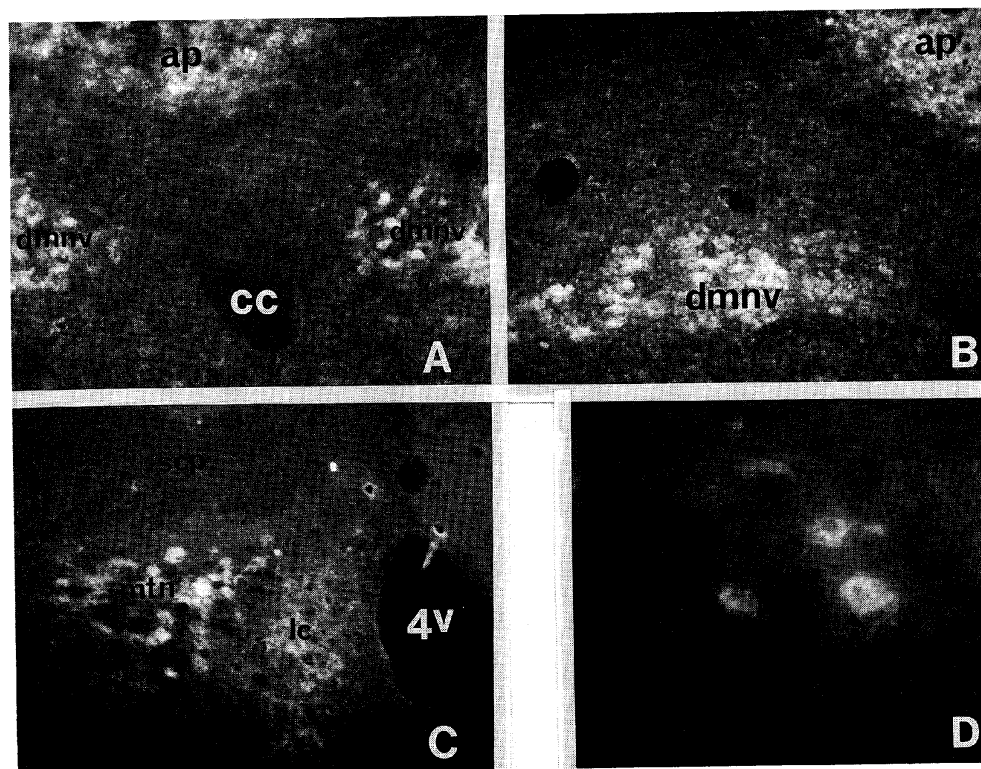
Following the injection of TB into the diaphragm, the examination of cervical spinal cord sections showed that bilateral labeling of neurons occurred in the PMN. Figure 1 shows the labeled neurons in a cross-section tissue slice (A) and a longitudinal tissue slice (B). Figure 1A also highlights the periphery of the spinal ventral gray matter. Neurons were also labeled bilaterally in the nucleus ambiguus (NA), as shown in Figure 1C and D. D shows a low magnification of the NA within the unlabeled brainstem reticular formation. Labeled cells were also identified in the dorsal motor nucleus of vagus (DMNV) and



**Figure 1** Photomicrographs of neurons labeled in the phrenic motor nucleus (PMN) (A and B) and the nucleus ambiguus (NA) (C and D) following the injection of True Blue (TB) into the diaphragm. (A) Cross section of the PMN; (B) Longitudinal section at a higher magnification. (D) Lower magnification of (C), highlighting the unlabeled reticular formation surrounding the NA.

the area postrema (AP) (Figure 2A and B) as well as in the caudal regions of the mesencephalic trigeminal nucleus (MTN) ventral to the superior cerebral peduncle and adjacent to the locus ceruleus (Fig. 2C and D). Cells were also labeled in the dorsal root ganglia.

Following the injection of TB into the diaphragm in rats with unilaterally transected phrenic nerves, neurons were labeled unilaterally in the PMN, on the side corresponding to the intact phrenic nerve. Bilaterally, labeled neurons were seen in the NA, DMNV, AP, and MTN (Table 1). The results showed that the labeling of brain neurons following the injection of TB into the diaphragm was not by way of the PMN. When the vagus nerve was transected



**Figure 2** Photomicrographs of neurons labeled in the dorsal motor nucleus of vagus (DMNV) and area postrema (AP) (A and B) and in the mesencephalic trigeminal nucleus (MTN) (C and D) following the injection of TB into the diaphragm. (A) The bilateral DMNV and the ventral AP are highlighted; (B) The entire mediolateral extent of the DMNV is highlighted. Essential landmarks for these neurons are the central canal (cc) seen in (A), and the superior cerebral peduncle (scp), the locus ceruleus (lc) and the fourth ventricle (4v) seen in (C). (D) Higher magnification, identifying the cell nucleus and labeled processes (arrowheads) of neurons labeled in the MTN. The visibility of the lc is due to autofluorescence and not to the TB tracer.

**Table 1** Nuclei Labeled Postinjection of Retrograde Neuronal Tracer into Rats with Transected Phrenic Nerves

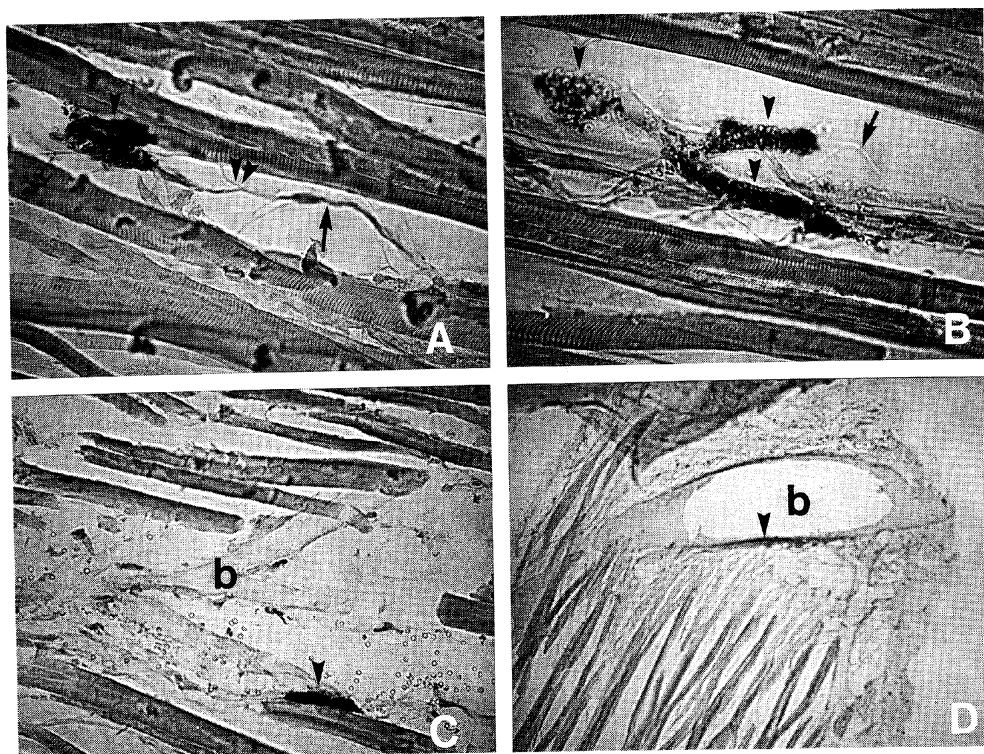
Nuclei	Ipsilateral	Contralateral
Phrenic motor nucleus	No	Yes
Nucleus ambiguus	Yes	Yes
Dorsal motor nucleus of vagus	Yes	Yes
Mesencephalic trigeminal nucleus	Yes	Yes
Area postrema	Yes	Yes

and rats were injected into the diaphragm with TB, labeled neurons were seen in the bilateral PMN and the MTN but only in the ipsilateral NA, DMNV, and AP; that is, labeling occurred on the same side as the intact vagus nerve (Table 2). This suggests that the labeling of the NA, DMNV, and AP occurs by way of the vagus nerve. The injection of TB into the thoracic cavity or abdominal cavity did not cause TB labeling of these neurons.

To determine the types of terminals that may be associated with the likely vagal projections to the diaphragm, HRP was injected into the crushed vagus nerve, with the objective of labeling nerve endings. Following the injection of HRP into the crushed vagus nerve and processing of slices of the diaphragm for HRP reaction products, it was shown that reaction products occurred in nerve ending-like structures (Fig. 3A, B, and C). Neutral red-stained nerve processes can be seen coursing among the muscle fibers. Some of the fibers bifurcate proximal to their terminals, which contain the HRP reaction products. Large blood vessels (Fig. 3D, b) also seem to contain HRP-reactive products, but not the smaller blood vessels (Fig. 3C, b). These results suggest that the vagus nerve projects to the diaphragm. The determination of ChAT-like immunoreactivity in slices from the diaphragm revealed the occurrence of ChAT-like immunoreactivity in larger blood vessels (Fig. 4A, b) but not in small thin-walled blood vessels (Fig. 4B, b). Nerve fibers containing ChAT-like immunoreactive substances were also detected (Fig. 4D).

**Table 2** Nuclei Labeled Postinjection of Neuronal Tracer into Rats with Transected Vagus Nerves

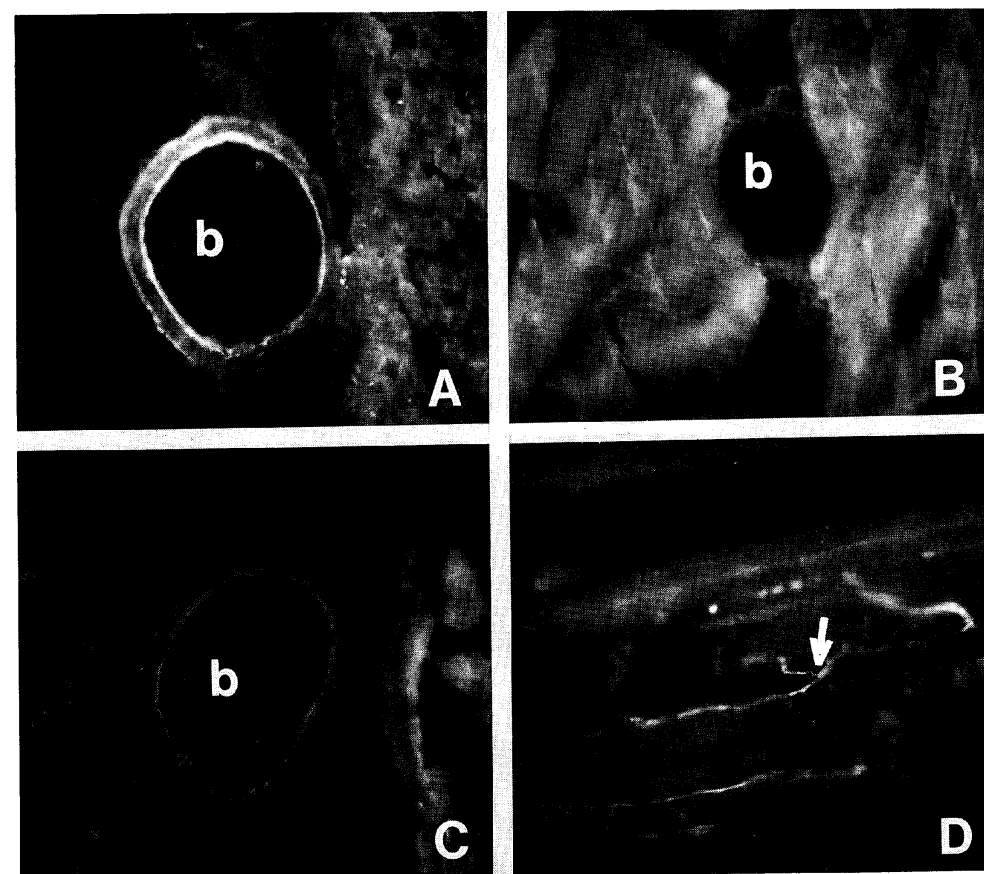
Nuclei	Ipsilateral	Contralateral
Phrenic motor nucleus	Yes	Yes
Nucleus ambiguus	No	Yes
Dorsal motor nucleus of vagus	No	Yes
Mesencephalic trigeminal nucleus	Yes	Yes
Area postrema	No	Yes



**Figure 3** Nerve endings (A, B and C, arrowheads) and a blood vessel (D, arrowhead) containing HRP reaction products following the injection of HRP-conjugate into the crushed vagus nerve and assayed for HRP reaction products in the rat's diaphragm. Small blood vessels (e.g., in panel C did not show HRP reaction products). Nerve fibers (arrows) proximal to the HRP-containing nerve endings and a bifurcated fiber (double arrowheads) can be seen in panels A and B.

#### IV. Discussion

The labeling of brain neurons following the injection of TB into the diaphragm suggests that direct projections from the brain to the diaphragm exist, probably similar to those observed for the liver, kidney, lung, and intestines. Axons originating in the NA, DMNV, and AP apparently traverse the vagus nerve to the diaphragm. Clearly, the labeling was not due to transsynaptic transport via the phrenic nerve, because transection of the phrenic nerve, which prevented the labeling of neurons in the PMN on the transected side, did not interfere with the labeling of brain neurons. Since the unilateral transection of the vagus nerve prevented the labeling of neurons in the NA, DMNV, and AP ipsilateral to the transected vagus nerve, we may conclude that neurons located in these nuclei send projections to the diaphragm via the vagus nerve. The labeled



**Figure 4** (A) Choline acetyl transferase (ChAT)-like immunoreactivity in the rat's diaphragm. (B) ChAT-like immunoreactivity in a large arterylike blood vessel is highlighted; (C) Significant reduction of ChAT-like immunoreactivity in a control diaphragm slice reacted with normal rabbit serum instead of the immune serum. The photomicrograph in panel B was overexposed to demonstrate the absence of ChAT-like immunoreactivity in vein-like small blood vessels. (D) ChAT-like immunoreactivity in nerve fiber-like processes. These processes resemble and may be identical to similar fibers that were shown to contain HRP-like reaction products; note the bifurcation in panel D (arrow) and in Fig. 3A (double arrowheads).

neurons in the caudal MTN were not affected by either transection, which means that the route of transport or the mechanism for labeling the MTN was not through the phrenic nerve or the vagus nerve.

The NA and DMNV are known to contain cholinergic vagal cell bodies; therefore the neurons labeled in these nuclei are probably cholinergic neurons, which send fibers to the diaphragm. The functions for these brain-to-diaphragm connections are yet to be identified. However, blood vessels associated cholinergic

fibers in the diaphragm had been reported (1), and in this study ChAT immunoreactivity was also identified in large artery-like blood vessels. This means that the vagal projections to the diaphragm may be cholinergic and serve to regulate large-vessel blood perfusion, because HRP-like reaction products were confined to large but not to small, thin-walled vein-like blood vessels. By virtue of the brain neuronal localization, these connections seem to be different from the cholinergic sympathetic vasodilator system, originating from neurons in the sympathetic chain. Because these projections may originate in the NA, the ventral respiratory nucleus, which includes the NA, may control respiratory functions, not only by modulating breathing but logically also by regulating vascular perfusion mechanisms.

Vagal nerve endings not associated with blood vessels were also seen. The HRP-labeled nerve endings seen in the diaphragm are distinct from the somatic nerve endings coming from the phrenic nerve, because HRP was specifically injected into the vagus nerve. It is of interest that the HRP-labeled nerve fibers were similar to the nerve fibers that contain ChAT-like immunoreactivity; note, for example, that both labeled fibers bifurcate. The HRP-labeled nerve endings may be presynaptic vagal endings located in the diaphragm. Although HRP-reactive products were also found in the nodose ganglion following the injection of HRP into the crushed vagus nerve, this does not, by itself, support the occurrence of sensory endings in the diaphragm, because fibers originating in the nodose ganglia and traversing the vagus nerve may have picked up the HRP from the injection site. The vagal endings in the diaphragm could also serve a motor function. It is intriguing to propose that these fibers may serve to directly modulate the diaphragm during physiological operations that demand the incorporation of the diaphragm—for example, swallowing, vomiting, talking, and sneezing. Since the NA innervates most laryngeal muscles, the NA may directly coregulate the functions of the larynx and diaphragm. For example, it may cause the abductor laryngeal muscle to close the larynx during coughing and at the same time regulate the diaphragm function to facilitate the coughing reflexes. Similarly, the NA may be involved in regulating the laryngeal muscles during speech as well as the participation of the diaphragm in speech. A dual function may also be expected of the AP, because the chemoreceptive emetic trigger zone is located in the AP. Thus the AP may control the role of the diaphragm in emesis.

The DMNV-to-diaphragm connection could help to control the function of the diaphragm directly. Thus it could facilitate the coordination of the respiratory functions with cardiovascular functions via the vagus nerve. The neurons labeled in the area of the MTN are also located within the vicinity of the so-called pneumotaxic region (19), and the MTN nucleus labeled in this study could have been the specific respiratory-responsive region. The MTN control muscles that play a principal role in gasping, in which the diaphragm is intimately involved. Finally, the labeling of neurons in the brain following the

injection of TB into the diaphragm may not be exclusive only to the diaphragm, because preliminary results showed that the injection of TB into other skeletal muscles—for example, the triceps—caused labeling of neurons in the brain. If the tracers are transported retrogradely, and not transsynaptically, direct connections from the brain to skeletal muscles exist, the function of which is more likely to be of a general nature, like the regulation of blood perfusion. Such direct neuronal connections from the brain to skeletal muscles would help to explain the highly discrete and localized manner in which peripheral blood flow can be regulated.

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