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The phrenic motor nucleus in the adult mouse

K. Qiu¹, M.A. Lane², K.Z. Lee¹, P.J. Reier², and D.D. Fuller¹

¹ University of Florida, College of Public Health and Health Professions, Department of Physical Therapy, PO Box 100154, 100 Newell Dr Gainesville, FL 32610

² University of Florida, College of Medicine, McKnight Brain Institute, Department of Neuroscience, PO Box 100244, 100 Newell Dr Gainesville FL 32610

Abstract

The present study was performed to establish an anatomical context for studies of phrenic motor function in mouse models of central nervous system trauma and disease. Application of cholera toxin β -subunit to the diaphragm of adult C57BL/6 mice revealed a columnar organization of phrenic motoneurons (PhMNs) which extended from rostral C3 to C6. Injection of Miniruby into the ventrolateral medulla revealed decussating, anterogradely labeled axons in the cervical spinal cord. In addition, application of the transneuronal tracer pseudorabies virus (PRV) to the right hemidiaphragm demonstrated a population of putative pre-phrenic interneurons at the level of the infected PhMN pool. These neuroanatomical features of the mouse phrenic nucleus are consistent with those described in other species and provide a foundation for studies of neuroplasticity and repair in relation to a functionally and anatomically identified spinal network.

Phrenic motoneurons (PhMNs) innervate the diaphragm (i.e., the primary inspiratory muscle of the body), and impaired phrenic motor function is commonly associated with respiratory insufficiency and morbidity (for review, see Lane et al. 2008a). Although the phrenic motor nucleus has been described in species ranging from rat to man, the overall distribution and organization of PhMNs in mice has not been well documented. With the exception of one prior study (Gaytan et al. 2002), which focused primarily on medullary circuitry, little effort has been made to examine the spinal circuitry innervating the diaphragm in adult mice. Given the increasing popularity of mouse models for genomic studies of circuit development and neuronal composition (Grillner et al. 2009), a more detailed description of phrenic neuroanatomy is essential. The mouse has also been recently shown to be amenable to studies of phrenic motor control, respiratory-related diseases and neurological injury (Minor et al. 2006; Stettner et al. 2007; DeRuisseau et al. 2009; Seeds et al. 2009). Therefore, the primary purpose of the current work was to characterize the phrenic circuit in adult mice using antero- and retrograde transneuronal tracing methods (Lane et al. 2008b).

Experiments were approved by the University of Florida Institutional Animal Care and Use Committee. C57BL/6 mice purchased from Harlan Inc. (Indianapolis, IN, USA) were used in all experiments. Cholera toxin β subunit (CT- β ; Biological Laboratories, Inc), and Pseudorabies virus (PRV; PRV152, Bartha strain), were used to retrogradely trace circuitry associated with the diaphragm. These methods were adapted from our published techniques in rats (Lane et al. 2008b; Lane et al. 2009).

Corresponding Author: David D. Fuller, Ph.D., Phone: (352) 273 6634, Fax: (352) 273 6109, ddf@php.ufl.edu.

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Mice were anesthetized with 2–3% isoflurane. After a midline abdominal incision the abdominal walls were incised and either CT- β (0.2% in 0.1M PBS; N=20) or PRV (8.0–9.9 $\times 10^8$ pfu/ml; N=9) were topically applied to the inferior surface of the diaphragm. A total volume of 20 μ l was distributed evenly over the diaphragm for CT- β (i.e. 10 μ l per side); for PRV experiments the 20 μ l volume was applied only to the right side of the diaphragm. The abdominal muscles were sutured and the skin was closed. Additional experiments were conducted to anterogradely trace bulbospinal projections as previously described (Lane et al. 2008b). Mice were placed in a Kopf stereotaxic frame and an incision was made at the base of the skull to expose the caudal medulla. A Carbostar-3 micropipette/electrode (Kation Scientific) filled with Miniruby (10,000 kDa biotin dextran amine conjugated with tetramethylrhodamine, 10% w/v in sterile saline) was connected to an extracellular amplifier (ExAmp 20KD, Kation Scientific) for recording neural activity. The electrode was lowered into the ventral lateral medulla in the region of the ventral respiratory column (VRC) (Gaytan et al. 2002) using a micro-manipulator. Once phasic activity that correlated with inspiration was detected, the pipette was connected to an iontophoretic device and Miniruby was delivered to the VRC over 15 minutes (5.5mA, alternating 7 seconds on, 7 seconds off). Following all surgical procedures, mice received buprenorphine (0.1 mg/kg, s.q.).

Spinal cord tissue samples were obtained 24–64 hours following PRV application and 24–72 hours following CT- β . Animals were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and then euthanized by transcardial perfusion (50 ml 0.9% saline followed by 200 ml 4% paraformaldehyde). Retrogradely labeled spinal neurons were subsequently visualized in transverse or horizontal 40 μ m vibratome sections. For horizontal sectioning and identification of the rostral-caudal extent of the PhMN pool, a pin hole was made in fixed specimens at each cervical dorsal root entry level from C2 to C8 (Goshgarian and Rafols 1981).

The distribution of CT- β and PRV was demonstrated immunohistochemically in vibratome sections (Lane et al. 2008b). Sections were washed in PBS (0.1M, pH 7.4), blocked against endogenous peroxidase activity (30% methanol, 0.6% hydrogen peroxide), and blocked against nonspecific protein labeling (10% normal serum PBS containing 0.03% Triton X-100). The sections were then incubated at 4°C overnight with primary antibodies against CT- β (polyclonal goat anti-serum to purified CT- β , List Biological Laboratories, 1:10,000) or PRV (rabbit anti-PRV; R b133/134 provided by Dr. Lynn Enquist; 1:10,000). Sections were subsequently incubated at room temperature in a biotinylated secondary antibody (donkey anti-goat or donkey anti-rabbit IgG, Jackson Immunochemicals; 1:200). Finally, tissues were incubated in an avidin-biotin complex and then reacted with 3,3'-diaminobenzidine tetrahydrochloride (DAB). The distribution of Miniruby labeled fibers was examined by staining for the presence of conjugated biotin (using the avidin-biotin-peroxidase method). Brightfield photographs were taken with a Zeiss AxioPhot microscope equipped with an AxioCam HRc digital camera. The total number of CT- β labeled PhMNs were counted on one side of horizontal sections in five mice (e.g. Fig. 1A, B). Cell counts were made on serial horizontal sections (40 μ m thick). Only those labeled neurons which displayed a nucleus were counted and an Abercrombie correction was used (Guillery et al. 1997; Lane et al. 2008b). The total number of cells was expressed as mean $\pm$ SEM.

CT- β labeling showed that PhMNs were distributed in a discontinuous column approximately 100 μ m wide which extended from rostral C3 to C6 (Fig. 1A–B). The phrenic nucleus was tangentially oriented (Goshgarian and Rafols 1981) and gradually shifted from a lateral to medial position in the ventral horn (Fig. 1B). The greatest density of labeled PhMN cell bodies was observed at C3–4 (Fig. 1B, C). Gaps between labeled neurons along the rostral-caudal axis were more prevalent at caudal levels (Fig. 1B). At the C3–4 segments, the phrenic motor nucleus could be found at a depth of approximately 0.9–1.0 mm from the dorsal surface of the spinal cord, and 0.6–0.8 mm lateral to the spinal midline (Fig. 1C–D). These general coordinates are

useful guidelines for targeted drug or gene delivery to the phrenic motor nucleus in adult mice (Fuller et al. 2010). CT- β was delivered to both sides of the diaphragm, and PhMN cell counts were done on the side with the most robust labeling. On average, we observed a total of 191 ± 9 labeled PhMN neurons (range of 182–205). The average somal area of cells was $667 \pm 123 \mu\text{m}^2$ (range 424–898 μm^2).

The majority of PhMN dendrites were organized rostrocaudally along the boundaries of the phrenic nucleus (Fig. 1A). The dendritic field of adjacent PhMNs overlapped extensively, and some dendrites extended medially and laterally (Figs. 1A, 2A). However, no PhMN dendritic arborizations were observed crossing the spinal midline.

Mice were sacrificed at 24–64 hours following application of PRV to the diaphragm. In a previous study in rats (Lane et al. 2008b), we reported that following diaphragm PRV application, a population of cervical interneurons (INs) became infected with PRV after PhMN infection, but before infection of brainstem neurons. We concluded that these INs were “pre-phrenic” and provided synaptic innervation to PhMNs (Duffin et al. 1996; Tian et al. 1996; Lane et al. 2008b; Lane et al. 2009). In the present study, no PRV-positive neurons were observed in the spinal cord at 24 hrs post diaphragm application ($N=2$, not shown). By 37–42 hours ($N=2$), 1st order infection of PhMNs was readily apparent (Fig. 2A). However, at this time point, the PhMN labeling was relatively sparse with substantial gaps between labeled neurons (Fig. 2A). At this same time point post-PRV delivery, a few labeled neurons were observed within lamina VII and X of the cervical spinal cord (INs) and in the brainstem reticular formation (not shown), but no labeling was noted in the region of the ventral respiratory column (VRC, Gaytan et al. 2002). When tissues were harvested at 55–64 hours ($N=5$), there was more robust labeling of cell bodies in the phrenic motor nucleus (Fig. 2C). In addition, labeling of cervical INs and medullary neurons was seen. The cervical INs were localized to lamina X, VII and IX (Fig. 2B, 2D), and brainstem neurons were seen throughout the ventral medulla with robust labeling in the midline reticular formation and some PRV-positive neurons in the region of the VRC (not shown). Collectively, the PRV data are consistent with the PhMN labeling patterns seen with CT- β (Fig. 1), and also indicate the presence of cervical INs with potential connections to PhMNs in gray matter regions where CT- β positive neurons were never seen.

In our final experiments, the anterograde tracer Miniruby was iontophoretically injected into the ventrolateral medulla at sites where rhythmic inspiratory bursting could be detected (Lane et al. 2008b). Histological analyses of cervical spinal tissues (C3–C4) from these mice revealed projections that terminated in the contralateral ventral gray matter (laminae IX) in the vicinity of putative PhMNs (Fig. 2E). Projections were also observed in lamina VII and X (Fig. 2F). In all mice with successful tracer delivery ($N=3$), projections that spanned the spinal midline were seen anterior (Fig. 2G) and posterior (not shown) to the central canal.

These collective neuroanatomical findings are consistent with previous descriptions of the phrenic nucleus in the rat and other species (see Ullah 1978; Goshgarian and Rafols 1981, 1984; Takahashi et al. 1985). While there is some variability in the reported rostral-caudal distribution of PhMNs, most studies have identified PhMNs between C3–C6/7 (Mitchell et al. 1956; Ullah 1978; Takahashi et al. 1980; Goshgarian and Rafols 1981; Takahashi et al. 1985; Johnson et al. 1988; Mantilla et al. 2009). Another consistent finding has been the “clustering” of PhMN cell bodies within the phrenic motor nucleus (e.g. Fig. 1A) (Goshgarian and Rafols 1981, 1984; Berger et al. 1984; Furicchia et al. 1987). We did note, however, that the rostral pole of the phrenic nucleus extends slightly more rostrally in the C57BL/6 mouse. In addition, we consistently observed that the greatest density of labeled neurons occurred in the C3–C4 spinal cord (Fig. 1), whereas the highest density of PhMNs appears to be C4–C5 in the rat (Goshgarian and Rafols 1981, 1984).

One of the more striking and consistently reported features of the PhMN pool is the predominant rostral-caudal “bundling” of dendritic projections (Goshgarian and Rafols 1981; Berger et al. 1984; Takahashi et al. 1985; Johnson et al. 1988). Our data from the adult mouse are consistent with these prior descriptions (i.e. Fig. 1A). Some radially-oriented dendrites were also seen extending toward lateral and medial white matter. However, consistent with the adult rat, none were observed extending across the spinal midline. Crossed PhMN dendrites are present in neonatal rats (Lindsay et al. 1991; Prakash et al. 2000; Zimmer et al. 2005), but are extremely rare in adult rats (Prakash et al. 2000).

The total number of CT- β labeled PhMNs (~ 200) in our study was considerably lower compared to observations in other species (i.e. ~ 300–700 PhMNs) (Webber et al. 1979; Goshgarian and Rafols 1981; Berger et al. 1984; Johnson et al. 1988; Boulenguez et al. 2007). There are at least two explanations for this discrepancy. First, it is possible that the total number of PhMNs is in fact considerably less in the adult mouse spinal cord. Equally possible, however, is that this particular approach of applying retrograde tracer to the diaphragm failed to label the entire phrenic motor pool. Intrapleural delivery of conventional retrograde tracers may represent one alternative approach for future studies (Mantilla et al. 2009).

As illustrated by our combined anterograde tracing of bulbospinal VRC projections in conjunction with transneuronal PRV tracing in the mouse and rat (Lane et al. 2008), the phrenic motor system offers important experimental opportunities to explore mechanisms of spontaneous and therapeutically induced recovery in the context of a functionally and anatomically identifiable neural substrate. Our findings assume added significance in light of recent studies that have demonstrated respiratory plasticity in mice (Minor et al. 2006; Minor et al. 2008; Seeds et al. 2009) similar to the “crossed phrenic phenomenon” (reviewed in Goshgarian 2003) in rats and other species. Our anterograde tracing results confirm the existence of crossed spinal projections from the ventral medulla to the cervical spinal cord in adult mice, as suggested by Minor and colleagues (Minor et al. 2006), which are thought to be the primary substrate mediating spontaneous recovery of PhMN function ipsilateral to C2 hemisections.

Several studies have provided correlative evidence for polysynaptic descending inputs to PhMNs which involves cervical INs (Dobbins et al. 1994; Lane et al. 2008b, 2009). In addition, recent evidence raises the possibility of a cervical IN relay as part of the ipsilateral phrenic recovery process following C2 hemisection injury in rats (Lane et al. 2008; Lane et al. 2009; Sandhu et al. 2009). While more detailed neurophysiological confirmation of whether cervical interneurons could serve a similar role in mice is not yet available, the transynaptic tracing in the present study is evidence for pre-phrenic cervical INs in the adult mouse spinal cord. The existence of these cells in a number of species, and the possibility of their involvement in human respiratory circuitry (Butler et al. 2007; Murray et al. 2010), warrants more in depth analysis of their role in modulating phrenic function normally and following respiratory dysfunction.

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Figure 1. Representative examples of CT- β labeling of PhMNs

Panel **A** shows a longitudinal tissue section from the C3 spinal cord illustrating the primarily rostrocaudal orientation of PhMN dendrites. Panel **B** shows montages of longitudinal tissue sections through the entire cervical spinal cord. The approximate locations of the cervical dorsal roots are indicated by the arrows. The small schematic diagrams of transverse spinal cord sections demonstrate the approximate horizontal level at which each image was obtained. Transverse spinal cord sections are depicted from C3 (panel **C**) and C5 (panel **D**). Scale bars: 50 μ m in A; 100 μ m in B; 200 μ m in C–D.

Figure 2. Representative examples of PRV and Miniruby labeling in the cervical spinal cord

These tissue sections were obtained at specific intervals after PRV delivery to the diaphragm. Longitudinal sections are shown in Panels **A** (C3–C4, 37 hours) and **C** (C3–C4, 55 hours) and **D** (C3, 60 hours, * indicates location of central canal). A transverse section is shown in Panel **B** (C3, 60 hours). Following delivery of Miniruby to the medulla, anterogradely labeled axons (arrows) were observed in transverse sections of the cervical spinal cord at the level of PhMNs (**E**), near the central canal (**F**), and crossing the spinal midline (**G**). CC: central canal. Scale bars: 50 μ m in A–F; 100 μ m in G.