Published in final edited form as:

J Neurosci Methods. 2014 January 30; 222: 156–164. doi:10.1016/j.jneumeth.2013.11.003.

The pattern and extent of retrograde transsynaptic transport of WGA-Alexa 488 in the phrenic motor system is dependent upon the site of application

Harry G. Goshgarian and Janelle L. Buttry

Department of Anatomy and Cell Biology, Wayne State University, School of Medicine, 540 East Canfield Avenue, Detroit, MI 48201, USA

Introduction

It is well known that when wheat germ agglutinin conjugated to horseradish peroxidase (WGA-HRP) is injected into muscle it will be retrogradely transported back to the motor neuron cell bodies and also transsynaptically to other neurons within the synaptic chain (Harrison et al., 1984, 1986; Porter et al., 1985; Moreno et al., 1992). Furthermore, it has been shown that the transsynaptic transport is selectively mediated across physiologically active connections rather than silent or less active connections (Harrison et al., 1984, 1986; Jankowska, 1985). Taking advantage of the retrograde transsynaptic capability of WGA-HRP, several years ago we injected the conjugate into a hemidiaphragm ipsilateral to a C2 spinal cord hemisection after recovery of the hemidiaphragm was induced by the “crossed phrenic phenomenon” (CPP, Moreno et al., 1992). We found WGA-HRP labeling not only in phrenic motor neurons (PMNs), but also bilaterally in the medullary center that provides the excitatory drive to PMNs during inspiration, the rostral ventral respiratory group (rVRG). Thus, the paper demonstrated that the retrograde transsynaptic transport of WGA-HRP also occurs in the phrenic motor system (Moreno et al., 1992).

In recent years, investigators have become interested in identifying the intracellular molecular mechanisms and intracellular pathways which may mediate respiratory plasticity and recovery of the diaphragm after spinal cord injury (e.g. Kajana and Goshgarian, 2008a, 2008b, 2009; MacFarlane and Mitchell, 2008; MacFarlane et al., 2009; Wilkerson and Mitchell, 2009). In our own laboratory work is underway to investigate the possibility that certain drug-induced recovery in the respiratory motor system after spinal cord injury is mediated by the activation of the cAMP-PKA intracellular pathway. In order to study such intracellular molecular mechanisms it would be advantageous to specifically identify the physiologically active respiratory neurons in the spinal cord and brainstem that are most

© 2013 Published by Elsevier B.V.

Corresponding author: Harry G. Goshgarian, PhD., Department of Anatomy and Cell Biology, Wayne State University, School of Medicine, 540 East Canfield Avenue, Detroit, MI 48201, USA, Phone: 313-577-1045, Fax: 313-577-3125, hgoshgar@med.wayne.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

likely responsible for mediating the plasticity. This could be accomplished by using the retrograde transsynaptic transport capability of WGA-HRP following injections of the tracer into the recovered hemidiaphragm. The identified neurons could then be isolated (e.g. by laser microdissection) and subjected to intracellular molecular analysis. However, visualizing WGA-HRP histochemically requires exposure of the tissue sections to chemicals and reagents (for staining) that may reduce the quality of the mRNA captured by laser microdissection (Gjerdrum et al., 2004; Mouledous et al., 2002; Wang et al., 2006). Several laboratories have documented this problem (Gjerdrum et al., 2004; Mouledous et al., 2002; Wang et al., 2006) and in our own laboratory we found this to be the case. Thus, we set out to investigate an alternative tracer, WGA-Alexa 488, which may have the same retrograde transsynaptic capabilities as WGA-HRP, but without the requirement of visualizing the tracer by histochemical staining. In this case, WGA is conjugated to a fluorochrome (Alexa 488) and can be visualized simply with a fluorescent microscope (Model et al., 2009).

WGA-Alexa tracers are well established (Panchuk-Voloshina et al., 1999), are exceptionally bright and are excellent for tracing axonal pathways in the central nervous system (Reeber et al., 2011; Yamazaki et al., 2009; Panchuk-Voloshina et al., 1999). Both anterograde (Reeber et al., 2011) and retrograde (Yamazaki et al., 2009) track tracing studies have been conducted with WGA-Alexa 488. Transneuronal/transsynaptic transport of WGA-Alexa 488, however, has never been demonstrated *in vivo*. Thus, the first aim of the present study was to determine if WGA-Alexa 488 would undergo retrograde transsynaptic transport in the phrenic motor system (i.e., labeling both PMNs and the rVRG).

Finally, to partly address the underlying mechanism for transsynaptic transport, the second aim of the present study was to investigate the pattern and extent of labeling that occurs when WGA-Alexa 488 is applied to the cervical phrenic nerve as compared to intradiaphragmatic injection. In the former case, the tracer diffuses into the exposed phrenic axons, while in the latter; WGA-Alexa 488 is actively taken up at the phrenic myoneural junction by absorptive receptor-mediated endocytosis. Our goal was to characterize the pattern of WGA-Alexa 488 labeling based on the site of application in the respiratory system following a C2 spinal cord hemisection.

Experimental Procedure

Experimental animals

All animal studies were approved by the Wayne State University School of Medicine Institutional Animal Care and Use Committee. Moreover, the work described in this article was carried out in accordance with the *Code of Ethics of the World Medical Association* for experiments involving animals. Twelve adult male Sprague Dawley rats (360 – 630g) were used in these studies. WGA-Alexa 488 was injected into the left hemidiaphragm of six rats or applied to the intact left phrenic nerve in the remaining six animals. The body weight, and age at time of surgery for each rat is shown in table 1. All animals were pre-anesthetized with atropine sulphate (0.05mg/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), animals were

subjected to either a left C2 spinal cord hemisection (N=9), or a sham hemisection (laminectomy and durotomy, but no spinal cord lesion, N=3).

After anesthesia induction the dorsal aspect of the neck was shaved and prepared for aseptic surgery in all anesthetized rats. A left C2 spinal cord hemisection or sham spinal cord hemisection surgical procedure was carried out as previously described (Moreno et al., 1992). The functional completeness of the hemisection was then confirmed by EMG analysis of the diaphragm (see below). After paralysis of the left hemidiaphragm was confirmed, the paravertebral muscles were sutured with 4-0 absorbable sutures (Vicryl, Ethicon) and the skin was closed with wound clips.

Electromyography (EMG) analysis and Intradiaphragmatic injection

Immediately after spinal cord hemisection, paralysis of the ipsilateral hemidiaphragm was confirmed by EMG analysis. The abdominal surface of the diaphragm was exposed by a 6–8 cm horizontal incision made approximately 0.5 cm caudal and parallel to the costal margin. Bipolar platinum wire electrodes (Grass E-2B) were placed on each side of the diaphragm and recordings were made. Signals were amplified (20,000X) and band pass-filtered (30Hz-3kHz) by Grass amplifiers (model P511 AC, Astro-Med, Inc., West Warwick, RI) and raw EMG signals were recorded by a Cambridge Electronic Design (CED, Cambridge, England) data acquisition system integrated with CED Spike 2® software. Recordings were taken from three areas of the left hemidiaphragm: anterior (sternal), lateral (costal) and posterior (crural). Only animals that showed an absence of activity in all three areas were included in the study. Previously, we correlated an anatomically complete hemisection with complete paralysis of the ipsilateral hemidiaphragm muscle (Moreno et al. 1992). After EMG verification of a functionally complete hemisection, 50µl of 2% WGA-Alexa 488 solution (Life Technologies, Eugene, OR., Cat. # W11261) was injected (5 injections, 10µl each) into the left hemidiaphragm of six of the nine hemisected animals using a Hamilton syringe. After WGA-Alexa 488 hemidiaphragmatic injection, the abdominal muscles were sutured with 4-0 absorbable suture and the skin was closed with 4-0 non-absorbable nylon suture (Ethilon, Ethicon).

Intact phrenic nerve soaks

In the remaining six animals (three with C2 spinal cord hemisection and three with sham surgery of the spinal cord) 2% WGA-Alexa 488 was applied to the isolated left phrenic nerve for 1 hour immediately after the spinal cord surgeries. The cervical phrenic nerve was approached ventrally and isolated from the brachial plexus with fine forceps and cotton swabs. Photographic images of the nerve were captured at 40X on an Olympus C-5050 digital camera attached to a Zeiss surgical microscope (fig. 1). A cup created by cutting off the bottom of a 1.5ml microcentrifuge tube was placed under the phrenic nerve. Small pieces of bench top paper were placed around the cup to absorb fluid produced by the animal during the 1 hour treatment. 1.5µl of 2% WGA-Alexa 488 was placed in the cup with a micropipette. The surgical field was then covered with a piece of gauze moistened in sterile saline to prevent tissues from drying out during treatment. After 1 hour, the remaining WGA-Alexa 488, the cup and the bench paper were removed and the area was gently

swabbed to remove excess fluid (fig. 1). The muscles were sutured with 4-0 absorbable suture followed by 4-0 non-absorbable nylon suture to close the skin.

Post-operative care

Following the cleaning of the cervical and abdominal incisions, the animals were injected with buprenorphine (0.01mg/kg, s.c.) in 10ml saline for pain control. After a short period of recovery on a heating blanket and when the animals were able to ambulate, they were returned to clean soft liter-lined cages with food and water provided ad libitum. The animals were also given peanut butter and cereal as an enticement to eat. The lower abdomen was gently compressed while the animals were still anesthetized to induce micturition and defecation. Bowel and bladder reflexes appeared to be normal within 24 hours after surgery.

Tissue preparation

Two days after intradiaphragmatic or phrenic nerve tracer application, animals were anesthetized with urethane (1.6 g/kg, ip) and transcardially perfused with 50ml saline followed by 350ml of 4% paraformaldehyde in 0.1M phosphate-buffered saline (pH 7.4). The cervical spinal cord and medulla were removed and an insect pin was inserted along the longitudinal axis of both on the right to differentiate the left from the right side. The medullary and spinal tissue was then post-fixed in the perfusate overnight and cryoprotected for 2–3 days in 30% sucrose in 0.1M PBS. Transverse sections (40µm) through the medulla and spinal cord were cut with a cryostat 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). Images were taken using the Axio Vision Rel. 4.8 Program.

Fluorescent microscopy

Neurons labeled with WGA-Alexa 488 are exceptionally bright and can be easily seen at low magnification (e.g. figs. 3A and 4A). Unlike WGA-FITC which has been described to have a high level of autofluorescence (Crossland, 1985), autofluorescence is not an issue when using WGA-Alexa 488. Positively labeled neurons always have distinct granules that can be seen at higher magnification (e.g. figs 3D, 3E). Non-labeled neurons do not show granulation (e.g. figs 4D, 4E). Identification of spinal cord and medullary centers (i.e., the phrenic nucleus and rVRG) was based on our previous experience labeling these centers (Goshgarian and Rafols, 1981; Moreno et al. 1992).

Results

Electromyographic (EMG) analysis

In every animal subjected to a left C2 spinal cord hemisection, paralysis of the left hemidiaphragm was confirmed by EMG analysis (fig. 2). We analyzed the entire hemidiaphragm including the anterior (sternal), lateral (costal) and posterior (crural) regions. Figure 2 shows an example of hemidiaphragmatic paralysis in the lateral region of the left hemidiaphragm. In a few animals (< 10%), residual activity persisted in one or more of the above regions. In these cases, we found that if we extended the spinal cord lesion slightly more laterally, the residual activity disappeared.

Intradiaphragmatic injection

In all 6 rats receiving a left hemidiaphragmatic injection of WGA-Alexa 488, bilateral labeling of the phrenic nucleus was observed (fig. 3A). In pilot studies we tried to: i) inject the most lateral aspects of the left hemidiaphragm or ii) “paint” the left hemidiaphragm (Lane et al., 2008, 2009, 2012) rather than inject it to achieve ipsilateral labeling only, but the results consistently yielded bilateral phrenic nucleus labeling. Furthermore, the observed spinal cord labeling was restricted to the phrenic nucleus only; there were no other motor neurons, propriospinal neurons or dorsal horn neurons labeled at the C3 – C6 level of the spinal cord (fig. 3A).

In addition to bilateral phrenic nucleus labeling, intradiaphragmatic injection of WGA-Alexa 488 resulted in bilateral labeling of the rostral ventral respiratory group (rVRG) in the medulla. The left (ipsilateral to injection) rVRG of one animal is shown in 3B, 3D while the right rVRG (contralateral to injection) in the same animal is shown in 3C, 3E. Note also the selectivity of the medullary labeling. The rVRG is the only center labeled. Moreover, the rVRG was always labeled bilaterally (Figs. 3B, 3C). In particular, we noted no labeling of gigantocellular reticulospinal neurons nor raphe neurons known to project to the phrenic nucleus (Dobbins and Feldman, 1994).

Intact phrenic nerve soaks

In contrast, when WGA-Alexa 488 was applied to the intact isolated left phrenic nerve in 6 animals (three with C2 spinal cord hemisection and three with a sham hemisection) as described in the methods, only ipsilateral phrenic nucleus labeling was observed (fig. 4A). The ipsilateral phrenic nucleus labeling was observed in all 6 animals. No contralateral phrenic motor neuron labeling was ever observed, nor was there any other spinal cord neurons labeled at the level of the phrenic nucleus (C3–C6, fig. 4A). Here again, we carried out pilot studies in which we initially : i) injected the transected or intact phrenic nerve with the tracer, ii) applied WGA-Alexa 488-soaked gelfoam to the exposed phrenic nerve or iii) attempted to isolate the phrenic nerve with Parafilm® before applying the tracer. All of these attempts yielded spurious labeling of phrenic and non-phrenic neurons in the cervical spinal cord. It was only when we isolated the phrenic nerve that we achieved consistent ipsilateral phrenic nucleus labeling exclusively.

Following the application of WGA-Alexa 488 to the isolated phrenic nerve, there was no retrograde transsynaptic labeling to the rVRG or any other medullary neuronal center observed in any of the 6 animals (figs 4B–E). Figures 4B and 4C are sections through the rVRG from an animal in which the phrenic nerve was soaked in WGA-Alexa 488. The unlabeled left and right rVRG can be seen at low magnification (figs. 4B, 4C respectively) and a higher power view of each is shown (figs 4D, 4E). The medullary level is comparable to the level showing the labeled rVRGs in (figs. 3B–3E) in which WGA-Alexa 488 was injected into the diaphragm. Initially, because of the activity-dependent nature of transsynaptic transport using WGA-HRP (Harrison et al., 1984;1986; Moreno et al, 1992), we hypothesized that there may be a difference in the labeling pattern occurring when WGA-Alexa 488 was applied to the *quiescent* phrenic nerve (i.e. ipsilateral to a C2 hemisection) versus an *active* phrenic nerve (ipsilateral to sham hemisection). However, as

mentioned previously, there was no notable difference between the two groups. The only labeled neurons detected were ipsilateral phrenic neurons (fig. 4A) regardless of whether the phrenic nerve was quiescent or active.

Discussion

The value of quantitation

Several laboratories, including our own, have estimated the number of phrenic motor neurons in the adult rat using retrograde tracers applied by either i) application to the transected or intact phrenic nerve (Goshgarian and Rafols, 1981; Boulenguez et al. 2007; Mantilla et al. 2009), ii) injection into the diaphragm (Boulenguez et al. 2007; Mantilla et al. 2009; Nicaise et al. 2012) or iii) intrapleural injection (Mantilla et al. 2009). There has been a wide range of phrenic neuron numbers reported in the uninjured adult rat from these investigators. For instance, our laboratory was the first to determine quantitatively the number of phrenic motor neurons (415 ± 18) in the rat phrenic nucleus by applying horseradish peroxidase to the transected phrenic nerve (Goshgarian and Rafols, 1981). Subsequently, other laboratories have reported different numbers of rat phrenic neurons using other tracers or different methods of tracer application (Nicaise et al., 2012; Mantilla et al. 2009; Boulenguez et al. 2007). For instance, Mantilla et al. (2009), using the B subunit of cholera toxin conjugated to Alexa 488, reported a range of labeled phrenic neurons depending upon the method of applying the tracer. They found that Intradiaphragmatic injection resulted in 178 ± 23 whereas applying the tracer to the transected phrenic nerve resulted in a mean of 242 ± 20 cells being labeled. The number of cells labeled after Intrapleural injection (224 ± 21) was very similar to phrenic nerve application (Mantilla et al. 2009). Interestingly, Boulenguez et al. (2007) also reported a range of labeled phrenic neurons (255–359) which depended not only upon the specific tracer used but also the site of application. The highest number of cells, 359 ± 17 , was estimated when 3% fluorogold (FG) was applied to the transected phrenic nerve (Boulenguez et al. 2007). The point being made here is that there are several different factors that will contribute to variability in the quantitative assessment of phrenic neurons between laboratories. In addition to the factors indicated above, the amount of tracer used, the sampling size (i.e. the number of sections on which phrenic neurons are counted), the method of estimation, the diffusible nature of the tracer (Boulenguez et al. 2007), whether there is an accounting for split cells, and the investigator themselves will contribute to variability. Thus, the efficacy of a new tracer in the phrenic motor system should never be judged solely by the number of motor neurons labeled from an individual laboratory. For this reason, we did not determine the number of phrenic neurons labeled with WGA-Alexa 488. The value of quantitation in the phrenic motor system is best appreciated when normal or control numbers are compared with experimental numbers by a single investigator in a laboratory who uses identical methods of assessment in both groups (see for example, Nicaise et al., 2012). It is important to appreciate that this comparison in neuron numbers is not transferable between laboratories for the reasons stated above.

Intradiaphragmatic injection

The present study shows that retrograde transsynaptic transport of WGA-Alexa 488 occurs in the phrenic motor system (i.e., labeling phrenic lower motor neurons and rVRG pre-motor neurons) when the tracer is injected intradiaphragmatically. Following left C2 hemisection and tracer injection into the ipsilateral hemidiaphragm, bilateral phrenic nucleus labeling occurred in every animal. The bilateral phrenic nucleus labeling was most likely due to WGA-Alexa 488 diffusing across the midline from the left hemidiaphragm to the right and being taken up by the axon terminals in both phrenic nerves since peripheral crossing of phrenic axons has not been demonstrated in the rat diaphragm (Laskowski and Sanes, 1987). The right phrenic nerve was not cut in the present study as it was in our previous study (Moreno et al. 1992). The primary reason the right phrenic nerve was not cut was based on the observation that the crossed phrenic phenomenon (induced by C2 hemisection and contralateral phrenicotomy) contributes little to the overall ventilation of the animal (Fuller et al. 2006) and thus, we did not want to subject the experimental animals to unnecessary respiratory stress. Initially, we had planned to inject the paralyzed hemidiaphragm (ipsilateral to hemisection) and then the functionally active hemidiaphragm (contralateral to hemisection) in separate groups of animals to assess the activity-dependent nature of transsynaptic transport, but this plan was changed when bilateral phrenic nucleus labeling occurred in all animals.

Bilateral phrenic nucleus labeling following an ipsilateral diaphragm injection is not a unique finding. Boulenguez et al. (2007) showed that dependent upon the monosynaptic retrograde fluorescent tracer injected unilaterally in the rat diaphragm, phrenic motor neurons would be labeled either bilaterally, ipsilaterally, or not at all. For instance, when FG was injected into one side of the diaphragm, most of the resultant labeling was seen in the ipsilateral phrenic nucleus, but there was also a “less intense artifactual labeling” observed in the contralateral phrenic nucleus and in the medulla (mainly in the area postrema and cranial motor nuclei). Very similar results were observed after horseradish peroxidase (HRP) injection, but there was no labeling observed at all after the injection of nuclear yellow or diamino yellow into the diaphragm. Furthermore, injection of the dextran amine, fluororuby (FR) and the carbocyanine, DiAsp, into one hemidiaphragm, always resulted in the exclusive labeling of ipsilateral phrenic motor neurons. The authors suggested that the lipophilic properties of DiAsp and the high molecular weight of FR may prevent their diffusion to adjacent tissues and into the bloodstream which may have caused the artifactual labeling observed with FG and HRP (Boulenguez et al., 2007). The present results show that WGA-Alexa 488 is an excellent retrograde transsynaptic fluorescent tracer in the phrenic motor system, however there is consistent diffusion of the tracer in the diaphragm.

Phrenic nerve soak

To address the issue of WGA-Alexa 488 diffusing across the midline of the diaphragm after a unilateral hemidiaphragm injection, we applied the tracer directly to the left phrenic nerve in both C2 hemisected and non-hemisected rats. Our goal was to achieve unilateral phrenic motor neuron labeling in order to assess possible differences between quiescent and functionally active phrenic motor neurons. In spite of the fact that there was WGA-Alexa 488 labeling of the ipsilateral phrenic motoneurons following application of the tracer to the

nerve, we did not observe transsynaptic labeling to the rVRG. Thus, there was no difference in transsynaptic transport between functionally quiescent (ipsilateral to hemisection) and active phrenic motor neurons (ipsilateral to sham spinal cord injury). This was an unexpected finding. Based on our experience with several other tracers in the phrenic motor system (e.g. HRP, Goshgarian and Rafols, 1981, 1984; CT-HRP, Furicchia and Goshgarian, 1987; and WGA-HRP, Moreno et al., 1992), we have never detected a difference in labeling pattern dependent on the site of tracer administration (i.e. nerve versus diaphragm). The reason for this difference in labeling pattern is unknown; initially, it suggested to us that unlike WGA-HRP, WGA-Alexa 488 may not be transsynaptically transported across physiologically active synapses. However, a careful examination of the literature suggested that the difference in labeling pattern may be due to differences in the underlying mechanisms of uptake and transport of WGA in the nervous system when the tracer is injected into muscle versus tracer application to the nerve (see below).

Transsynaptic transport of WGA

Some aspects of the mechanism of transsynaptic transport of WGA have been delineated in the literature. WGA has an affinity to N-acetyl-D-glucosamine and sialic acid which are components of glycoconjugates found on most neuronal cell membrane (Robertson, 1990; Fabian and Coulter 1985). These sugars act as receptors when WGA is injected into muscle (Robertson, 1990). Thus, WGA is taken up and retrogradely transported as a consequence of binding to these specific receptor sites on the motor nerve terminal membranes. Once WGA enters the axon, it is retrogradely transported within smooth vesicles, cisternae and tubules and once in the cell body, the above membrane compartments fuse and the transported lectin is incorporated into lysosomes (Schwab et al., 1979). In the electron microscopic studies of Schwab et al. (1979), the selective release of WGA from postsynaptic dendrites followed by the rapid uptake of the lectin into presynaptic nerve terminals was observed. However after counting the number of presynaptic terminals labeled the authors concluded that the retrograde transsynaptic transport capability of WGA was far less than other macromolecules such as tetanus toxin (Schwab et al 1979).

The precise mechanism for the release of WGA from postsynaptic dendrites into the synaptic cleft is still unknown. Since the 1980s it has been suggested that there is a preferential release from postsynaptic dendrites of physiological active synaptic connections (Harrison et al., 1984, 1986; Jankowska 1985). This was initially determined by observing the location and number of labeled motor neurons and interneurons (propriospinal neurons) in the spinal cord of cats and rats following the injection of WGA-HRP into motor nerves of chronically anesthetized and awake animals (Harrison et al., 1984; Jankowska 1985). Furthermore, the extent of interneuronal labeling was enhanced when the motor nerves were stimulated which facilitated synaptic activity to the motor neurons (Harrison et al., 1984; Jankowska, 1985). Since Schwab et al. (1979) never observed WGA in the synaptic cleft, but observed the lectin in the pre-synaptic terminals contacting retrogradely labeled neurons and postsynaptic dendrites, they concluded that release and transsynaptic transfer occurs rapidly.

The present finding that transsynaptic transport does not occur when WGA-Alexa 488 is applied to the isolated phrenic nerve is not easily explained. If activity were the only factor involved, we should have detected a difference in uptake and transport between the C2 hemisectioned animals (in which the phrenic nerve was quiescent) and the non-hemisectioned animals (in which the phrenic nerve was phasically discharging). However, both groups of animals showed the same results; that is, only ipsilateral phrenic neurons were labeled. The different results obtained after intradiaphragmatic injection and phrenic nerve application suggest different uptake mechanisms. Since we stripped the phrenic nerve of its facial covering and applied WGA-Alexa 488 directly to the nerve, the tracer may have entered the nerve primarily by simple diffusion (i.e., not by a receptor-mediated uptake mechanism). Once within the nerve, the retrograde transport of the tracer back to the cell body most likely occurred as originally described by Schwab et al. (1979), however, transsynaptic transport did not occur. Since it has been shown that lectin binding sites are not distributed uniformly on individual neurons (Hatten et al., 1979), it may be that WGA-Alexa 488 must be applied at the phrenic myoneural junction where there may be a high concentration of WGA receptors in order for transsynaptic transport to occur (Fabian and Coulter, 1985; Robertson, 1990).

The results of the present study show for the first time that HRP is not necessary for retrograde transsynaptic transport in the phrenic motor system; WGA alone is sufficient to mediate the transport. This was not apparent in our previous study involving the intradiaphragmatic injection of WGA-HRP (Moreno et al., 1992). We and others have shown that HRP alone will be retrogradely transported to phrenic motor neurons when injected into the diaphragm (Webber et al., 1979; Boulenguez et al., 2007) or applied to the cut phrenic nerve (Goshgarian and Roubal, 1986; Goshgarian and Rafols, 1981, 1984), but HRP alone does not mediate retrograde transsynaptic transport. There is a possibility that the Alexa 488 component of the conjugate used could mediate transsynaptic transport, but this is unlikely since Mantilla et al. (2009) demonstrated that Alexa 488 conjugated to the β subunit of cholera toxin does not mediate transsynaptic transport in the phrenic motor system. Furthermore, our own work has indicated that this same toxin is not transsynaptically transported when injected into the diaphragm (Furicchia and Goshgarian, 1987).

Phrenic motor neurons are unique in that they maintain a phasic discharge regardless of whether an animal is anesthetized or awake. Neurons in the rVRG are responsible for maintaining this discharge (Ellenberger and Feldman, 1988) and this is the presumed basis for the activity-dependent retrograde transsynaptic labeling of both phrenic motor neurons and rVRG neurons when WGA is injected into the diaphragm (Moreno et al., 1992 and the present study). It is important to point out that no spinal interneurons were labeled when a WGA conjugate was injected into the diaphragm either during the CPP as in our previous study (Moreno et al., 1992) or during normal respiration or after hemidiaphragmatic paralysis as in the present study. In all of these studies, however, it needs to be stressed that the tracers were injected into animals with acutely injured (2–3 days) spinal cords. It is presently unknown if propriospinal neurons are recruited in relaying respiratory drive in animals with chronically injured (several weeks) spinal cords. Recently, Reier and colleagues (Lane et al., 2008, 2009, 2012) demonstrated the synaptic relationships of

phrenic neurons and interneurons following the retrograde transsynaptic transport of pseudorabies virus and suggested that propriospinal neurons may be recruited as part of the plasticity leading to the spontaneous recovery of the diaphragm after chronic C2 spinal cord hemisection (Nantwi et al. 1999). We agree with the authors that additional studies are needed to substantiate this possibility. A study is presently underway in our laboratory utilizing WGA-Alexa 488 injection into the diaphragm to determine if there are differences in propriospinal labeling following injection of the tracer in acutely versus chronically injured spinal cords.

Conclusions

In summary, the present results show that WGA-Alexa 488 can be used to anatomically identify motor and pre-motor neurons in the respiratory circuitry without subjecting tissue sections to chemical reagents that may not be compatible with biochemical or molecular analyses of these neurons. Thus, the study provides investigators with a new tool to study plasticity in the respiratory system after spinal cord injury. The following novel findings are reported herein: i) retrograde transsynaptic transport of WGA-Alexa 488 occurs in the phrenic motor system when the tracer is injected intradiaphragmatically, ii) only retrograde intracellular transport of WGA-Alexa 488 occurs following the application of the tracer to the isolated phrenic nerve, iii) Thus, the pattern and extent of labeling of WGA-Alexa 488 in the phrenic motor system is dependent upon the site of tracer application, iv) HRP is not necessary for retrograde transsynaptic transport in the phrenic motor system, v) the present anatomical results suggest that descending respiratory drive from the rVRG to phrenic motor neurons is not relayed by propriospinal neurons after acute spinal cord injury, but whether this is true after chronic spinal cord injury remains to be determined.

Acknowledgments

This study was supported by a grant from the Craig H. Neilsen Foundation and NIH grant HD-31550.

References

- Boulenger P, Gestreau C, Vinit S, Stamegna JC, Kastner A, Gauthier P. Specific and artifactual labeling in the rat spinal cord and medulla after injection of monosynaptic tracers into the diaphragm. *Neurosci Lett*. 2007; 417(2):206–211. [PubMed: 17412505]
- Crossland WJ. Anterograde and retrograde axonal transport of native and derivatized wheat germ agglutinin in the visual system of the chicken. *Brain Res*. 1985; 347(1):11–27. [PubMed: 2413966]
- Dobbins EG, Feldman JL. Brainstem network controlling descending drive to phrenic motoneurons in rat. *J Comp Neurol*. 1994; 347(1):64–86. [PubMed: 7798382]
- Ellenberger HH, Feldman JL. Monosynaptic transmission of respiratory drive to phrenic motoneurons from brainstem bulbospinal neurons in rats. *J Comp Neurol*. 1988; 269(1):47–57. [PubMed: 3361003]
- Fabian RH, Coulter JD. Transneuronal transport of lectins. *Brain Res*. 1985; 344(1):41–48. [PubMed: 2412649]
- Fuller DD, Golder FJ, Olson EB Jr, Mitchell GS. Recovery of phrenic activity and ventilation after cervical spinal hemisection in rats. *J Appl Physiol*. 2006; 100(3):800–806. [PubMed: 16269524]
- Furicchia JV, Goshgarian HG. Dendritic organization of phrenic motoneurons in the adult rat. *Exp Neurol*. 1987; 96:621–634. [PubMed: 3446220]

- Gjerdrum LM, Abrahamsen HN, Villegas B, Sorensen BS, Schmidt H, Hamilton-Dutoit SJ. The influence of immunochemistry on mRNA recovery from micodissected frozen and formalin-fixed, Paraffin-embedded sections. *Diagn Mol Pathol*. 2004; 13(4):224–33. [PubMed: 15538113]
- Goshgarian HG, Rafols JA. The phrenic nucleus of the albino rat: A correlative HRP and Golgi study. *J Comp Neurol*. 1981; 201:441–456. [PubMed: 7276259]
- Goshgarian HG, Rafols JA. The ultrastructure and synaptic architecture of phrenic motor neurons in the spinal cord of the adult rat. *J Neurocytol*. 1984; 13:85–109. [PubMed: 6707715]
- Goshgarian HG, Roubal PJ. Origin and distribution of phrenic primary afferent nerve fibers in the spinal cord of the adult rat. *Exp Neurol*. 1986; 92:624–638. [PubMed: 3709737]
- Harrison PJ, Hultborn H, Jankowska E, Katz R, Storai B, Zytnicki D. Labeling of interneurons by retrograde transsynaptic transport of horseradish peroxidase from motoneurons in rats and cats. *Neurosci Lett*. 1984; 45:15–19. [PubMed: 6547216]
- Harrison PJ, Jankowska E, Zytnicki D. Lamina VIII interneurons in crossed reflex pathways in the cat. *J Physiol*. 1986; 371:147–166. [PubMed: 3701648]
- Hatten ME, Schachner M, Sidman RL. Histochemical characterization of lectin binding in mouse cerebellum. *Neuroscience*. 1979; 4:921–35. [PubMed: 552613]
- Jankowska E. Further indications for enhancement of retrograde transneuronal transport of WGA-HRP by synaptic activity. *Brain Res*. 1985; 341:403–408. [PubMed: 4041803]
- Kajana S, Goshgarian HG. Administration of phosphodiesterase inhibitors and an adenosine A1 receptor antagonist induces phrenic nerve recovery in high cervical spinal cord injured rats. *Exp Neurol*. 2008a; 210(2):671–680. [PubMed: 18289533]
- Kajana S, Goshgarian HG. Spinal activation of the cAMP-PKA pathway induces respiratory motor recovery following high cervical spinal cord injury. *Brain Res*. 2008b; 1232:206–213. [PubMed: 18656458]
- Kajana S, Goshgarian HG. Systemic administration of rolipram increases medullary and spinal cAMP and activates a latent respiratory motor pathway after high cervical spinal cord injury. *J Spinal Cord Med*. 2009; 32(2):175–182. [PubMed: 19569465]
- Lane ME, White TE, Coutts MA, Jones AL, Sandhu MS, Bloom DC, Bolser DC, Yates BC, Fuller DD, Reier PJ. Cervical pre-phrenic interneurons in the normal and lesioned spinal cord of the adult rat. *J Comp Neurol*. 2008; 511(5):692–709. [PubMed: 18924146]
- Lane MA, Lee KZ, Fuller DD, Reier PJ. Spinal circuitry and respiratory recovery following spinal cord injury. *Respir Physiol Neurobiol*. 2009; 169(2):123–132. [PubMed: 19698805]
- Lane MA, Lee KZ, Salazar K, O'Steen BE, Bloom DC, Fuller DD, Reier PJ. Respiratory function following bilateral mid-cervical contusion injury in the adult rat. *Exp Neurol*. 2012; 235(1):197–210. [PubMed: 21963673]
- Laskowski MB, Sanes JR. Topographic mapping of motor pools onto skeletal muscles. *J Neurosci*. 1987; 7(1):252–260. [PubMed: 3543250]
- MacFarlane PM, Mitchell GS. Respiratory long-term facilitation following intermittent hypoxia requires reactive oxygen species formation. *Neurosci*. 2008; 152(1):189–197.
- MacFarlane PM, Satriotomo I, Windelborn JA, Mitchell GS. NADPH oxidase activity is necessary for acute intermittent hypoxia-induced phrenic long term facilitation. *J Physiol*. 2009; 587(pt 9):1931–1942. [PubMed: 19237427]
- Mantilla CB, Zhan W-Z, Sieck GC. Retrograde labeling of phrenic motoneurons by intrapleural injection. *J Neurosci Meth*. 2009; 182:244–249.
- Model MA, Reese JL, Fraizer GC. Measurement of wheat germ agglutinin binding with a fluorescent microscope. *Cytometry Part A*. 2009; 74A:874–881.
- Moreno DE, Yu X-J, Goshgarian HG. Identification of the axon pathways which mediate functional recovery of a paralyzed hemidiaphragm following spinal cord hemisection in the adult rat. *Exp Neurol*. 1992; 116:219–228. [PubMed: 1375167]
- Mouledous L, Hunt S, Harcourt R, Harry JL, Williams KL, Gutstein HB. Lack of compatibility of histological staining methods with proteomic analysis of laser-capture microdissected brain samples. *J Biomol Tech*. 2002; 13(4):258–64. [PubMed: 19498991]

- Nantwi KD, El-Bohy A, Schrimsher GW, Reier PJ, Goshgarian HG. Spontaneous functional recovery in a paralyzed hemidiaphragm following upper cervical spinal cord injury in adult rats. *Neurorehab Neural Repair*. 1999; 13(4):225–234.
- Nicaise C, Hala TJ, Frank DM, Parker JL, Authelet M, Leroy K, Brion J-P, Wright MC, Lepore AC. Phrenic motor neuron degeneration compromises phrenic axonal circuitry and diaphragm activity in a unilateral cervical contusion model of spinal cord injury. *Exp Neurol*. 2012; 235(2):530–552.
- Panchuk-Voloshina N, Haugland RP, Bishop-Stewart J, Bhargat MK, Millard PJ, Mao F, Leung WY. Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates. *J Histochem Cytochem*. 1999; 47(9):1179–1188. [PubMed: 10449539]
- Porter JD, Guthrie BL, Sparks L. Selective retrograde transneuronal transport of wheat germ agglutinin-conjugated horseradish peroxidase in the oculomotor system. *Exp Brain Res*. 1985; 57:411–416. [PubMed: 3838281]
- Reeber SL, Gebre SA, Sillitoe RV. Fluorescence mapping of afferent topography in three dimensions. *Brain Struct Funct*. 2001; 107(s00429-011-0304-2)
- Robertson B. Wheat germ agglutinin binding in rat primary sensory neurons: a histochemical study. *Histochem*. 1990; 94:81–85.
- Schwab ME, Suda K, Thoenen H. Selective retrograde transsynaptic transfer of a protein, tetanus toxin, subsequent to its retrograde axonal transport. *J Cell Biol*. 1979; 82:798–810. [PubMed: 92475]
- Wang H, Owens JD, Shih JH, Li MC, Bonner RF, Mushinski JF. Histological staining methods preparatory to laser capture microdissection significantly affect the integrity of the cellular RNA. *BMC Genomics*. 2006; 7:97. [PubMed: 16643667]
- Webber CL Jr, Wurster RD, Chung JM. Cat phrenic nucleus architecture as revealed by horseradish peroxidase mapping. *Exp Brain Res*. 1979; 35(3):395–406. [PubMed: 456448]
- Wilkerson JE, Mitchell GS. Daily intermittent hypoxia augments spinal BDNF levels, ERK phosphorylation and respiratory long-term facilitation. *Exp Neurol*. 2009; 217(1):116–123. [PubMed: 19416672]
- Yamazaki H, Ohi Y, Haji A. μ -opioid and N-methyl-D-aspartate receptors are localized at laryngeal motoneurons of guinea pigs. *Biol Pharm Bull*. 2009; 32(2):293–96. [PubMed: 19182392]

Highlights

- Article introduces WGA-Alexa 488, a fluorescent retrograde transsynaptic tracer
- Methods of phrenic administration: Intradiaphragmatic and phrenic nerve application
- Nerve application labels ipsilateral phrenic motor neurons only
- Bilateral phrenic and rVRG cells labeled exclusively after intradiaphragmatic injection

Figure 1.

Photographs of the surgical exposure of the left phrenic nerve and the application of WGA-Alexa 488 to the nerve for 1 hour. In all photographs, caudal is at the top, rostral is at the bottom, lateral is to the left and medial is to the right. A, surgical exposure of the phrenic nerve (PN) as it lays on the ventral surface of the brachial plexus (BP) before the application of the tracer. V, vagus nerve; C, carotid artery; J, jugular vein. B, during the application of the tracer, a cup containing 1.5 μ l of 2% WGA-Alexa 488 is placed so that the nerve is bathed in the solution. Bench top paper (B) is placed in the surrounding tissue to absorb fluid from the animal during the application period. C, after the 1 hour application period, the cup and the bench top paper is removed and the tissue is gently swabbed. Note that the phrenic nerve (PN) has a yellow color indicating that the tracer diffused into the nerve.

Figure 2.

Electromyograms taken from both sides of the diaphragm from an animal that was subjected to left C2 spinal cord hemisection. Note that the right hemidiaphragm is not significantly affected by the hemisection and displays EMG bursting every time the animal takes a breath. The left hemidiaphragm, however, is completely paralyzed and does not display EMG activity. The spikes on the left are EKG (electrocardiogram) waves. Calibration bar: 1 second.

Figure 3.

Labeling pattern following injection of 50 µl of 2% WGA-Alexa 488 into the left hemidiaphragm from an animal that was subjected to left C2 spinal cord hemisection. A, low power view (4X objective) of a section through the C3 level of the spinal cord. The photograph shows bilateral phrenic nucleus labeling exclusively. Note that there is no other neuron labeling in the spinal cord gray matter and suggests that the tracer diffuses across the midline of the diaphragm to label both phrenic nuclei. This same pattern of labeling was seen in all animals with a left hemidiaphragm injection. The pinhole (P) marks the right side of the cord (contralateral to injection). B, C Each animal receiving intradiaphragmatic

injections of WGA-Alexa 488 also displayed bilateral labeling of the rVRG indicating retrograde transsynaptic transport. B, low power view (5X objective) of the labeled left rVRG (circled area). SNV, spinal nucleus of V, STV, spinal tract of V are indicated as landmarks. C, same magnification as in B showing the right rVRG (circled area) in the same animal. Note that there is no other medullary center labeled. Small arrows in both B, C point to small labeled non-neuronal cells associated with blood vessels. Calibration bar in C, also pertains to B. D, higher power view (20X objective) of the labeled left rVRG shown in B. E, higher power view of the right rVRG shown in C. Note granulation in both rVRG centers indicating positively labeled cells. Calibration bar shown in E also pertains to D.

Figure 4.

Labeling pattern shown following WGA-Alexa 488 application for 1 hour to the left phrenic nerve from an animal that was subjected to left C2 spinal cord hemisection. A. Low power view of the C4 level of the spinal cord showing phrenic nucleus labeling only on the left side (ipsilateral to nerve application). The pinhole (P) marks the right side of the spinal cord. Note that no other neurons are labeled in the spinal cord other than the ipsilateral phrenic nucleus. Also note that the phrenic nucleus is as intensely labeled as it is following diaphragm injection of the tracer (compare this figure with fig. 3A). Left (B) and right (C) sides of the medulla at the level of the rVRG (circled areas in both B and C) from an animal in which WGA-Alexa 488 was applied to the phrenic nerve. Note that the rVRGs are not labeled suggesting that retrograde transsynaptic transport does not occur when the tracer is applied to the phrenic nerve. SNV, spinal nucleus of V; STV, spinal tract of V are included as landmarks. These sections are comparable to the sections shown in 3B and 3C. Magnification in B is the same as in C. D, higher power view of the rVRG shown in B (circled area). E, higher power view of the rVRG shown in C (circled area). Note that there is no granulation in the rVRG neurons indicating that they are not labeled. In order to see these unlabeled centers, the intensity of the fluorescent scope was increased substantially.

Compare background intensity shown in figs 3D, 3E with these figures. Magnification of D is the same as E.

Table 1

Weight and Age of Experimental Animals		
WG-A-Alexa 488 Application	Weight (grams)	Age at time of Surgery
C2Hx intradiaphragmatic L hemidiaphragm	630g	182 days
C2Hx intradiaphragmatic L hemidiaphragm	470g	99 days
C2Hx intradiaphragmatic L hemidiaphragm	540g	120 days
C2Hx intradiaphragmatic L hemidiaphragm	520g	142 days
C2Hx intradiaphragmatic L hemidiaphragm	510g	142 days
C2Hx intradiaphragmatic L hemidiaphragm	360g	81 days
Sham soak intact L phrenic nerve	410g	85 days
Sham soak intact L phrenic nerve	440g	93 days
Sham soak intact L phrenic nerve	455g	107 days
C2Hx soak intact L phrenic nerve	420g	85 days
C2Hx soak intact L phrenic nerve	440g	93 days
C2Hx soak intact L phrenic nerve	455g	109 days