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Retrograde Labeling of Phrenic Motoneurons by Intrapleural Injection

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Abstract

Studies of motoneuron plasticity during development or in response to injury or disease rely on the ability to correctly identify motoneurons innervating specific muscle groups. Commonly, injections of retrograde tracer molecules into a target muscle or into a transected nerve are used to label specific motoneuron pools. However, intramuscular injection does not consistently label all motoneurons in the target pool; and either injection site may involve additional surgical procedures and muscle or nerve perturbations. For instance, retrograde labeling of phrenic motoneurons by injection into the diaphragm muscle is commonly employed in studies of plasticity in respiratory motor control. Diaphragm intramuscular injection involves a laparotomy, and this additional surgery may limit the ability to conduct labeling studies particularly in small animals. In the present study, we provide validation of a novel method for phrenic motoneuron labeling using intrapleural injection of Alexa 488-conjugated cholera toxin subunit B. Only phrenic motoneurons were labeled in the cervical spinal cord as verified by co-staining with rhodamine-conjugated dextran injected into the diaphragm muscle or applied via phrenic nerve dip. Thoracic intercostal motoneurons and some dorsal root ganglia neurons were also labeled by intrapleural injection, but there was no evidence of trans-synaptic labeling. Phrenic motoneuron labeling was not present if the phrenic nerve was transected prior to intrapleural injection. This novel method is ideally suited for morphological studies and analyses of mRNA expression in isolated phrenic motoneurons using techniques such as laser capture microdissection.

Keywords

phrenic motoneurons; diaphragm muscle; respiratory plasticity; retrograde tracer

1. Introduction

Structural and functional characterization of motoneurons relies on the ability to unambiguously identify motoneurons innervating specific muscle groups. Commonly, retrograde tracers either injected into a target muscle or applied to a transected nerve are used to specifically label motoneurons (Gordon and Richmond, 1990; Yates et al., 1999; Zhan et al., 2000; Boulenguez et al., 2007). However, both approaches require surgery, and have additional experimental limitations. For example, intramuscular injections do not consistently

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label all motoneurons in the target pool. Transection of the nerve is obviously a major perturbation that may affect both structural and functional characteristics of motoneurons.

Retrograde labeling of phrenic motoneurons by injection into the diaphragm muscle is a common method used in studies of plasticity in respiratory motor control (Yates et al., 1999; Prakash et al., 2000; Boulenguez et al., 2007). This procedure usually involves a laparotomy followed by intra-diaphragmatic injection of retrograde tracers such as lipophilic dyes, cholera toxin subunit B or conjugated dextrans. Laparotomy itself has been shown to depress respiration (Shannon and Freeman, 1981; Sieck and Fournier, 1989) and thereby may impact structural and functional properties of phrenic motoneurons. Similarly, phrenicotomy may also affect phrenic motoneuron properties.

In the present study, we provide validation of a novel method for labeling phrenic motoneurons using intrapleural injection of fluorescently-conjugated cholera toxin subunit B. This method does not require anesthesia, surgery or phrenic nerve transection. Labeling of phrenic motoneurons in the cervical spinal cord was verified by direct comparison to labeling via intramuscular injection and application to transected phrenic nerves (i.e., “nerve dip”). Results demonstrated co-staining of phrenic motoneurons by intrapleural injection and by either intramuscular injection or direct application to transected nerves. In addition to phrenic motoneurons, thoracic intercostal motoneurons were labeled by intrapleural injection. No other spinal cord labeling was observed, but some dorsal root ganglia neurons were also labeled. Thus, there was no evidence of trans-synaptic labeling with cholera toxin subunit B. Phrenic motoneurons were not labeled by intrapleural injection if the phrenic nerve was transected prior to intrapleural injection. This novel method is ideally suited for analyses of mRNA expression in isolated phrenic motoneurons in applications such as laser capture microdissection.

2. Methods

2.1. Experimental animals

Adult male, Sprague-Dawley rats (280–300 g in body weight; n=12) were used. All procedures were approved by the Institutional Animal Care and Use Committee, and were conducted in strict accordance with the American Physiological Society guidelines (2006).

Animals were anesthetized with a mixture of ketamine (10 mg/kg) and xylazine (60 mg/kg) for labeling procedures involving intramuscular injection or phrenicotomy. Intrapleural injections were performed in awake, lightly restrained animals (see section 2.2). At the terminal experiment, animals were deeply anesthetized as above and euthanized by exsanguination following administration of heparin (100 U). Thereafter, animals were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) prior to tissue collection.

2.2. Labeling techniques

Retrograde labeling of phrenic motoneurons is well established, using either diaphragm intramuscular injection (Prakash et al., 1993; Kinkead et al., 1998; Prakash et al., 2000) or phrenic nerve dip (Zhan et al., 1989). We compared intrapleural tracer injection to these two techniques. In addition, phrenic nerve transection (necessary for the nerve dip procedure) was used to verify phrenic nerve transport of intrapleurally-injected tracer molecules (Fig. 1). Animals were assigned to one of three groups: (1) bilateral intrapleural injection followed 3 days later by unilateral phrenic nerve dip with dextran conjugate (n=3); (2) unilateral phrenic nerve dip immediately followed by bilateral intrapleural injection (n=3); and (3) concomitant bilateral injections of cholera toxin subunit B intrapleurally and dextran conjugate into the diaphragm muscle (n=6). In all cases, tissues were collected 3 days after the last injection.

2.2.1. Intrapleural injection—Awake animals were lightly restrained slightly tilted on their side with the help of an assistant. While stabilizing the animal's back, the rib cage was palpated in order to identify the fifth intercostal space at the anterior axillary line. Using sterile conditions and appropriate antisepsis, a 50- μ l Hamilton syringe was used to inject 15–20 μ l of a 0.2% Alexa Fluor 488-conjugated cholera toxin subunit B solution (Invitrogen Corp., Carlsbad, CA). Injections were performed transcutaneously into the thoracic cavity on each side (5–7 mm deep, from skin). Animals were monitored closely for any signs of respiratory compromise such as that following unintentional pneumothorax, but none were evident in this study.

2.2.2. Phrenic nerve dip—The procedure for phrenic nerve dissection and labeling has been previously described (Zhan et al., 1989). Briefly, the phrenic nerve was dissected in the neck via a ventral approach. After isolating at least 5 mm, the phrenic nerve was cut as far distally as possible. The proximal nerve stump was carefully de-sheathed and immersed in a microdish containing ~5 μ l of 5% tetramethylrhodamine-conjugated dextran (Invitrogen). The dish was covered with petroleum jelly to avoid contamination of surrounding tissues. After 45–60 min, the microdish was removed and tissues were sutured in layers.

2.2.3. Intradiaphragmatic injection—Using a previously described technique (Prakash et al., 1993; Kinkead et al., 1998; Prakash et al., 2000), anesthetized animals underwent laparotomy and the diaphragm muscle was exposed. A total of 15–20 μ l of a solution containing 5% tetramethylrhodamine-conjugated dextran (Invitrogen) was injected into randomly selected sites in the diaphragm muscle. On the right or left side, 4–5 spots were injected (~4 μ l each) in either the sternal or costal regions.

2.3. Tissue preparation

At the terminal experiment, following transcardial fixation, the entire spinal cord was exposed via a dorsal laminectomy. Spinal cord segments including cervical, thoracic and lumbar were removed en block and post-fixed in 4% paraformaldehyde in PBS overnight. Dorsal root ganglia were also carefully dissected and placed in fixative solution. Spinal cord segments were then immersed in 24% sucrose in PBS for cryoprotection prior to sectioning using a Reichert-Jung Frigocut cryostat (Reichert Microscope Services; Depew, NY) in 50 μ m-thick longitudinal (horizontal) sections. Sections were placed sequentially on gelatin-subbed slides carefully maintaining side orientation. Subsequently sections were treated with graded alcohols and xylene, and coverslipped with DPX mountant (Fluka; Sigma-Aldrich, St. Louis, MO).

2.4. Confocal microscopy

Spinal cord sections were visualized using an Olympus FluoView 200 laser scanning confocal microscope (Olympus America Inc., Melville, NY) mounted on an upright Olympus BX50WI microscope and equipped with Argon (488 nm) and HeNe (543 nm) lasers. Spinal cord sections were scanned at low magnification first to identify regions with labeled neurons. The sections within each segment were then imaged sequentially starting with the most rostral section. The 488 nm and 543 nm illumination lines were used to excite Alexa 488 and tetramethylrhodamine, respectively. Simultaneous imaging was possible using a 540 nm dichroic mirror and appropriate emission filters (510–540 nm and 585–625 nm for Alexa 488 and tetramethylrhodamine, respectively). Images were obtained using either a 20 $\times$ 0.8 NA or 40 $\times$ 1.4 NA oil immersion lens. Three-dimensional image stacks were collected in an 800 $\times$ 600 array, with a 1.6 μ m step size for the 20 $\times$ lens (XY pixel dimensions: 1.0 μ m) and a 0.8 μ m step size for the 40 $\times$ lens (XY pixel dimensions: 0.5 μ m). Acquisition parameters (e.g., laser intensity, confocal aperture, photomultiplier gain and dark current) were kept fixed across preparations (Prakash et al., 1993; Sieck et al., 1999).

Labeled motoneurons were counted manually and dual-labeled neurons were determined by co-staining with fluorescent tracer molecules in maximal projection images from each confocal stack. No thresholding or post-imaging processing algorithms were applied. In a subset of intrapleurally-injected and phrenic nerve dip animals (Table 1), complete motoneuron numbers were determined by counting all labeled motoneurons in consecutive sections on one side of the spinal cord. All motoneurons were counted on the same side of the injection for the intramuscular labeling group. Statistical analyses were performed using JMP statistical software (SAS Institute, Cary, NC). Differences in the extent of motoneuron labeling across techniques were determined by comparing counts of the total number of motoneurons using one-way ANOVA. Post hoc comparisons were performed using Tukey-Kramer's honestly significant difference test ($p < 0.05$).

3. Results

3.1. Phrenic motoneuron labeling by intrapleural injection

Large neurons in the ventral horn of the C3, C4, C5 and C6 segments of the cervical spinal cord (which correspond to the location of the phrenic motoneuron pool) were labeled extensively following intrapleural injection of Alexa 488-conjugated cholera toxin subunit B (Fig. 2A). Labeled neurons were organized in a column with cephalocaudal orientation roughly parallel to the long axis of the spinal cord, as previously reported for the rat phrenic motor nucleus (Goshgarian and Rafols, 1981; Furicchia and Goshgarian, 1987; Lindsay et al., 1991; Kinkad et al., 1998; Prakash et al., 2000). Clusters of neurons generally corresponded with cervical spinal cord segments (Fig. 2A). Within a cluster, each labeled neuron had morphological characteristics similar to those described for phrenic motoneurons, including the major axis in a rostrocaudal direction, with 6–8 primary dendrites and bundling of the dendritic trees in the major axis orientation (Fig. 2A, inset). In general, ~220 neurons were labeled in the cervical ventral horns on each side of the spinal cord after intrapleural injection (Table 1). This number is in general agreement with previous studies reporting the number of phrenic motoneurons in the adult rat using other labeling techniques such as direct application of retrograde tracers including dextrans, wheat germ agglutinin, and several fluorescent tracers (Furicchia and Goshgarian, 1987; Gordon and Richmond, 1990; Prakash et al., 2000; Boulenguez et al., 2007).

In addition to labeled neurons in the cervical ventral horn, neurons were also labeled in the ventral horn of thoracic spinal cord segments following intrapleural injection (Fig. 3), consistent with the location of intercostal motoneurons (Zhan et al., 2000). Similar to ventral horn neurons found in the cervical spinal cord segments, labeled neurons in the thoracic ventral horn were organized in a medially-located column with morphological characteristics similar to those described for intercostal motoneurons (Zhan et al., 2000), and were thus likely motoneurons. Labeled neurons were not found in any other spinal cord regions outside of the ventral horn in cervical and thoracic segments. However, some neurons were also labeled in cervical and thoracic dorsal root ganglia (Fig. 4), consistent with retrograde transport of cholera toxin subunit B via the phrenic and intercostal nerves. Labeling of dorsal root ganglia neurons was relatively sparse, limited primarily to a subset of larger neurons.

3.2. Comparison of motoneuron labeling via intrapleural injection vs. direct application to the transected phrenic nerve

Consistent with previous reports (Goshgarian and Rafols, 1981; Lindsay et al., 1991), phrenic nerve dip (Fig. 1A) resulted in extensive labeling of phrenic motoneurons. Motoneurons were found in clusters, generally corresponding with each spinal cord segment from C3 to C6. Although individual motoneurons within each cluster were sometimes difficult to identify, up to ~240 phrenic motoneurons could be labeled ipsilateral to the phrenic nerve dip (Table 1),

consistent with previous estimates of phrenic motoneuron numbers in the adult rat (Goshgarian and Rafols, 1981; Lindsay et al., 1991). Importantly, phrenic motoneurons were labeled exclusively unilaterally after nerve transection. Ipsilateral intrapleural injection resulted in largely overlapping labeling of phrenic motoneurons when compared to direct application to the transected nerve (Fig. 4). In all cases ($n = 499$ neurons), cervical ventral horn neurons labeled by intrapleural injection (Alexa 488) were co-labeled by rhodamine dextran applied directly to the transected phrenic nerve. Over 90% of phrenic motoneurons labeled by direct tracer application to the phrenic nerve were labeled by intrapleural injection. In addition, neurons were co-labeled throughout the ventral spinal cord segments corresponding to the phrenic motor nucleus, with no evidence of compartmentalization of labeling across segments with either method of tracer injection.

When unilateral phrenicotomy immediately preceded bilateral intrapleural injection of cholera toxin subunit B (Fig. 1B), no labeling was observed in the spinal cord ventral horn segments ipsilateral to the phrenicotomy (Fig. 5), while labeling was present on the contralateral side (not shown). These results are consistent with the exclusively unilateral innervation of the diaphragm muscle by cervical spinal cord segments, and indicate that intrapleural labeling of motoneurons using cholera toxin subunit B depends on transport along the phrenic nerve. Thoracic intercostal motoneurons were still labeled by intrapleural tracer injection in these animals but not by tracer application to the transected phrenic nerve, demonstrating appropriate tracer uptake (not shown).

3.3. Comparison of motoneuron labeling via intrapleural vs. intramuscular injection

Consistent with previous reports (Kinkead et al., 1998; Prakash et al., 2000), phrenic motoneurons were labeled reliably by multiple intramuscular injections into the diaphragm muscle (Fig. 1C). In general, ~180 phrenic motoneurons per side were labeled by intramuscular injection (Table 1). Following intramuscular injection, phrenic motoneuron clusters were labeled throughout the phrenic motor pool from C3 to C6. Motoneurons were labeled such that individual phrenic motoneurons were easily identified. Labeling of phrenic motoneurons by intrapleural injection consistently was more extensive when compared to intramuscular injection ($p < 0.05$), although the two groups were not completely overlapping: some neurons were labeled by only one dye. In 408 neurons examined, ~60% of phrenic motoneurons labeled by intrapleural injection were labeled by intramuscular injection and ~80% of neurons labeled by intramuscular injection were labeled by intrapleural injection. These results are consistent with selective and more widespread, albeit not complete, labeling of the phrenic motor nucleus by intrapleural injection, and sparser labeling by intramuscular injection.

4. Discussion

In the present study, we provide validation of a novel method for labeling phrenic motoneurons using intrapleural injection of fluorescently-conjugated cholera toxin subunit B. Labeling of phrenic motoneurons in the cervical spinal cord was verified by direct comparison to labeling via application to transected phrenic nerves (i.e., “nerve dip”) and intramuscular injection. Selective and extensive labeling of phrenic motoneurons within the cervical spinal cord was possible by intrapleural injection. Transport of fluorescently-conjugated cholera toxin subunit B via the intact phrenic nerve is necessary for phrenic motoneuron labeling since nerve transection completely prevented labeling by intrapleural injection. Consistent with previous studies providing detailed anatomical analyses of the phrenic motor nucleus using alternative tracer injections (Goshgarian and Rafols, 1981; Furicchia and Goshgarian, 1987; Lindsay et al., 1991; Kinkead et al., 1998; Yates et al., 1999; Prakash et al., 2000), phrenic motoneurons are arranged in columnar fashion in the medial aspect of the ventral horn in the cervical spinal cord (C3–C6), with an axis approximately parallel to the long axis of the spinal cord. Results

demonstrated co-staining of phrenic motoneurons by intrapleural injection vs. both intramuscular injection and direct application to transected nerves, and indicated widespread, near complete labeling of the phrenic motor nucleus by intrapleural injection. A similar pattern of motoneuron labeling was obtained following intrapleural injection in neonatal rats (postnatal day 7) and adult mice (data not shown).

Boulenguez et al. (2007) recently reported important differences in retrograde labeling of motoneurons depending on the tracer molecule used, suggesting that some tracers may lack selectivity in retrograde labeling of motoneurons. The number of phrenic motoneurons labeled by intrapleural injection of cholera toxin subunit B was similar to that previously reported in the rat by our laboratory using phrenic nerve dip (Prakash et al., 1993, 1994). In fact, we found near complete concordance of labeling by intrapleural injection of cholera toxin subunit B followed by nerve dip of rhodamine-conjugated dextrans. This extensive co-labeling of phrenic motoneurons is consistent with the fact that the number of phrenic motoneurons labeled by intrapleural cholera toxin subunit B injection was also comparable to that reported by other laboratories using nerve dip of other retrograde tracers including dextrans, wheat germ agglutinin, and fluorescent dyes (Furicchia and Goshgarian, 1987; Gordon and Richmond, 1990; Prakash et al., 2000; Boulenguez et al., 2007). Together, these results suggest that there are no differences in retrograde labeling of phrenic motoneurons based on the tracers used in this study.

In contrast to phrenic nerve dip, the number of phrenic motoneurons labeled by diaphragm intramuscular injection of rhodamine-conjugated dextran was less than that labeled by intrapleural injection of cholera toxin subunit B. These results are consistent with our previous studies where we found less extensive labeling of phrenic motoneurons using diaphragm intramuscular injection of fluorescent dextrans or cholera toxin subunit B (Kinkead et al., 1998; Prakash et al., 2000; Zhan et al., 2000). Thus, if sampling of phrenic motoneurons is an issue, e.g., laser capture microdissection studies examining phrenic motoneuron mRNA expression, the intrapleural labeling technique would be preferred. In such studies, the only alternative would be phrenic nerve dip; however, axotomy itself may influence mRNA expression in motoneurons (MacLennan et al., 1999).

The demonstration of retrograde transport following intrapleural injection raises the possibility of applying this technique for purposes other than retrograde identification of phrenic motoneurons. For example, intrapleural injection may be used to target siRNA constructs or viral vectors to phrenic motoneurons by retrograde transport, with possibly much greater yields than with intramuscular or intraneural approaches. Manipulation of protein expression in phrenic motoneurons has a variety of potential applications, not least of which is manipulation of motoneuron excitability under conditions of hypoventilation.

In addition to phrenic motoneurons, thoracic intercostal motoneurons were labeled. While this technique could be used to label intercostal motoneurons, it would be difficult to unambiguously distinguish between motor pools innervating internal, middle or external intercostal muscles (De Troyer and Farkas, 1989; Zhan et al., 2000). However, it is likely that motoneurons innervating internal intercostal muscles are more extensively labeled, and that motoneurons innervating external intercostal muscles are labeled less extensively. The extent of labeling of intercostal motoneurons by intrapleural injection was not systematically evaluated as in the case of phrenic motoneurons. Therefore, it is difficult to assess the utility of this method for labeling intercostal motoneurons.

It is very important to note that other than motoneurons in the ventral horn, no other neurons in the spinal cord were labeled following intrapleural injection of cholera toxin subunit B. However, some neurons in cervical and thoracic dorsal root ganglia were also labeled by

intrapleural injection. Labeled neurons in dorsal root ganglia were mainly larger neurons, likely as a result of differential ganglioside expression (Gong et al., 2002). Cholera toxin subunit B binds to GM1 gangliosides which are present in both motor and sensory neurons (Wolf et al., 1998). Taken together, these results are consistent with lack of trans-synaptic transport of cholera toxin subunit B. Some retrograde tracers are trans-synaptically transported (e.g., tetanus toxin fragment C, pseudorabies and rabies virus). Thus, if these retrograde tracers were injected intrapleurally, the possibility of trans-synaptic labeling of neurons in the central sensory pathway and interneurons would have to be considered. Indeed, this may limit the usefulness of intrapleural injection of these trans-synaptic labeling compounds.

5. Conclusions

In the present study, we provide validation of a novel and simple method for labeling phrenic motoneurons using intrapleural injection of fluorescently-conjugated cholera toxin subunit B. This method does not require anesthesia, surgery or phrenic nerve transection, and thus may be ideally suited for analyses of mRNA expression in isolated phrenic motoneurons in applications such as laser capture microdissection since extensive, direct labeling is possible.

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Figure 1.

Schematic depicting experimental setup for validation of intrapleural labeling of phrenic motoneurons. Cholera toxin B (green arrows) was injected intrapleurally. Animals were assigned to 3 groups. **A.** Unilateral phrenic nerve dip with dextran conjugate was performed 3 days after bilateral intrapleural injection of cholera toxin B (green arrows). **B.** Unilateral phrenic nerve dip immediately preceded bilateral intrapleural injection. **C.** Dextran conjugate (red arrows) was injected into the diaphragm muscle at the time of intrapleural injection. The time line for these injections is shown at the bottom. Tissues were collected 3 days after the last injection. See text for details.

Figure 2.

A. Longitudinal section of the cervical spinal cord showing labeling of motoneurons by intrapleural injection of Alexa 488-conjugated cholera toxin subunit B (green) and phrenic nerve dip labeling with rhodamine-conjugated dextran (red). Bar represents 100 μm . Image is a montage of individual confocal stacks, oriented in cephalad to caudad direction from top to bottom. Insert: Representative higher magnification merged image of representative motoneurons from box in A showing extensive co-labeling (yellow). Bar represents 30 μm .

B. Longitudinal section of the cervical spinal cord showing labeling of motoneurons by intrapleural injection of Alexa 488-conjugated cholera toxin subunit B (green) and diaphragm intramuscular injection with rhodamine-conjugated dextran (red). Bar represents 30 μm .

Figure 3.

Longitudinal section of the thoracic spinal cord showing labeling of motoneurons by intrapleural injection of Alexa 488-conjugated cholera toxin subunit B. Image is a montage of individual confocal stacks, oriented in cephalad to caudad direction from left to right. Bar represents 30 μm .

Figure 4. Representative image of cervical dorsal root ganglia showing labeling of few sensory neurons by intrapleural injection of Alexa 488-conjugated cholera toxin subunit B (asterisks). Background autofluorescence was collected to obtain an outline of the dorsal root ganglion in gray. Bar represents 30 μ m.

Figure 5.

Longitudinal section of the cervical spinal cord showing labeling of phrenic motoneurons via phrenic nerve dip with tetramethylrhodamine-conjugated dextran (A; red). No labeling by intrapleural injection occurred ipsilateral to the phrenic nerve dip (B; green) if phrenic nerve transection immediately preceded intrapleural injection. Bar represents 100 μm .

Table 1

Retrograde labeling of phrenic motoneurons by different techniques.

Intraleural (n=6)	Phrenic nerve dip (n=4)	Intramuscular (n=6)
224 ± 21 *	242 ± 20 *	178 ± 23

n, indicates number of animals used for total motoneuron counts on one side of the cervical spinal cord (mean ± standard deviation).

* indicates statistically significant difference from intramuscular technique (ANOVA; $F_{15,2} 11.9$, $p=0.0012$). *Post hoc* comparisons performed using Tukey-Kramer's HSD test ($p<0.05$).