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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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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.

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ABSTRACT

Background: Intrapleural injection of CTB-Alexa 488, a retrograde tracer, provides an alternative labeling technique to the surgically invasive laparotomy required for intradiaphragmatic injection. However, CTB-Alexa 488 is incapable of crossing synapses restricting the tracer to the phrenic nuclei and the intercostal motor nuclei in the spinal cord.
New method: Intrapleural injection of WGA-Alexa 488, a transsynaptic tracer, provides a method to label the respiratory motor pathway in both the spinal cord and medulla. Intradiaphragmatic injection of WGA-Alexa 594 and vagal nerve injections of True blue were used to confirm the phrenic nuclei and to differentiate between the rVRG and the NA in the medulla.
Results: Following intrapleural injection, WGA-Alexa 488 was retrogradely transported to the phrenic nuclei and to the intercostal motor nuclei. Subsequently WGA-Alexa 488 was transsynaptically transported from the phrenic motoneurons to the pre-motor neurons in the rVRG that provide the descending drive to the phrenic neurons during inspiration. In addition WGA-Alexa 488 was identified in select cells of the NA confirmed by a dual label of both WGA-Alexa 488 and True blue.
Comparison with existing method: WGA-Alexa 488 demonstrates retrograde transsynaptic labeling following intrapleural injection whereas the previous method of injecting CTB-Alexa 488 only demonstrates retrograde labeling.
Conclusions: Intrapleural injection of WGA-Alexa fluor conjugates is an effective method to transsynaptically label the phrenic motor system providing an alternative for the invasive laparotomy required for intradiaphragmatic injections. Furthermore, the study provides the first anatomical evidence of a direct synaptic relationship between rVRG and select NA cells.

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 hemisected 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

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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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 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 et al. (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 (0.04 mg/kg, im) 10 min prior to anesthesia induction to reduce mucus secretions during the subsequent aseptic survival surgery. Following anesthesia induction with a mixture of ketamine (70 mg/kg, ip) and xylazine (7 mg/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 2–3 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 μl 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 6 cm 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 μl was administered to each rat in 10 μl increments. The amount of tracer injected was based on a previous study (Moreno et al., 1992) which determined that five 10 μl 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

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 and 7 notes the anatomical plane the medulla and spinal cord tissue samples were sectioned.

Animal ID	Weight	Vagus injection (right)	Intrapleural 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	Horizontal	Horizontal
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