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Retrograde labeling of phrenic motoneurons by intrapleural injection

Carlos B. Mantilla*, Wen-Zhi Zhan, Gary C. Sieck

Departments of Physiology & Biomedical Engineering, and Anesthesiology, Mayo Clinic, USA

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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.

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 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.

* Corresponding author at: Jo 4W-484F, 200 First St SW, Rochester, MN 55905. Tel.: +1 507 255 7481; fax: +1 507 255 7300.
E-mail address: mantilla.carlos@mayo.edu (C.B. Mantilla).

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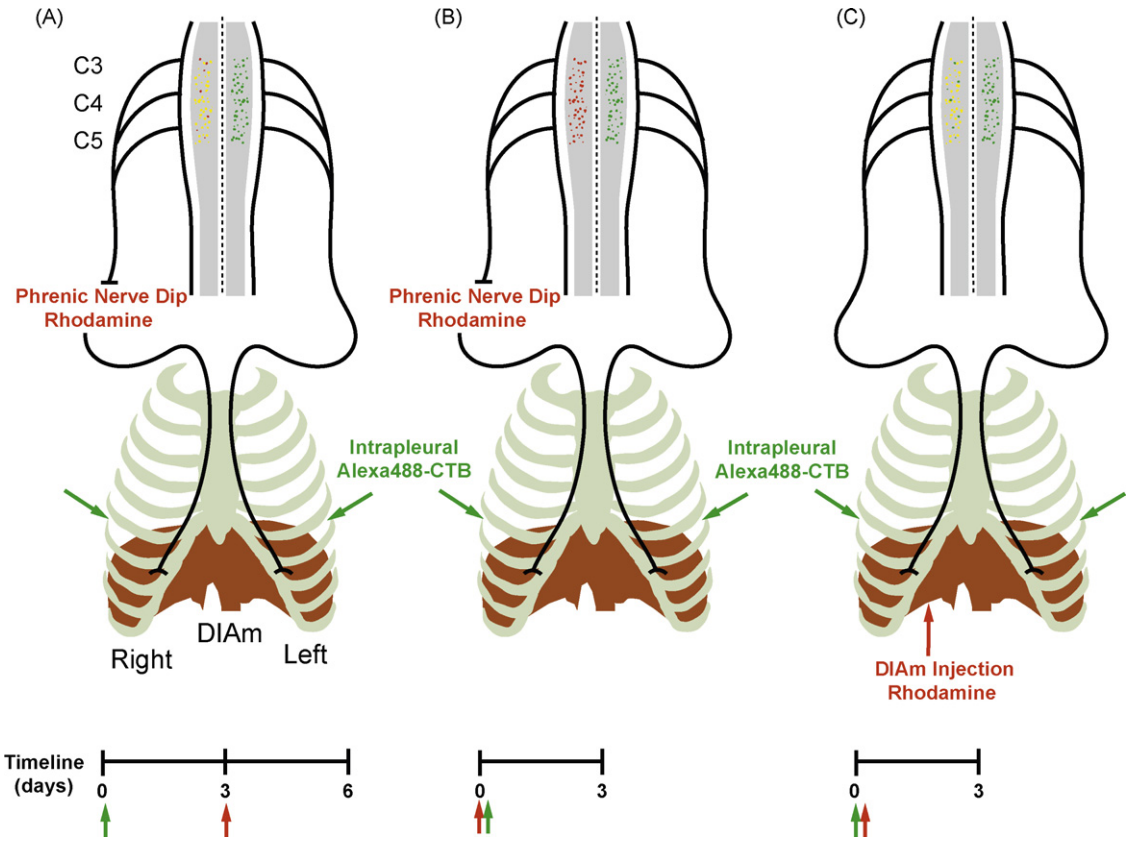

Fig. 1. Schematic depicting experimental setup for validation of intrapleural labeling of phrenic motoneurons. Cholera toxin B (green arrows) was injected intrapleurally. Animals were assigned to 3 groups. (A) Unilateral phrenic nerve dip with dextran conjugate was performed 3 days after bilateral intrapleural 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.

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, 2000; Kinkead et al., 1998) 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-μl Hamilton syringe was used to inject 15–20 μl 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 desheathed and immersed in a microdish containing ~5 μl 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.