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WGA-Alexa Transsynaptic Labeling in the Phrenic Motor System of Adult Rats: Intrapleural Injection versus Intradiaphragmatic Injection

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Keywords

Intrapleural injection; intradiaphragmatic injection; WGA-Alexa; respiratory pathway; phrenic nuclei; rostral ventral respiratory groups

1. Introduction

The ability to transsynaptically label phrenic motoneurons and pre-motor rVRG neurons responsible for descending respiratory drive (Ellenberger and Feldman, 1988) provides an anatomical picture to compare various states of the phrenic motor system following spinal cord injury (Goshgarian et al., 1991). Recently, in our lab we demonstrated in acutely injured cervical hemisectioned rats, that intradiaphragmatic injection of wheat germ agglutinin conjugated to Alexa 488 (WGA-Alexa 488) selectively labels phrenic motoneurons in the phrenic nuclei (PN) in the cervical spinal cord, and then is transsynaptically transported to the rostral ventral respiratory groups (rVRGs) in the medulla over select physiologically active synaptic connections (Goshgarian and Buttry, 2014). Unfortunately intradiaphragmatic injection requires a laparotomy, an invasive surgical exposure, shown to adversely affect respiration, therefore potentially altering functional outcomes (Sieck and Fournier, 1989; Barbalho-Moulim et al., 2011). In 2009 Mantilla and colleagues introduced a new method of labeling phrenic motoneurons by injecting cholera toxin subunit beta (CTB) conjugated to Alexa 488 bilaterally into the intrapleural space. Delivery of a retrograde neuronal tracer by way of intrapleural injection to label the phrenic motor system eliminates the need for anesthesia and the laparotomy required for intradiaphragmatic injections (Mantilla et al. 2009). Following intrapleural injection the authors reported labeling of phrenic motoneurons as well as thoracic intercostal motoneurons and dorsal root

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ganglion neurons, but there was no evidence of transsynaptic labeling within the spinal cord tissue examined (Mantilla et al., 2009).

When investigating anatomical changes of the respiratory motor pathway following spinal cord injury, both the pre-motor neurons in the rVRGs that are responsible for descending respiratory drive as well as the motoneurons in the PN must be considered. Mantilla and colleagues (2009) did not report any evidence of transsynaptic transport in the medulla presumably because CTB-Alexa 488 is not a transsynaptic tracer (Cabot et al., 1994, Lee et al., 2009). Therefore the possibility of transsynaptic transport of a neuronal tracer to the rVRGs in the medulla following intrapleural injection is still a question that has yet to be resolved. Thus the first objective of this study was to inject WGA-Alexa 488, a known retrograde transsynaptic tracer (Goshgarian and Buttry, 2014) intrapleurally to determine if transsynaptic labeling would occur. In carrying out this experiment we found it necessary to differentiate rVRG neurons from nucleus ambiguus (NA) neurons. The neurons of these two nuclei are intermingled in the ventrolateral medulla (Feldman and Ellenberger, 1988; Ellenberger and Feldman, 1990; Hayakawa et al., 2004; Spyer, 2009) and it is difficult to differentiate one type of neuron from the other. The identification of WGA-Alexa 488 labeled rVRG neurons would suggest retrograde transsynaptic labeling from the phrenic nuclei to the rVRG following intrapleural injection. However, since the NA innervates some targets in the thoracic cavity via the vagus nerve (lung parenchyma, Hadziefendic and Haxhiu, 1999; bronchus, Fontán et al., 2000; trachea, Pérez Fontán and Velloff, 2001), this potential route of tracer uptake following intrapleural injection must be considered. Exposure of the vagus nerve or vagal nerve targets to WGA-Alexa 488 following intrapleural injection resulting in the labeling of NA cells would indicate retrograde labeling (not transsynaptic labeling). To identify the cell bodies that contribute to the vagus nerve, True blue, a retrograde tracer incapable of crossing synapses can be injected directly into the vagus nerve (Payne, 1987). The second objective of this study therefore was to differentiate labeled neurons identified as rVRG cells and NA cells using three different tracers injected into three different locations to determine to what extent retrograde transsynaptic transport versus retrograde transport occurs following intrapleural injection.

2. Materials and Methods

2.1 Injections

All rat studies were approved by the Wayne State University School of Medicine Institutional Animal Care and Use Committee. The following procedures were carried out in accordance with the *Code of Ethics of the World Medical Association* for experiments involving animals. A total of 11 adult male Sprague Dawley rats were injected with atropine sulphate (0.04mg/kg, im) 10 minutes prior to anesthesia induction to reduce mucus secretions during the subsequent aseptic survival surgery. Following anesthesia induction with a mixture of ketamine (70mg/kg, ip) and xylazine (7mg/kg, ip) the ventral aspect of the neck and abdomen were shaved and prepared for aseptic surgery. Each of the 11 rats received a series of 2 to 3 injections described below and summarized in table 1.

In all 11 rats the right vagus nerve was exposed by an incision of the ventral neck. The vagus nerve was identified based on the anatomical relationship with the jugular vein and

the carotid artery. A Hamilton syringe was used to inject 1 μ l of a neuronal tracer into the right vagus nerve; 9 of the 11 rats were injected with 5% True blue chloride (TB, Santa Cruz Biotechnology, Inc. Cat. # sc-216026) aqueous solution. Due to the hazy appearance of True blue, and the need to differentiate autofluorescence from real labeling, 2 additional rats were injected with 2% WGA-Alexa 594 (Life Technologies Cat. # W11262) saline solution into the right vagus nerve to validate the presence of labeled NA cells following intradiaphragmatic injection. In all 11 rats the surrounding area was examined for any signs of leakage. The neck muscles were sutured with 4 – 0 absorbable sutures (Vicryl) followed by closure of the skin with 4 – 0 non-absorbable nylon sutures (Ethilon).

Ten of the 11 rats underwent a laparotomy to expose the abdominal surface of the diaphragm (table 1). A horizontal incision 6cm in length was made approximately 0.5 cm caudal and parallel to the costal margin on the left side. A 28 gauge Hamilton syringe (catalog # 7637-01, and # 7803-02) was used to inject either 2% WGA-Alexa 594 saline solution (N=8) or 2% WGA-Alexa 488 (Life Technologies Cat. # W11261) saline solution (N=2) into the left hemidiaphragm. A total of 50 μ l was administered to each rat in 10 μ l increments. The amount of tracer injected was based on a previous study (Moreno et al., 1992) which determined that five 10 μ l injections distributed from the anterior to posterior region of the hemidiaphragm were sufficient to label the phrenic nuclei that spans the C3 – C6 segments of the cervical spinal cord. The abdominal muscles were sutured with 4 – 0 absorbable sutures followed by closure of the skin with skin staples (Fine Science Tools Cat. # 12030).

Nine of the 11 rats received a transcutaneous intrapleural injection administered through the fifth intercostal space into the thoracic cavity on the right side according to the method described by Mantilla et al., 2009. All 9 rats received 50 μ l of 2% WGA-Alexa 488 saline solution administered with a 25 gauge by 5/8” needle syringe. It is important to note that advancing the needle too far will result in an injection into the lung parenchyma.

2.2 Post Procedure Care

Incisions were cleaned and the rats were given buprenorphine (0.02mg/kg, sc) in 10mL saline for pain management and yohimbine (2mg/kg, ip) to reverse the xylazine. The rats recovered on a heating blanket. Once rats were able to ambulate, they were returned to clean soft litter-lined cages with food and water provided ad libitum on the floor of the cage. Buprenorphine (0.02mg/kg, sc) was given every 8 – 12 hours for pain management.

2.3 Tissue Preparation for Fluorescent Microscopy

Forty eight hours after injections, all 11 rats were anesthetized with urethane (1.6 g/kg, ip) and transcardially perfused with 150 mL saline followed by 300 mL of 4% paraformaldehyde in 0.1M phosphate-buffered saline (pH 7.4). The spinal cord (cervical through thoracic) and medulla were removed and an insect pin was inserted along the longitudinal axis of both on the left to differentiate the left from the right side. The medullary and spinal tissue was then post-fixed in the perfusate overnight and cryoprotected 2 – 3 days in 30% sucrose in 0.1M PBS until the tissue sank. Transverse, horizontal, or sagittal sections through the medulla and spinal cord were cut in 40 μ m thick sections with a

cryostat and collected in 0.1M PBS (table 1). Every section from the medulla was saved, whereas every 3rd spinal cord section was saved. Sections were then washed in 0.1M PBS and mounted on poly-L-lysine treated slides. The sections were cover slipped with Fluoromount (Sigma cat. # F4680) and examined with a Zeiss Axiophot fluorescent microscope using the 488 filter (green), 594 filter (red), and the Dapi filter (blue). Images were taken using the Axio Vision Rel. 4.8 Program. Autofluorescence is not problematic when examining WGA-Alexa tracers due to the distinct granulation within the WGA-Alexa labeled cells (Goshgarian and Buttry, 2014). However, True blue labeled cells have a hazy appearance throughout the cell body. To reduce the chance of counting false positive True blue labeled cells, images were captured with exposure times that resulted in a dark background. Cells were considered labeled with True blue if the cell body was clearly identifiable and brightly illuminated compared to the dark background. Cells on every other section from the medulla were counted since every section was saved after sectioning the tissue; this was to avoid counting the same cell body twice. Since every 3rd section from the spinal cord was saved, cells were counted on every spinal cord section saved. A cell was considered labeled if the cell body with a nucleus was identifiable.

3. Results

3.1 Spinal Cord

Injection of WGA-Alexa 488 (green) into the right intrapleural cavity resulted in retrograde transport to produce bilateral labeling of phrenic motoneurons in the cervical spinal cord (C3 – C6) in 7 of the 9 rats (fig. 1A, 1E, table 2). The remaining 2 rats lacked WGA-Alexa 488 labeling following intrapleural injection in all spinal sections examined. We believe this was due to an injection directly into the lung parenchyma, not the intrapleural space. At the time of perfusion the 2 rats that lacked labeling displayed a site of trauma on the right upper portion of the lung and the lung tissue was notably yellow, the color of WGA-Alexa 488 saline solution. Due to this observation, the 2 rats; TB4 and TB6, were excluded from the quantitative analysis.

Intradiaphragmatic injection of WGA-Alexa 594 (red, N=8) or WGA-Alexa 488 (green, N=2) into the left hemidiaphragm also resulted in bilateral labeling of phrenic motoneurons in all rats examined (fig. 1A, 1E). At the level of the phrenic nuclei (C3 – C6), both intrapleural and intradiaphragmatic injection resulted in isolated labeling of the phrenic nuclei in all cervical spinal cord sections examined. There was no labeling in any other neuronal center at C3 – C6 (fig. 1, 2). Moreover, there was no evidence of spinal interneurons labeled with either WGA-Alexa 488 or 594 following intrapleural or intradiaphragmatic injection. The lack of spinal interneurons confirms the findings in our previous study examining WGA-Alexa 488 labeling following intradiaphragmatic injection (Buttry and Goshgarian, 2014). Of the WGA-Alexa 488 labeled cells observed in the PN following a right intrapleural injection, $86 \pm 17.9\%$ displayed a dual label with WGA-Alexa 594 (left intradiaphragmatic injection) (table 2). This data confirm that intrapleural injection is an effective method to label phrenic motoneurons and is comparable to intradiaphragmatic injection (Mantilla et al., 2009).

In addition, following intrapleural injection WGA-Alexa 488 was detected bilaterally in the thoracic spinal cord in the intercostal motor nuclei (fig. 3A) confirming the findings of Mantilla and colleagues (2009). However, following intradiaphragmatic injection, neither WGA-Alexa 594 (red, N=8) or WGA-Alexa 488 (green, N=2) were detected in the thoracic spinal cord (fig. 3B) confirming the results of our previous study (Buttry and Goshgarian, 2014). The labeling of intercostal motoneurons following intrapleural injection, but not after intradiaphragmatic injection indicates that the tracer is exposed to phrenic axons exclusively when injected into the diaphragm (Buttry and Goshgarian, 2014). In contrast, intrapleural injection results in the tracer being exposed to the entire intrapleural space, in which the tracer is able to diffuse freely before coming into contact with multiple muscle targets including the diaphragm and intercostal muscles.

3.2 Medulla

Rat TB1 received a right intrapleural injection of WGA-Alexa 488 followed by an injection of True blue into the right vagus nerve. Forty-eight hours after injection TB1 demonstrated labeled cells in the intermingled region of the NA and rVRG. Some cells were labeled with either WGA-Alexa 488 or True blue. In addition, there were numerous cells dually labeled with both WGA-Alexa 488 and True blue demonstrating that intrapleural injection results in the labeling of NA ambiguous cells.

Six of the 8 rats that received 3 tracers displayed individually labeled, dual labeled and triple labeled cells in the region of the rVRG and NA (fig. 4 – 6, table 3). TB4 and TB6 were excluded due to the lack of WGA-Alexa 488 labeling in the spinal cord (see section 3.1). The following qualitative and quantitative data includes a total of 82 sections spanning the medulla from 6 rats (summary in table 3).

The left half of the medulla displayed WGA-Alexa 488 and WGA-Alexa 594 labeling isolated to the rVRG and NA following right intrapleural (WGA-Alexa 488) and left intradiaphragmatic (WGA-Alexa 594) injection. Of these cells $18.1 \pm 10.8\%$ were observed with only WGA-Alexa 488 (green filter, fig. 4A), $13.6 \pm 7.1\%$ with only WGA-Alexa 594 (red filter, fig. 4B), and $68.2 \pm 8.9\%$ were dually labeled with both tracers (yellow in appearance, fig. 4D); a quantitative summary is located in table 3. Left intradiaphragmatic injection of WGA-Alexa 594 resulted in uptake of the tracer by the phrenic nerve, retrograde transport to the phrenic nuclei, followed by transsynaptic transport to the rVRGs as demonstrated in our previous study investigating WGA-Alexa 488 (Goshgarian and Buttry, 2014). Cells containing WGA-Alexa 488 following intrapleural injection were labeled by one or more of the following three potential routes; 1) transsynaptic transport following tracer uptake by the phrenic nerve, 2) transsynaptic transport following tracer uptake by the intercostal nerves, or 3) retrograde transport following tracer uptake from the vagus nerve and its extensive branches that innervate targets in the thoracic cavity. It is important to note that there was a complete absence of True blue in the left half of the medulla following True blue injection into the right vagus nerve (fig. 4C).

In comparison, the right half of the medulla displayed WGA-Alexa 488 and WGA-Alexa 594 labeling isolated to the rVRG and NA (fig. 4–6) following intrapleural (WGA-Alexa 488) and intradiaphragmatic (WGA-Alexa 594) injection. A total of 189 cells were

identified as rVRG neurons based on the presence of WGA-Alexa 488 alone, WGA-Alexa 594 alone, or a dual label of 488 and 594 and a complete absence of True blue. Of the right rVRG cells, $9.2 \pm 8.6\%$ were observed with WGA-Alexa 488 (green filter, fig. 4E), $9.3 \pm 6.9\%$ with WGA-Alexa 594 (red filter, fig. 4F), and $81.5 \pm 11.8\%$ were dually labeled with both WGA-Alexa 488 and WGA-Alexa 594 (yellow in appearance, fig. 4H, table 3). However, unique to the right half of the medulla, True blue (TB) a retrograde tracer incapable of crossing synapses (Payne, 1987) was injected into the right vagus nerve to identify the ipsilateral NA cells (fig. 4G & H). A total of 1026 NA cells contained TB. Of these NA cells, $65.9 \pm 15.4\%$ contained TB alone, $3.0 \pm 3.5\%$ contained TB with WGA-Alexa 594, $5.1 \pm 5.1\%$ contained TB with WGA-Alexa 488, and $26.0 \pm 9.7\%$ contained all three tracers (fig. 4H, 5D, 6; table 3). In addition to the right NA, TB alone was identified in a scattering of cells ventral to the NA (fig. 5C, 5D, & 6), and in the dorsal motor nucleus of vagus (DMX) (fig. 7), both isolated to the right side. The anatomical relationship of the rVRG and NA can be appreciated in both transverse (fig. 4 & 6) and sagittal (fig. 5) planes where both nuclei intermingle within the same cell column in the ventrolateral medulla as described previously (Feldman and Ellenberger, 1988; Ellenberger and Feldman, 1990; Hayakawa et al., 2004; Spyer, 2009).

To validate the result of identifying WGA-Alexa 594 in True blue labeled NA cells following intradiaphragmatic injection, two additional animals, NA1 and NA2 were added. True blue has a hazy appearance and can present difficulties when examining tissue at the cellular level with a fluorescent microscope. In contrast WGA-Alexa fluorochromes have a distinct granulation resulting in easily identifiable labeled cells. NA1 and NA2 received right vagus nerve injections of WGA-Alexa 594 followed by left intradiaphragmatic injections of WGA-Alexa 488. Both animals displayed individually labeled cells of either WGA-Alexa 594 or WGA-Alexa 488, and dually labeled cells. This again validates that intradiaphragmatic injection of WGA-Alexa fluorochromes results in both rVRG and NA labeled cells.

Following intradiaphragmatic injection of WGA-Alexa 594, the observation of NA cells containing both TB and WGA-Alexa 594 suggests that WGA-Alexa 594 was transsynaptically transported from rVRG neurons to NA neurons. It is important to stress that this is the first anatomical report of a direct synaptic relationship between rVRG and select NA cells. Further support of the selectivity of this synaptic relationship is the fact that all other locations of TB labeled cells, i.e. the DMX (fig. 7) and the cells scattered ventral to the main column of the NA and rVRG (fig. 5C, 5D, and 6) had a complete lack of WGA-Alexa 594. Similar to intradiaphragmatic injection of WGA-Alexa 594, following intrapleural injection WGA-Alexa 488 was also absent in the DMX and in the region ventral to the NA suggesting that WGA-Alexa 488 was transsynaptically transported from the PN to the rVRG and then to only select cells of the NA.

4. Discussion

As pointed out in the results section, a potential technical problem of injecting tracers into the intrapleural space is injecting the tracer into lung parenchyma. This will compromise the

results and since this happened in our study, we decided to note this problem so that it can be avoided by others in the future.

Mantilla et al. (2009) demonstrated retrograde transport of CTB-Alexa 488 to phrenic motoneurons and intercostal motor neurons after intrapleural injection, but there was no evidence of transsynaptic transport. The present study demonstrates that intrapleural injection of an Alexa 488 conjugate is not a barrier to retrograde transsynaptic transport in the phrenic motor system. Rather, retrograde transsynaptic transport following intrapleural injection is dependent upon the specific tracer conjugated to Alexa 488. In this study we demonstrated that intrapleural injection of WGA-Alexa 488 not only labels phrenic motoneurons and intercostal motor neurons similar to CTB-Alexa 488 (Mantilla et al., 2009), but is also transsynaptically transported in a retrograde fashion to the rVRG and select NA cells in the medulla.

The rVRG and NA have been described as an intermingled column of neurons in the ventrolateral medulla indistinguishable based on size or morphology (Feldman and Ellenberger, 1988; Ellenberger and Feldman, 1990; Hayakawa et al., 2004; Spyer, 2009). In order to distinguish the NA from the rVRG, True blue, a retrograde tracer incapable of crossing synapses, was injected into the right vagus nerve (Sawchenko and Swanson, 1981; Stuesse and Powell, 1982; Cunningham and Sawchenko, 1989). Following intrapleural injection, WGA-Alexa 488 was identified in both the rVRG and select NA cells. Right rVRG neurons were identified based on a dual label of WGA-Alexa 488 (intrapleural injection) and WGA-Alexa 594 (intradiaphragmatic injection) and a complete absence of TB (vagus nerve injection). NA cells were identified by TB labeling. We also saw labeling of WGA-Alexa 488 and TB in the same cell. There are two potential mechanisms as to how the NA cells became labeled with WGA-Alexa 488; by retrograde transport via targets of the vagus nerve in the thoracic cavity or transsynaptic transport via the phrenic motor pathway. We speculate the latter; following intrapleural injection select cells of the NA were labeled with WGA-Alexa 488 after transsynaptic transport within the phrenic motor pathway from the PN to the rVRG and subsequently to the NA. In support, WGA-Alexa 488 labeling was restricted to specific NA cells intermingled with rVRG cells. WGA-Alexa 488 was never identified in the other two locations of TB labeling: the DMX and the cells scattered ventral to the NA.

Following TB injection into the right vagus nerve, the right DMX and a scattering of cells ventral to the right NA were labeled with TB in addition to the right NA. The DMX has been shown to provide preganglionic parasympathetic innervation to the esophagus (Hadziefendic and Haxhiu, 1999; Hayakawa et al., 2002), larynx (Basterra et al., 1987; Kobler et al., 1994), trachea (Haxhiu and Loewy, 1996; Atoji et al., 2005), lung parenchyma (Hadziefendic and Haxhiu, 1999), and myocardium (Stuesse and Powell, 1982). The cells ventral to the NA are associated with the myocardium (Stuesse and Powell, 1982). These cells of the DMX and the area ventral to the NA are not related directly to respiration. If the only route of WGA-Alexa 488 transport to the NA was via uptake by the vagus nerve, the DMX and cells ventral to the NA would also be expected to exhibit a dual label of TB and WGA-Alexa 488. However, in all rats WGA-Alexa 488 was isolated to the rVRG and the intermingled cells of the NA demonstrating a functional significance of the transsynaptic

transport of WGA-Alexa 488 from rVRG to select NA cells. Using physiological techniques, Ellenberger and Feldman (1990) demonstrated that some of the NA cells intermingled with the rVRG cells exhibit respiratory related discharge patterns.

In the past we have demonstrated the selective transsynaptic transport of WGA-Alexa 488 over select physiologically active synapses of the respiratory motor pathway in acute and chronic spinal cord injured rats (Buttry and Goshgarian, 2014). Following intradiaphragmatic injection of WGA bound conjugates the tracer was described as being located strictly in the PN and rVRG (Goshgarian and Buttry, 2014; Buttry and Goshgarian 2014). However this study demonstrates the transsynaptic transport of WGA bound conjugates following intradiaphragmatic (as well as intrapleural) injection must be expanded beyond the rVRG to select cells of the NA in the non-injured rat. The connections between the NA and the rVRG have been described physiologically before (Shannon and Freeman, 1981) and suggest a role in the coordination of respiration with muscle control over airflow (Ellenberger and Feldman, 1990; Spyer, 2009) mediated by pharyngeal and laryngeal muscles. Past studies speculated a role of interneurons relaying information between the NA and rVRG (Ellenberger and Feldman, 1990). However, we provide anatomical evidence of a direct synaptic relationship between rVRG and select NA cells which further supports our contention that the transsynaptic transport occurs over physiologically active connections. Finally, this study shows that transsynaptic transport using WGA-Alexa fluor conjugates can occur over two synaptic connections linking phrenic motoneurons with rVRG cells and select NA cells.

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Abbreviations

PN	phrenic nuclei
rVRG	rostral ventral respiratory group
NA	nucleus ambiguus
WGA-Alexa 488	wheat germ agglutinin conjugated to Alexa 488
WGA-Alexa 594	wheat germ agglutinin conjugated to Alexa 594
TB	True blue
CTB	cholera toxin subunit beta
DMX	dorsal motor nucleus of vagus

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Highlights

- Intrapleural injection of WGA tracers label the respiratory motor pathway
- Transsynaptic transport of WGA tracers occur after intrapleural injection
- Intrapleural injection labels select cells in the nucleus ambiguus and rVRGs

Figure 1.

Transverse sections of the cervical spinal cord at the level of the phrenic nuclei in the same rat. A. displays a low magnification of the left phrenic nucleus (encircled), B-D display a higher magnification of A. B displays WGA-Alexa 488 (green filter) labeled cells following a right (contralateral) intrapleural injection of this tracer. C displays WGA-Alexa 594 (red filter) labeled cells following a left (ipsilateral) intradiaphragmatic injection. D is a merged image of B and C; note cells contain either WGA-Alexa 488 (arrow) or 594 alone (θ arrow), or a mixture of both tracers (noted with * in the nucleus). The dually labeled cells are easily

identified in B and C. E displays a low magnification of the right phrenic nucleus (encircled), F-H display a higher magnification of E. F displays WGA-Alexa 488 (green) labeled cells following a right (ipsilateral) intrapleural injection. G displays WGA-Alexa 594 (red) labeled cells following a left (contralateral) intradiaphragmatic injection. H is a merged image of F and G; note cells contain either WGA-Alexa 488 (arrows) or a mixture of both tracers (noted with * in the nucleus). Scale bars are 200 μ m (A and E) and 50 μ m (B-D and F-H).

Figure 2.

Sagittal section (insert of transverse section for reference) through the cervical spinal cord displaying WGA-Alexa 488 (green filter) and WGA-Alexa 594 (red filter) labeling within the right phrenic nucleus following right intrapleural injection of WGA-Alexa 488 and left intradiaphragmatic injection of WGA-Alexa 594. Notice cells contain either WGA-Alexa 488 (green, examples noted with # arrow) or WGA-Alexa 594 (red, examples noted with θ arrow) and cells with a dual label appear yellowish in color (examples noted with * arrows). Scale bar is 100 μ m.

Figure 3.

A, Low power magnification of a transverse section through the spinal cord at the T6 level showing bilateral WGA-Alexa 488 (green filter) labeling in the region of the intercostal motor nuclei (encircled areas) following right intrapleural injection of this tracer. B, image of the same section in A using the red filter to detect WGA-Alexa 594 following left intradiaphragmatic injection. The WGA-Alexa 594 label (red) is absent (encircled areas). Thus, intrapleural injection labels intercostal motoneurons, but Intradiaphragmatic injection does not. Scale bar is 200 μ m. P; pinhole marks left side.

Figure 4.

High power magnification of a transverse section through the medulla displaying both the left (A-D) and right (E-H) rostral ventral respiratory groups and the nucleus ambiguus. A and E display WGA-Alexa 488 (green filter) labeled cells following a right intrapleural injection of this tracer (A and E captured with equal exposure times). B and F display WGA-Alexa 594 (red filter) labeled cells following a left intradiaphragmatic injection of this tracer (B and F captured with equal exposure times). C and G display the absence on the left and presence on the right of True blue (blue filter) labeled cells following a right vagus nerve

injection of this tracer (C and G captured with equal exposure times). D is a merged image of A-C, notice cells contain either WGA-Alexa 488 (green, θ arrow), WGA-Alexa 594 (red, # arrow), or a dual label and appear yellow in color (noted with * in the nucleus). All rVRG cells are transsynaptically labeled. The image shows that not all rVRG cells are dually labeled; some are green only and some are red only. H is a merged image of E-G. Notice cells contain either True blue alone (nucleus ambiguus cells, $\bar{U}$ arrow) or a mixture of two or three of the labels in one cell. The yellow cells are dually labeled rVRG cells (WGA-Alexa 488 and WGA-Alexa 594, arrow), but the dually (True blue and WGA-Alexa 594, # arrow) and triple labeled cells (noted with * in the nucleus) suggests that rVRG and NA cells not only intermingle, but they have a synaptic relationship. The cells marked with an asterisk in H can be seen with the green, red and blue filter in E-G respectively. Scale bar is 50 μ m.

Figure 5.

Low power magnification of a sagittal section of the medulla displaying the right rostral ventral respiratory group and the intermingled cells of the nucleus ambiguus. A displays WGA-Alexa 488 (green filter) labeled cells following a right (ipsilateral) intrapleural injection of this tracer. B displays WGA-Alexa 594 (red filter) labeled cells following a left (contralateral) intradiaphragmatic injection. C displays True blue (blue filter) labeled cells following a right (ipsilateral) vagus nerve injection. D is a merged image of A-C, Note that the blue labeled NA cells (arrows) not only follow the main column of the rVRG, but there

is also a scattering of cells ventral to the main nuclei (arrows in C and D). Note also that many of the cells in the main column are dually labeled rVRG cells (WGA-Alexa 488 and WGA-Alexa 594, # arrows) or triple labeled NA cells (* arrows) demonstrating a synaptic relationship with the rVRG and NA cells. The ventrally extended cells (arrows in C and D) displayed only True blue label. Scale bar is 200 μ m.

Figure 6.

High power magnification of a transverse section through the medulla displaying the right rostral ventral respiratory group and the nucleus ambiguus (encircled) labeled after an ipsilateral intrapleural injection of WGA-Alexa 488 (green filter) and a contralateral intradiaphragmatic injection of WGA-Alexa 594 (red filter). True blue (blue filter) was injected into the ipsilateral vagus nerve to label the NA. The figure is a merged image of all three tracers; notice cells contain either True blue alone (NA cells; cells without arrows within the encircled area) or a mixture of two or three of the labels in one cell (# arrow, True blue and WGA-Alexa 594; θ arrow, True blue and WGA-Alexa 488; * arrow, triple label).

Notice ventrally extended cells (arrows for examples), displaying True blue only, extend far ventral to the encircled NA and rVRG nuclei. Scale bar is 100 μ m.

Figure 7.

Low power magnification of a transverse section through the dorsal medulla displaying the right dorsal motor nuclei of vagus (DMX) labeled with True blue (blue filter) after ipsilateral injection of True blue into the vagus nerve. A displays a complete lack of WGA-Alexa 488 (green filter) labeled cells following a right (ipsilateral) intrapleural injection of this tracer. B displays a complete lack of WGA-Alexa 594 (red filter) labeled cells following a left (contralateral) intradiaphragmatic injection. C displays True blue (blue filter) labeled cells within the right DMX (arrow) following a right (ipsilateral) vagus nerve injection. D is

a merged image of A-C. Notice WGA-Alexa 488 and 594 labeling is completely absent in the DMX. IV notes the 4th ventricle for reference. Scale bar is 500μm.

Table 1

List of the 11 rats included in this study and the locations and tracers injected into each rat. Column 1 lists the Animal Identification. Column 2 lists the weight at the time of injection for each rat. Columns 3–5 list the tracers injected into each location. Columns 6 & 7 notes the anatomical plane the medulla and spinal cord tissue samples were sectioned.

Animal ID	Weight	Vagus Injection (right)	Intrapeurial Injection (right)	Intradiaphragmatic Injection (left)	Medulla Sectioned	Spinal Cord Sectioned
TB1	373	True Blue	WGA-Alexa 488	None	Transverse	Transverse
TB2	360	True Blue	WGA-Alexa 488	WGA-Alexa 594	Transverse	Transverse
TB3	379	True Blue	WGA-Alexa 488	WGA-Alexa 594	Sagittal	Sagittal
TB4	364	True Blue	WGA-Alexa 488	WGA-Alexa 594	Transverse	Transverse
TB5	358	True Blue	WGA-Alexa 488	WGA-Alexa 594	Coronal	Coronal
TB6	361	True Blue	WGA-Alexa 488	WGA-Alexa 594	Transverse	Transverse
TB7	372	True Blue	WGA-Alexa 488	WGA-Alexa 594	Transverse	Transverse
TB8	374	True Blue	WGA-Alexa 488	WGA-Alexa 594	Transverse	Transverse
TB9	351	True Blue	WGA-Alexa 488	WGA-Alexa 594	Transverse	Transverse
NA1	406	WGA-Alexa 594	None	WGA-Alexa 488	Transverse	None
NA2	473	WGA-Alexa 594	None	WGA-Alexa 488	Transverse	None

Cell counts from 270 cervical spinal cord sections sampled from 5 rats. Column 1 lists the ID for each rat. Rats that received both intradiaphragmatic and intrapleural injections were included in this cell count. Column 2 lists the number of WGA-Alexa 594 labeled cells located in the right phrenic nucleus (PN). Column 3 lists the number of WGA-Alexa 488 labeled cells located in the right PN. Column 4 lists the number of dually labeled cells with WGA-Alexa 594 and WGA-Alexa 488 located in the right PN. Column 5 lists the number of WGA-Alexa 594 labeled cells located in the left PN. Column 6 lists the number of WGA-Alexa 488 labeled cells located in the left PN. Column 7 lists the number of dually labeled cells with WGA-Alexa 594 and WGA-Alexa 488 located in the left PN. Columns 8–10 list the total number of cells (right and left sides combined) labeled with WGA-Alexa 594, WGA-Alexa 488, or with a dual label of WGA-Alexa 594 and 488 respectively. The TB3 spinal cord was sectioned in a sagittal plane, left could not be differentiated from right therefore only total counts were recorded. TB1 was excluded from the count due the animal not receiving an intradiaphragmatic injection. TB4 and TB6 were excluded due to an unsuccessful intrapleural injection.

Table 2

Animal ID	Right PN 594	Right PN 488	Right PN 594+488	Left PN 594	Left PN 488	Left PN 594+488	Total 594	Total 488	Total Total 594 +488
TB2	4	1	174	88	12	105	92	13	279
TB3							159	9	123
TB7	11	0	102	48	0	56	59	0	158
TB8	0	39	33	44	12	30	44	51	63
TB9	1	3	96	55	18	37	56	21	133
TOTALS	16	43	405	235	42	228	410	94	756

Cell counts from a total of 82 medulla sections sampled from 6 rats. Column 1 lists the ID for each rat. Rats that received injections in all three locations (intradiaphragmatic, intrapleural and vagus nerve) were included in this cell count. Columns 2–8 list the numbers of labeled cells located in the right half of the medulla in the region of the NA and rVRG. Cells were identified as individually labeled (WGA-Alexa 594, WGA-Alexa 488, or True blue), dually labeled (any combination of 2 of the 3 tracers), or triple labeled (all 3 tracers in a single cell). Columns 9–11 list the numbers of labeled cells located in the left half of the medulla in the region of the NA and rVRG. Cells were identified as individually labeled (WGA-Alexa 594 or WGA-Alexa 488), or dually labeled with both WGA-Alexa 594 and WGA-Alexa 488. TB1 was excluded from the count due the animal not receiving an intradiaphragmatic injection. TB4 and TB6 were excluded due to an unsuccessful intrapleural injection.

Table 3

Animal ID	Right rVRG/NA 594	Right rVRG/NA 488	Right rVRG/NA 594+488	Right NA Trueblue 594	Right NA Trueblue+ 488	Right NA Trueblue+ 594+488	Left rVRG/NA 594	Left rVRG/NA 488	Left rVRG/NA 594+488
TB2	7	11	37	8	15	32	29	58	98
TB3	0	1	20	10	10	45	2	4	21
TB5	1	0	26	0	1	25	4	23	61
TB7	7	3	39	5	15	46	14	0	45
TB8	4	1	17	0	0	36	9	15	50
TB9	1	3	11	1	3	62	26	23	93
TOTALS	20	19	150	24	44	246	84	123	368