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## Functional and morphological assessment of diaphragm innervation by phrenic motor neurons --Manuscript Draft--

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<b>Abstract:</b>	In this protocol, we specifically focus on tools to assess phrenic motor neuron (PhMN) innervation of the diaphragm at both the electrophysiological and morphological levels. Compound muscle action potential (CMAP) recording following phrenic nerve stimulation can be used to quantitatively assess functional diaphragm innervation by PhMNs of the cervical spinal cord in vivo in anesthetized rats and mice. Because CMAPs represent simultaneous recording of all myofibers of the whole hemi-diaphragm, it is useful to also examine the phenotypes of individual motor axons and myofibers at the diaphragm NMJ in order to track disease- and therapy-relevant morphological changes such as partial and complete denervation, regenerative sprouting and reinnervation. This can be accomplished via whole-mount immunohistochemistry (IHC) of the diaphragm, followed by detailed morphological assessment of individual NMJs throughout the muscle. Combining CMAPs and NMJ analysis provides a powerful approach for quantitatively studying diaphragmatic innervation in rodent models of CNS and PNS disease.
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Re: Manuscript Submission - *Journal of Visualized Experiments*

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JoVE Editorial Board:

Enclosed for review at *JoVE* is the manuscript entitled "Functional and morphological assessment of diaphragm innervation by phrenic motor neurons."

In this protocol, we specifically focus on tools to assess phrenic motor neuron (PhMN) innervation of the diaphragm at both the electrophysiological and morphological levels. Compound muscle action potential (CMAP) recording following phrenic nerve stimulation can be used to quantitatively assess functional diaphragm innervation by PhMNs of the cervical spinal cord in vivo in anesthetized rats and mice. Because CMAPs represent simultaneous recording of all myofibers of the whole hemi-diaphragm, it is useful to also examine the phenotypes of individual motor axons and myofibers at the diaphragm NMJ in order to track disease- and therapy-relevant morphological changes such as partial and complete denervation, regenerative sprouting and reinnervation. This can be accomplished via whole-mount immunohistochemistry (IHC) of the diaphragm, followed by detailed morphological assessment of individual NMJs throughout the muscle. Combining CMAPs and NMJ analysis provides a powerful approach for quantitatively studying diaphragmatic innervation in rodent models of CNS and PNS disease including traumatic spinal cord injury and amyotrophic lateral sclerosis (ALS).

Dr. Wright (co-corresponding author) and I have extensive expertise in diaphragm electrophysiology and diaphragm NMJ morphological analysis, respectively. Melanie Martin (a student in Dr. Wright's lab) and Ke Li (a post-doctoral fellow in my lab) put together this manuscript and will take part in the filming of the protocol.

I have been in contact with Nandita Singh about this *JoVE* submission.

I look forward to the reviewer comments. If there are any questions I can answer, please feel free to contact me.

Sincerely,

Angelo C. Lepore, Ph.D.

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Compound muscle action potential, phrenic motor neuron, phrenic nerve, diaphragm, innervation, denervation, sprouting, neuromuscular junction, NMJ, spinal cord injury, SCI, amyotrophic lateral sclerosis, ALS, respiratory function

**Short Abstract:**

Compound muscle action potential recording quantitatively assesses functional diaphragm innervation by phrenic motor neurons. Whole-mount diaphragm immunohistochemistry assesses morphological innervation at individual neuromuscular junctions. The goal of this protocol is to demonstrate how these two powerful methodologies can be used in various rodent models of spinal cord disease.

**Long Abstract:**

This protocol specifically focuses on tools for assessing phrenic motor neuron (PhMN) innervation of the diaphragm at both the electrophysiological and morphological levels. Compound muscle action potential (CMAP) recording following phrenic nerve stimulation can be used to quantitatively assess functional diaphragm innervation by PhMNs of the cervical spinal cord *in vivo* in anesthetized rats and mice. Because CMAPs represent simultaneous recording of all myofibers of the whole hemi-diaphragm, it is useful to also examine the phenotypes of individual motor axons and myofibers at the diaphragm NMJ in order to track disease- and therapy-relevant morphological changes such as partial and complete denervation, regenerative sprouting and reinnervation. This can be accomplished via whole-mount immunohistochemistry (IHC) of the diaphragm, followed by detailed morphological assessment of individual NMJs throughout the muscle. Combining CMAPs and NMJ analysis provides a powerful approach for quantitatively studying diaphragmatic innervation in rodent models of CNS and PNS disease.

**Introduction:**

Amyotrophic Lateral Sclerosis (ALS) is a debilitating motor neuron disease associated with the loss of both upper and lower motor neurons and consequent muscle paralysis. Upon diagnosis, patient survival is on average only 2-5 years<sup>1</sup>. Phrenic motor neuron (PhMN) loss is a critical component of the pathogenesis of ALS. Patients ultimately die due to loss of PhMN innervation of the diaphragm, the primary muscle of inspiration<sup>2,3</sup>. Traumatic spinal cord injury (SCI) is also a serious problem with associated breathing difficulties. Approximately 12,000 new cases of SCI occur each year<sup>4</sup> due to traumatic damage to the spinal cord. Despite disease heterogeneity with respect to location, type and severity, the majority of SCI cases involve trauma to the cervical spinal cord, which often results in debilitating and persistent respiratory compromise. In addition to ALS and SCI, other central nervous system (CNS) diseases can be associated with diaphragmatic respiratory dysfunction<sup>5,6</sup>.

The phrenic nerve is an efferent motor nerve that innervates the ipsilateral hemi-diaphragm and that originates from PhMN cell bodies located in the C3-C5 levels of the ipsilateral cervical spinal cord. PhMN output is controlled by descending bulbospinal input from the brainstem in an area known as the ventral respiratory group (VRG) <sup>7</sup>. The VRG-PhMN-diaphragm circuit is central to the control of inspiratory breathing, as well as other non-ventilatory diaphragm behaviors. Various traumatic injuries and neurodegenerative disorders that affect this circuitry can lead to a profound decline in respiratory function and patient quality of life. Descending input to PhMNs from the VRG, PhMN survival, phrenic nerve integrity and proper innervation at the diaphragm neuromuscular junction (NMJ) are all necessary for normal diaphragm function. It is therefore important to employ techniques that can quantitatively evaluate this circuit *in vivo* in rodent models of ALS, SCI and other CNS diseases.

With this protocol, the goal is to describe experimental tools for assessing PhMN innervation of the diaphragm at both the electrophysiological and morphological levels. Compound muscle action potentials (CMAPs) are recorded by stimulating all efferent motor neuron axons of a given motor nerve and then analyzing the elicited depolarization response of the target myofibers. This technique can be used *in vivo* in anesthetized rats and mice to quantify functional innervation of the hemi-diaphragm by PhMNs <sup>8</sup>. Due to the fact that CMAPs represent simultaneous recording of all (or at least many/most) myofibers of the whole hemi-diaphragm, it is useful to also examine the phenotypes of individual motor axons and myofibers at the diaphragm NMJ in order to track disease- and therapy-relevant morphological changes such as partial and complete denervation, regenerative sprouting and reinnervation. This can be accomplished via whole-mount immunohistochemistry (IHC) of the diaphragm, followed by detailed morphological assessment of individual NMJs throughout the muscle <sup>9</sup>. Combining CMAPs and NMJ analysis provides a powerful approach for quantitatively studying diaphragmatic innervation in rodent models of CNS and PNS disease.

## **Protocol:**

Experimental procedures were approved by the Thomas Jefferson University institutional animal care and use committee and conducted in compliance with the European Communities Council Directive (2010/63/EU, 86/609/EEC and 87-848/EEC), the NIH Guide for the care and use of laboratory animals, and the Society for Neuroscience's Policies on the Use of Animals in Neuroscience Research.

### **1. Compound Muscle Action Potentials (CMAPs)**

#### **1.1. Preparing the animal**

1.1.1. Anesthetize the rat using an inhalant (isoflurane) at 1-2% delivered to effect or by injection (ketamine, xylazine, acepromazine). Dosage of injectable anesthesia is as follows: 95.0 mg/kg of ketamine, 10.0 mg/kg of xylazine, 0.075 mg/kg of acepromazine.

**NOTE:** We successfully use both inhalant and injectable anesthetic approaches when conducting this CMAP recording protocol, with no preference.

1.1.2. The animal must be maintained at the appropriate anesthetic depth before the surgery is started, through the conclusion of surgery, and until post-operative buprenorphine has taken effect. Confirm proper anesthetization by brief toe pinch to determine that a withdrawal response is not elicited. Additionally, test the corneal reflex with a cotton swab. Throughout the surgical procedure, determine that heart rate and respiratory rate have not increased, which suggests that anesthetic depth has become too light.

1.1.3. Apply a vet ointment on the animal's eyes to prevent dryness while under anesthesia.

1.1.4. The surgeon should wash her/his hands with a disinfectant (chlorhexidine scrub) before beginning surgery. The surgeon should wear sterile gloves, facemask, and gown or clean lab coat. All instruments should be washed and autoclaved before surgery. Add a sterilization indicator strip inside the instrument kit before autoclaving at the level of the instruments to verify that sterilization is complete. The surgeon should verify that the entire line on the strip is black before beginning surgery. Tape the outside of the box with indicating autoclave tape as well. If surgery is to be performed on several animals on the same day, clean the instruments between surgeries and sterilize in a glass bead sterilizer. If necessary, sterilize both ends of the instrument in the glass bead sterilizer by placing the handle of the instrument in the sterilizer for the appropriate time, removing the instrument with a sterile hemostat, cooling it in a sterile field and then placing the functional end of the instrument into the glass bead sterilizer for the appropriate period of time.

1.1.5. Once animal is anesthetized, place on surgical board with dorsal surface down and abdomen facing up so that tape can be used to secure animal's forelimbs to surgical board.

1.1.6. Shave ventral surface caudally beginning at the base of the skull, and ensure that the area around abdomen on either side of the midline is shaved depending on which hemi-diaphragm is being recorded. Apply povidone-iodine to the shaved surface of the skin.

## 1.2. Preparation for CMAP recordings

1.2.1. Following animal preparation, place ground electrode subcutaneously into tail (Figure 1).

1.2.2. Place the reference electrode subcutaneously into the contralateral lower abdominal region.

1.2.3. Place conductive gel onto self-adhesive surface strip or disk recording electrode (dimensions of surface strip electrode:  $0.5 \times 3$  cm), and then place surface electrode transversely along the unilateral costal margin.

1.2.4. Place stimulating electrodes transcutaneously 0.5 cm apart lateral to trachea and superior to the clavicle. Insert the electrodes approximately 1.0 cm deep through the skin. Secure the electrodes by hand so that their location does not move with subsequent stimulations.

**NOTE:** Angle stimulating electrodes  $90^\circ$  relative to the skin at the site of insertion.



### 1.3. Compound Muscle Action Potential (CMAP) recordings

1.3.1. Obtain electrophysiological recordings using a stimulator/amplifier followed by computer assisted data analysis.

1.3.2. The stimulus parameters of single pull stimulation should be 0.5 ms, 1.0 Hz supramaximal pulses. The amplitude of the stimulus should be between 6.0 and 8.0 mv (Figure 2A).

**NOTE:** Within an experiment, use a stimulus intensity with the same amplitude across animals. The objective is to maximize the response amplitude by using the minimal amplitude of stimulation. It is important to note that the initial response that occurs at the time of stimulation is a stimulation artifact. In addition, care should be taken to obtain a CMAP response that is preceded by a stable/flat baseline after the stimulation artifact, which is then followed by the rapid CMAP response. In order to obtain such a response, it may be necessary to reposition the stimulation and/or recording electrodes.

1.3.3. Wait 30 seconds between stimulations, and repeat 10 stimulations in a row to obtain the average response (Figure 2B-C). Measure the amplitude from baseline to peak (Figure 2A). Conduct phrenic nerve stimulation during the phase of the animal's respiratory cycle when the phrenic nerve/diaphragm is not activated. The procedure can be repeated on the same animal for the contralateral hemi-diaphragm.

1.3.4. After the final CMAP recording session, perfuse the animal if NMJ staining will be conducted. Conduct CMAP recordings repeatedly on the same animal approximately once every week.

1.3.5. Following survival CMAP recordings, allow animal to recover on a circulating warm water heating pad, and continuously monitor during recovery until awake and sternally recumbent. Do not return an animal that has undergone surgery to the company of other animals until fully recovered.

1.3.6. Once sternally recumbent, monitor the animal every 12 hours for the first 48 hours (once daily thereafter). If the animal appears to be dehydrated (flaccid skin, listlessness), administer fluids (lactated ringers solution; subcutaneously; 1-2 mL per injection, with the sites rotated; 2 times per day) until post-surgical weight and fluid loss stabilizes. Provide animals with softened food in small dishes during the acute post-operative period if necessary to encourage eating if they cannot reach the wire bar lid feeder.

1.3.7. Administer buprenorphine at a dose of 0.05 mg/kg before the end of surgery and then at 12 hour intervals for the first 24 hours (and dependent upon signs of pain / stress thereafter) via subcutaneous injection (site rotated). If signs of pain or distress are noticed thereafter, treat animals with buprenorphine. Signs indicative of pain / distress include lack of activity, vocalizations, lack of eating or drinking (dehydration), excessive weight loss, guarding, excessive gnawing or scratching at site of incision. Additional criteria used in determining pain and distress include unresponsiveness to extraneous stimuli, transient or unprovoked

vocalization, labored or abnormal breathing, persistent reddish-brown naso-ocular discharge, marked piloerection. Also monitor evidence of poor hygiene that would suggest ill animals. If animal is still observed to be in distress after 72 hours of treatment (according to the pain / stress criteria outline above, and after receiving the analgesic protocol described above) euthanize the animal.

## **2. Diaphragm neuromuscular junction (NMJ) analysis**

### **2.1. Animal dissection**

2.1.1. Administer the animal an overdose of injectable anesthesia (three times dose used above: ketamine at 285.0mg/kg, xylazine at 30.0mg/kg, acepromazine at 0.225 mg/kg; administered intraperitoneally) to minimize pain and distress. Following overdose, all animals should have a second method of euthanasia (thoracotomy) to ensure death. Death should be confirmed by observing cardiac and respiratory arrest and by noting the animal's fixed and dilated pupils.

2.1.2. Conduct a laparotomy with scalpel or dissection scissors extending the excision from the zyphoid process caudally along the midline. Carefully separate skin and connective tissue from underlying muscle using scalpel and forceps.

2.1.3. While pulling up on the zyphoid process, use dissection scissors to remove whole diaphragm, ensuring that the diaphragm remains attached to surrounding bone. This is important as it will prevent damage to the diaphragm muscle, will allow for successful dissection of the entire diaphragm and will aid in cleaning muscle for subsequent staining. **NOTE:** At this point, animal can be perfused if necessary.

### **2.2. Cleaning and staining of the diaphragm**

2.2.1. Pin down the whole dissected diaphragm, superior surface facing up, to silicone rubber in a glass 100 mm petri dish in 4% paraformaldehyde (made in 1% phosphate buffered saline - PBS) for 20 minutes using a stereomicroscope. Stretch the diaphragm to make it taut, and using insect pins, pin down the surrounding tissue to avoid pinning the diaphragm muscle itself (Figure 3).

2.2.2. Carefully clean off connective tissue from only the superior surface of muscle with #5 forceps and small scissors.

**NOTE:** Carry out all subsequent steps of this staining protocol slowly rocking at room temperature covered from light unless otherwise specified. Ensure that the diaphragm muscle is always completely submerged in liquid to avoid drying out.

2.2.3. Prior to staining, prepare all necessary reagents: 1X PBS, 2% bovine serum albumin (BSA)/PBS, 0.1 M glycine made in 2% BSA/PBS, 4% paraformaldehyde (PFA) in 1X PBS, and 0.2% TBP (0.2% Triton X100 in 2% BSA/PBS). Be sure to pre-cool methanol at -20°C.

2.2.4. Wash 3x for 10 minutes in 1x PBS.

2.2.5. Incubate in 0.1 M glycine (made in 2% BSA/PBS) for 30 minutes.

2.2.6. Incubate in RBTX (rhodamine-conjugated  $\alpha$ -bungarotoxin) solution for 15 minutes. Solution for RBTX is as follows: Rhodamine-conjugated alpha bungarotoxin 1:400 dilution in 1x PBS. **NOTE:** In this protocol, Rhodamine is used, but any fluorophore of choice can be used for staining.

2.2.7. Wash 3x for 10 minutes in 1x PBS.

2.2.8. In -20°C freezer, incubate with MeOH for five minutes. **NOTE:** Do not add MeOH before placing muscle in the freezer, and immediately remove solution following the incubation period.

2.2.9. Wash 3x for 10 minutes in 1x PBS.

2.2.10. Block in 0.2% TBP for 1 hour (0.2% Triton made in 2% BSA/PBS).

2.2.11. Incubate with primary antibody solution (in 0.2% TBP) at 4 °C overnight while rocking. Dilutions of antibodies as follows: SV2 (1:10) and SMI-312 (1:1000).

2.2.12. Wash 3x for 10 minutes with 0.2% TBP.

2.2.13. Incubate with secondary antibody using 1:100 dilution of FGM1 (FITC-conjugated goat anti-mouse IgG1) (made in 0.2% TBP) while rocking for 1 hour. **NOTE:** Protect from light for the rest of the protocol for staining.

2.2.14. Wash 3x for 10 minutes with 1x PBS

2.2.15. Re-clean surrounding connective tissue on the superior (see above) surface of muscle prior to mounting and coverslipping. **NOTE:** It is recommended to clean and then mount sample immediately after staining. However, if necessary, sample may be covered in 1x PBS, wrapped in parafilm to prevent evaporation and stored at 4°C for up to 1 week, with changing of PBS daily.

2.2.16. Upon mounting on glass slide, coverslip with non-hardset Vectashield. Seal edges of coverslip with clear nail polish to prevent drying out. Store slides lying flat in cardboard slide folders in -20°C for long term.

### 2.3. Analysis and Confocal Imaging

2.3.1. Quantitatively analyze the morphology of the stained and mounted hemi-diaphragm under 20X and 40X magnification using an upright epifluorescence microscope.

**NOTE:** Because the diaphragm is stained and analyzed in a whole-mount fashion, the muscle is quite thick. Consequently, most of the fluorescence staining is completely out of focus when a static image is taken using non-confocal fluorescence microscopy. For this reason, it is best to

conduct the NMJ analysis in real-time on the microscope as this allows you constantly focus “up” and “down” through the muscle to properly identify the morphology of each of the individual NMJs. Using this approach, one can easily follow the trajectory of individual motor axons as they move through the tissue in spatial relationship to the post-synaptic receptor clusters.

2.3.2. By measuring the ventral-to-dorsal length of the muscle, divide the muscle into three separate sections for analysis based on topographic innervation patterns from specific spinal cord levels (Figure 4H).

2.3.3. Beginning at the ventral region of the muscle and moving dorsally, analyze all en face NMJs located on surface muscle fibers. Do not analyze muscles fibers deeper than the first 3-4 fibers from the surface as interpretation may be difficult due to poorer antibody penetration (RBTX penetrates much deeper into the muscle due to the small size of the toxin compared to the larger antibodies used to label neurons).

2.3.4. Identify each NMJ as **intact** if the pre-terminal axon of the neuron is thick in diameter and all regions of the nerve terminal completely overlap with the underlying muscle acetylcholine receptors (AChRs).

2.3.5. Identify each NMJ as **partially denervated** if any region of the underlying muscle AChRs is unoccupied with corresponding nerve terminal.

2.3.6. Identify each NMJ as **completely denervated** if the muscle AChRs have no corresponding nerve terminal (it is also important to have other NMJs with labeled neurons in the same field of focus, so that one can be sure the lack of nerve terminal is not simply due to poor antibody penetration in that region of muscle).

2.3.7. Identify each NMJ as **multiply innervated** if there is more than one pre-terminal axon innervating an individual NMJ (this indicates that there has been some level of denervation and likely attempts of reinnervation at this junction).

2.3.8. Identify each NMJ as **thinly innervated** if the NMJ has complete overlap between nerve terminal and underlying AChRs but has a thin pre-terminal axon (this also is indicative of some level of denervation and attempts at reinnervation).

2.3.9. Conduct analysis for each of the three identified regions for all of the above NMJ categories for each animal. Average the data from each animal with results from other animals from the same experimental group. Approximately 200-300 junctions in rat hemi-diaphragm muscle and 150-200 in mouse hemi-diaphragm should be quantified in at least 3 (preferably 6) animals per experimental group.

2.3.10. Obtain high-resolution confocal images, used for publication, with a confocal microscope.

2.3.11. Obtain Z-stacks at 0.3  $\mu\text{m}$  step size for 20-40 $\mu\text{m}$  depths, and collect additional optical sections above and below each junction to ensure that the entire synaptic profile is included.

2.3.12. Use acquisition software to reconstruct z-series images into maximum intensity projections.

### **Representative results:**

Adult Sprague-Dawley rats received either laminectomy only (uninjured control) or unilateral hemi-contusion SCI at the C4 spinal cord level<sup>10-12</sup>. At 5 weeks post-surgery, peak CMAP amplitude recorded from the hemi-diaphragm ipsilateral to the laminectomy/injury site was significantly reduced in SCI rats (Figure 2C) compared to laminectomy-only control (Figure 2B). All NMJs in the hemi-diaphragm were completely intact in control non-diseased wild-type rats (Figure 4A, C). On the contrary, SOD1<sup>G93A</sup> rats (a rodent model of ALS) showed significant pathology at hemi-diaphragm NMJs (Figure 4B), including complete denervation (Figure 4G, arrowhead), partial denervation (Figure 4E, arrowhead; Figure 4G, NMJ on right), multiple innervation (Figure 4D) and thin pre-terminal axons (Figure 4F, arrowhead).

### **Figure legends:**

#### **Figure 1. CMAP electrode placement.**

The ground electrode is placed subcutaneously into the tail. The reference electrode is placed into the abdomen. The surface recording electrode is placed transversely along the unilateral costal margin. Stimulating electrodes are depicted as both red and black.

#### **Figure 2. CMAP recordings and analysis.**

(A) The amplitude of CMAP response can be measured from baseline to peak. A typical CMAP response should show both the stimulus artifact and response curve. (B-C) Representative average CMAP responses. Stimulation should be carried out at least ten consecutive times to obtain a repeated average of the response. Shown here are a laminectomy-only uninjured rat (B) and a comparison of CMAP responses for an injured rat that received a unilateral C4 hemi-contusion (C).

#### **Figure 3. Preparation of diaphragm for whole-mount staining.**

Insect pins should be placed into surrounding tissue to secure the hemi-diaphragm to Sylgard. Avoid placing pins into the muscle itself. In this image, both hemi-diaphragm muscles are shown and pinned down separately.

#### **Figure 4. NMJ phenotypes.**

Confocal imaging was conducted to visualize post-synaptic acetylcholine receptors (in red labeled with rhodamine-conjugated  $\alpha$  bungarotoxin) and axonal fibers and pre-synaptic terminals (in green labeled with SMI-32 and SV2 antibodies, respectively). All NMJs in hemi-diaphragm were completely intact in control non-diseased wild-type rats (A, C). On the contrary, SOD1<sup>G93A</sup> rats (a rodent ALS model) showed significant pathology at hemi-diaphragm NMJs (B), including complete denervation (G, arrowhead), partial denervation (E, arrowhead; G, NMJ on right), multiple innervation (D) and thin pre-terminal axons (F, arrowhead). The diaphragm muscle is sub-divided into three separate sections for NMJ morphology analysis (H)<sup>13</sup>. Scale bar: 25 $\mu\text{m}$ .

## Discussion:

As respiratory function is compromised in both traumatic SCI and ALS, developing therapies that target breathing and specifically diaphragm innervation are clinically relevant<sup>5,6</sup>. In order to comprehensively study respiratory function, a combined approach method should be used. CMAPs measure the degree of functional innervation of the diaphragm by way of external phrenic nerve stimulation, but not endogenous bulbospinal respiratory drive<sup>8</sup>. In addition, these recordings do not allow for examination of morphological changes at the NMJ, particularly at the level of individual motor axons and post-synaptic acetylcholine receptors. By using the described staining protocol, one can quantitatively assess relevant morphological phenotypes such as complete denervation, partial denervation and even regenerative sprouting and reinnervation at individual NMJs of the diaphragm muscle<sup>9</sup>.

Nevertheless, these two protocols still do not fully capture all aspects of respiratory pathology. For example, if injury occurs to the descending bulbospinal axons without any damage to the PhMN pool or phrenic nerve, CMAP amplitudes may still appear normal even though there is significant impairment in this circuitry because the phrenic nerve is externally stimulated<sup>14</sup>. Importantly, CMAPs do not allow for measurement of endogenous supraspinal respiratory drive. Furthermore, CMAPs do not provide a measure of overall ventilation. It may be beneficial to conduct, for example, whole body plethysmography to examine overall breathing behavior and spontaneous intra-muscular EMG recordings and phrenic nerve recordings to test endogenous PhMN activation. In addition, other approaches such as measuring blood gas levels can provide important information about respiratory function<sup>15</sup>. It may also be possible to employ the described diaphragm CMAP recording technique for motor unit number estimation<sup>16</sup>, though we have never attempted this. It is also important to note that these two protocols were described for only one respiratory muscle, the diaphragm. However, there are other inspiratory and expiratory respiratory muscles such as the intercostals that can be studied and that are affected by diseases such as SCI and ALS.

In this protocol, we describe diaphragm CMAP and NMJ analysis specifically in the rat model. Similarities between rats and mice make transfer of both these techniques to mice straightforward. We previously showed that partial loss of PhMNs following cervical contusion SCI does result in quantifiable decreases in diaphragm CMAP amplitude that correlate with morphological NMJ denervation. Furthermore, we have demonstrated these deficits in both mouse<sup>17</sup> and rat<sup>10-12</sup> SCI models. In addition, we have shown quantifiable diaphragm CMAP reduction in both mouse<sup>18</sup> and rat<sup>13,19</sup> models of ALS (i.e. the SOD1<sup>G93A</sup> model) that are associated with more subtle diaphragm denervation by PhMNs compared to cervical SCI, and we have demonstrated that we can detect relatively smaller therapeutic effects on CMAP amplitude of interventions such as stem cell transplantation in these ALS models<sup>19</sup>. Collectively, these findings demonstrate the utility of using these analyses to quantitatively assess both functional and morphological assessment of diaphragm innervation by PhMNs in both rat and mouse models of SCI and ALS.

By combining the histological and functional techniques detailed in this protocol to examine breathing and innervation at the NMJ, better insight can be gained into nervous system diseases associated with diaphragm dysfunction, thus providing greater awareness of how to target therapies in animal models and ultimately in patients.

**Acknowledgments:**

This work was supported by the NINDS (grant #1R01NS079702 to A.C.L.) and the SURP Program at Thomas Jefferson University (M.M.).

**Disclosures:**

We have nothing to disclose.

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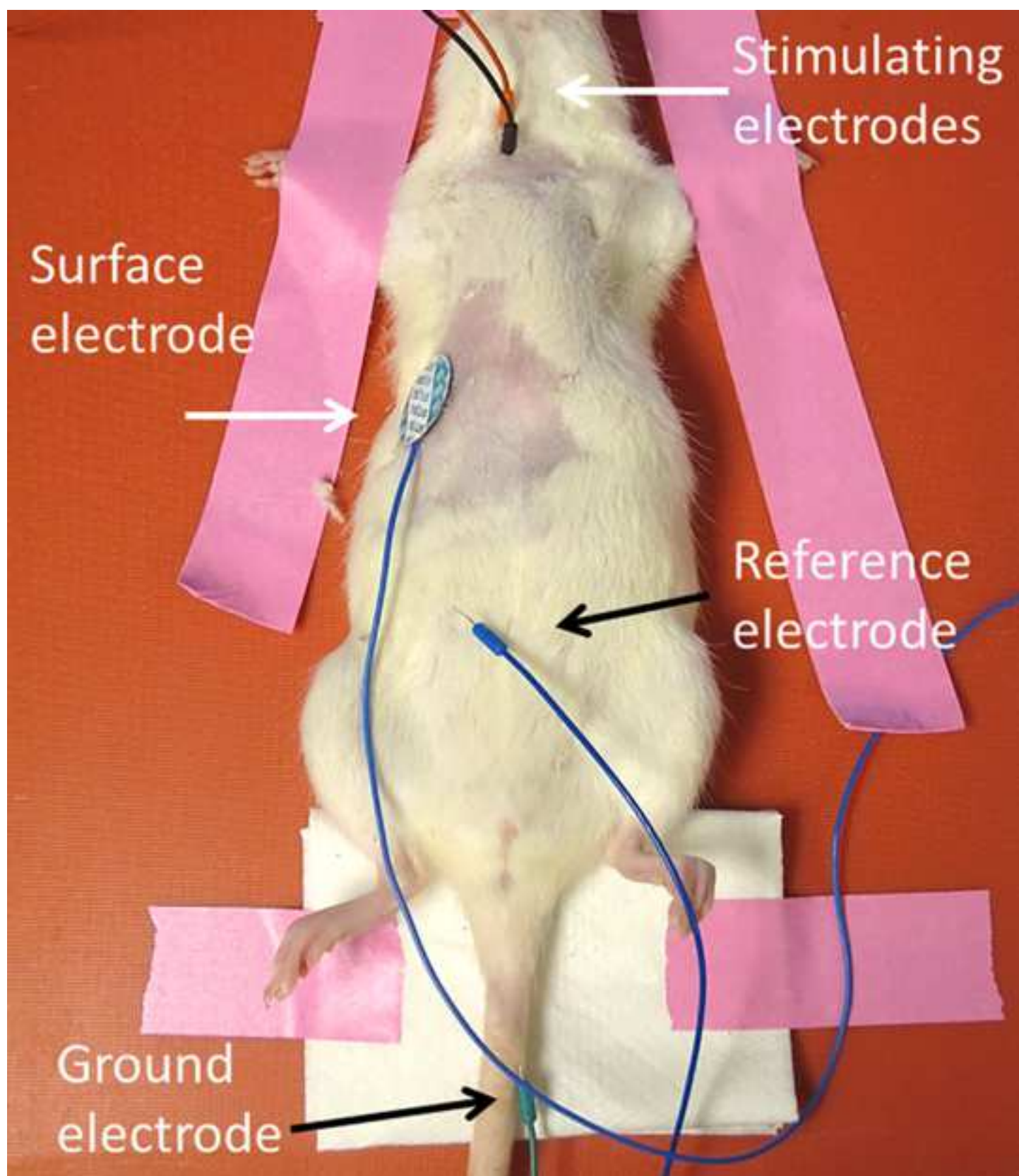


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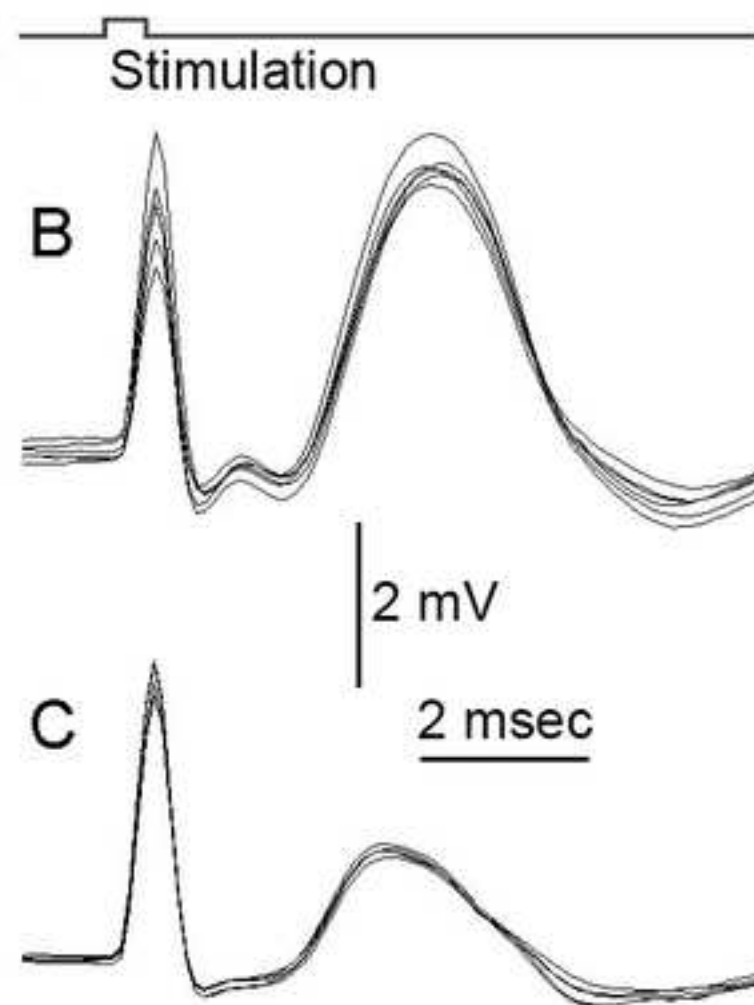
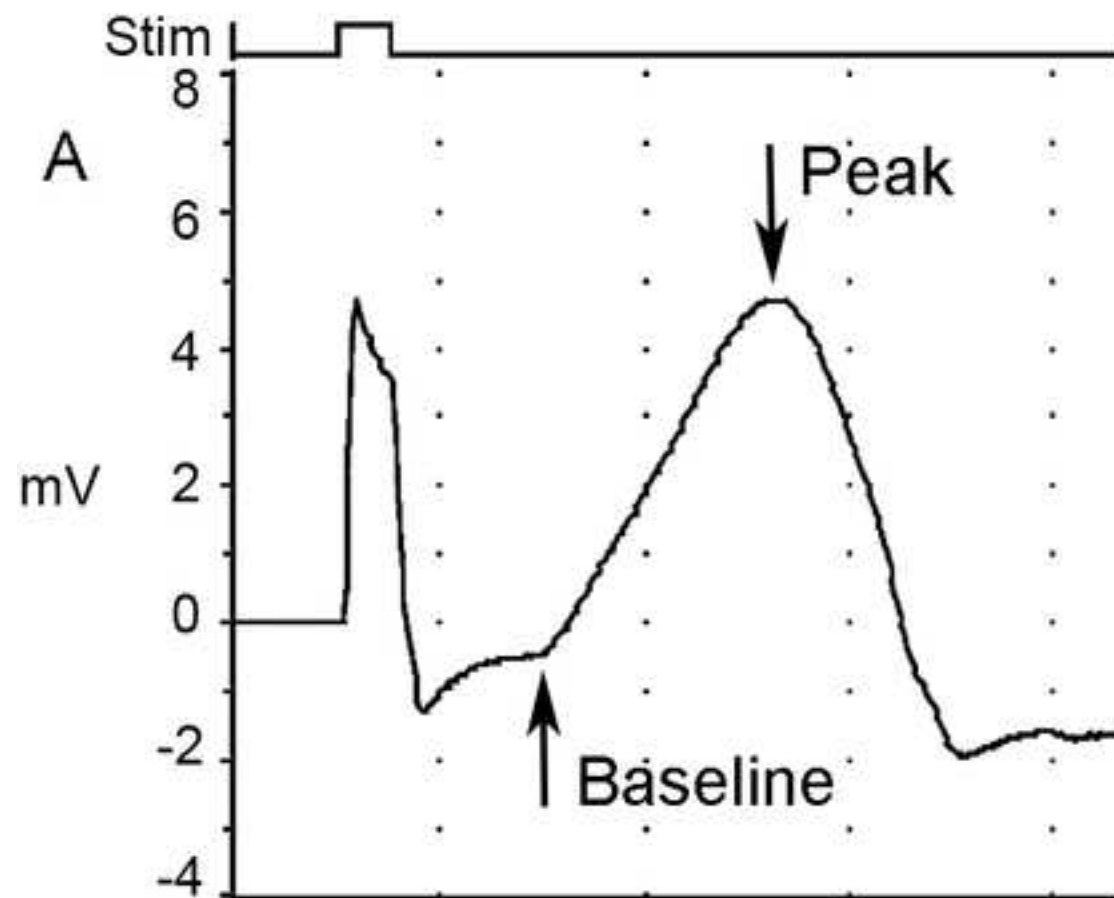


Figure 3

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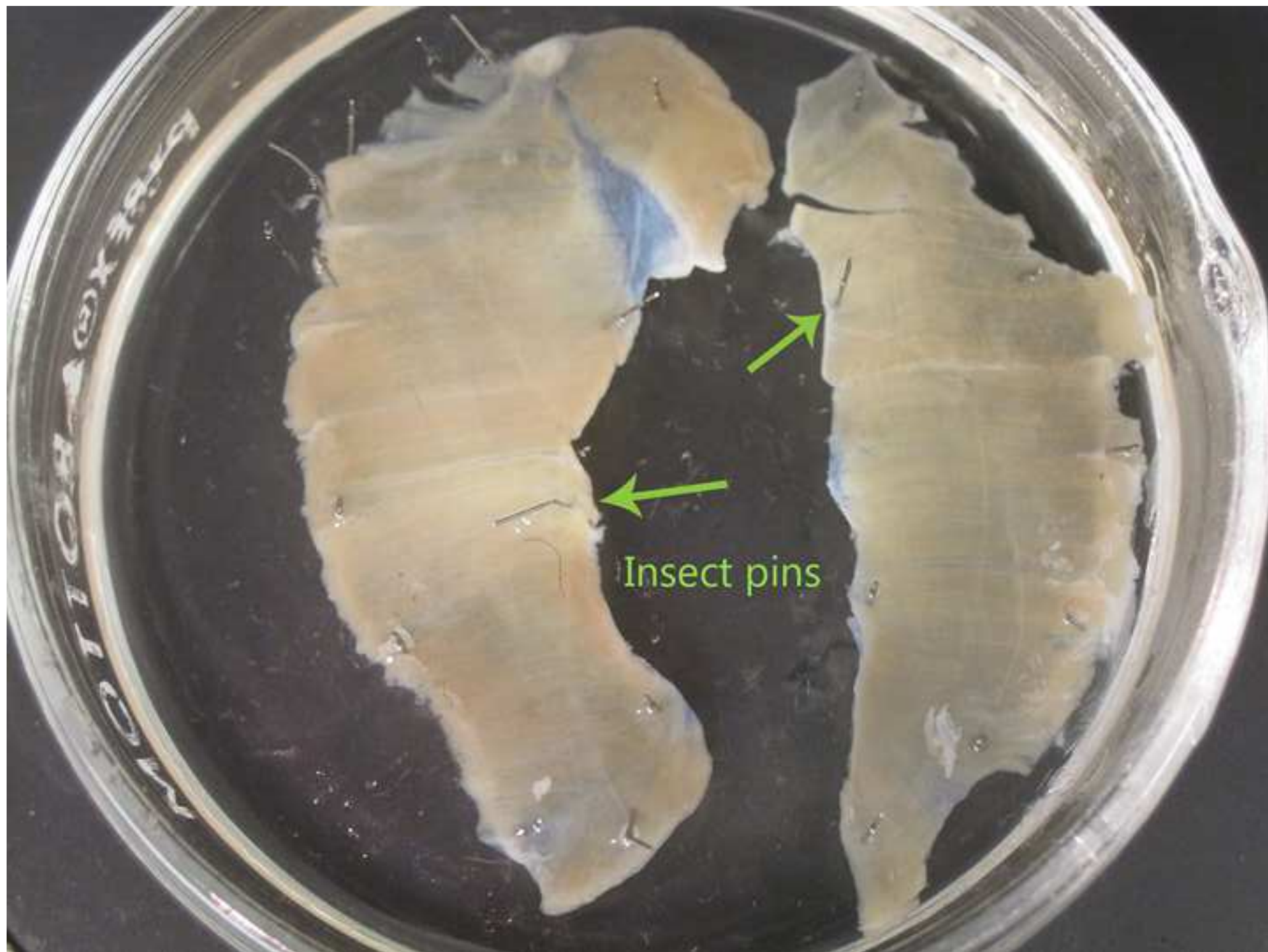
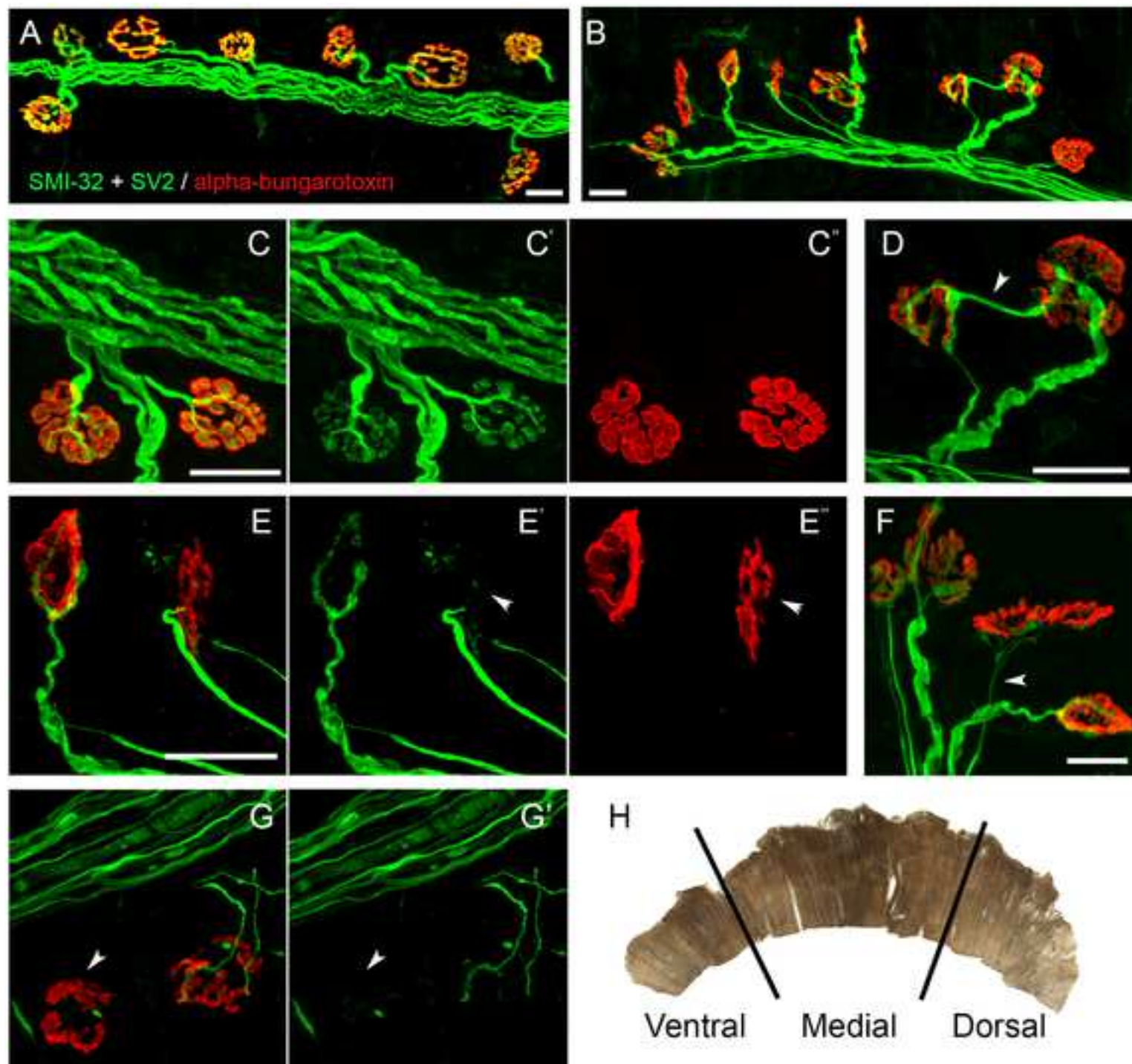




Figure 4  
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Name of Material/ Equipment	Company	Catalog Number
Paraformaldehyde	Fisher	T353-500
1X Phosphate Buffered Saline, pH 7.4	Invitrogen	10010049
2% Bovine serum albumin (2% BSA)	Sigma-Aldrich	A3059-100g
0.2% Triton X100 in 2% BSA/PBS (Blocking Buffer)	Sigma-Aldrich	T9284-100mL
0.1M Glycine	Sigma-Aldrich	G-7126
$\alpha$ -bungarotoxin	Invitrogen	T1175
SMI-312	Sternberger Monoclonals	SMI312
SV2	Developmental Studies Hybridoma Bank	SV2-Supernatant
FITC goat anti-mouse IgG1	Roche	3117731001
Silicone rubber	Sylgard, Dow Corning	Part # 184
Vectashield fluorescent mounting medium	Vector laboratories	H-1000
Small Spring Scissors	Fine Science Tools	15002-08
Dissection forceps	Fine Science Tools	11295-51
Software for CMAP recordings	Scope 3.5.6; ADI	
Disk surface electrodes	Natus neurology	019-409000
Subdermal needle electrodes	Natus neurology	019-453100
Conductive gel	Aquasonic	122-73720
Stimulator/recording system for CMAP recordings	ADI Powerlab 8SP stimulator	
Amplifier for CMAP recordings	BioAMP	

## Comments/Description

Make 10% solution first in de-ionized distilled water; make 4% with 1X PBS, adjust pH to 7.4

Dissolve 2g BSA into 100mL of 1X PBS

Dissolve 0.2ml/100mL 2% BSA/PBS

Add 0.185g to 25mL of 2% BSA/PBS

Concentration 1:400

Concentration 1:1,000

Concentration 1:10

Concentration 1:100

Follow instructions that come with kit: can use multiple sized culture dish (30mm, 60mm, 100mm) depending on needs

This is not a hard-set medium. You will need to secure the cover slip with clear nail polish.



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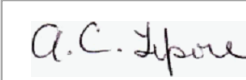
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Re: Manuscript Revision - *JoVE*

October 6<sup>th</sup>, 2014

Dr. Upponi:

Enclosed for review at *JoVE* is resubmission of the manuscript entitled "Functional and morphological assessment of diaphragm innervation by phrenic motor neurons."

We have addressed all of the comments of the three reviewers, as well as the editorial comments. We have enclosed along with the manuscript a point-by-point set of responses to each reviewer comment, and we have made all of the necessary changes to the manuscript text and figures. We appreciate the helpful comments of the reviewers, and we feel that the changes made based on their input have made for an improved manuscript.

I hope that you will be able to send this resubmission back to the original reviewers. If so, I look forward to their comments. If there are any questions that I can answer, please feel free to contact me.

Sincerely,

Angelo C. Lepore, Ph.D.

Dear Dr. Lepore,

Your manuscript JoVE52605R2 'Functional and morphological assessment of diaphragm innervation by phrenic motor neurons' has been peer-reviewed and the following comments need to be addressed.

Please keep JoVE's formatting requirements and the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

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## **Author Response to Editorial and Reviewer Comments**

The changes to the manuscript text in response to the editorial and reviewer comments have been noted in red font in the revision.

### **Editorial comments:**

**Reviewer comment:** Scale bar measurement missing in Figure legend 4.

**Response:** We have added the scale bar measurement to the figure legend.

**Reviewer comment:** Your current short abstract is above our 50 word limit; please make sure the short abstract is between 10 to 50 words.

**Response:** The short abstract is 48 words by our count.

**Reviewer comment:** Please re-write step 1.3.4 of your protocol section in imperative tense, as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.).

**Response:** We have modified the first sentence of this step to the imperative tense. However, the second sentence is not a protocol step per se. It is more a point being made that repeated measurements can be conducted on the same animal, if desired. It would not sound correct if written in the imperative tense.

**Reviewer comment:** Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

**Response:** We have maintained the changes based on the editorial comments from the prior revision.

**Reviewer comment:** Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

**Response:** We have proofread the manuscript.

**Reviewer comment:** If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as "Re-print with permission from (reference#)" or "Modified from.." etc. And please send a copy of the re-print permission for JoVE's record keeping purposes.

**Response:** The figures have not been previously published.

### **Reviewer #1:**

#### *Manuscript Summary:*

In the methods paper by Martin et al, the authors describe two methods of analysis for determining the functional motor innervation of the diaphragm in rodents, the first being CMAP of the diaphragm in a live animal, followed by a protocol for determining innervation of the diaphragm at the NMJ by histochemical methods after the animal is sacrificed. Although both methods are established techniques in use by many labs, CMAP of the diaphragm in rodents is less common, and technical tricks for good staining and imaging of the diaphragm can make or break the outcome of the experiment. Therefore clear explanation of a standardized and robust protocol will be of use to many labs investigating motor function, and this methods paper will be well received.

### *Major Concerns:*

**Reviewer comment:** .... more explanation on 1.2.4 (placement of stimulating electrodes) would be appreciated. How deep are they inserted? How are they secured?

**Response:** We have added these details to step 1.2.4.

**Reviewer comment:** Description of materials sufficient? Yes except for CMAP hardware: Which amplifier? Address for BioAMP?

**Response:** We agree that this is important information. In fact, we included these details in the original submission, but were asked to remove this commercial information by the editorial staff. Therefore, we have not added this information, despite the reviewer's request.

**Reviewer comment:** Are appropriate controls suggested? No controls are described for much of the procedure, although a C4 contusion spinal cord injury is used as a control for reduced phrenic function in rats. Since the protocol does not explain how to do the SCI, it would be good to reference a source of instruction for this as well as relevant animal welfare guidelines.

**Response:** We have added the appropriate reference to our previous publications describing the C4 hemi-contusion SCI model in the rat.

**Reviewer comment:** Although the description of the methods is sufficient to follow, the usefulness of the diaphragm CMAP experiments in rodents for studying neuromuscular disease is not clear mainly because you need massive loss in muscle innervation to see differences by CMAP. Diaphragm CMAP is altered in humans with neuromuscular disease, and the authors show altered CMAP in a rat following contusion spinal cord injury, presumably associated with massive phrenic dysfunction. However, despite showing altered NMJ innervation in SODG93A rats, they do not show the CMAP is sensitive enough to correlate with any specific degree of denervation. Do SOD rats have reduced diaphragm CMAP? Moreover, the authors suggest this can be used in mice, which have smaller muscles than rats and therefore generate a smaller CMAP, making the challenge even bigger. If the authors showed a comparison of a healthy animal and a diseased (rather than injured) one, and demonstrated a significant difference, this would validate the method and allow comparisons to a control.

**Response:** In this protocol, we describe diaphragm CMAP and NMJ analysis specifically in the rat. Similarities between rats and mice make transfer of these techniques to mice straightforward. We previously showed that partial loss of PhMNs following cervical contusion SCI does result in quantifiable decreases in diaphragm CMAP amplitude that correlate with NMJ denervation. Furthermore, we have shown these deficits in both mouse and rat SCI models. In addition, we have shown quantifiable diaphragm CMAP reduction in both mouse and rat models of ALS (i.e. the SOD1-G93A model) that are associated with more subtle diaphragm denervation by PhMNs compared to cervical SCI, and we have shown that we can detect relatively smaller therapeutic effects on CMAP amplitude of interventions such as stem cell transplantation in these ALS models. Collectively, these findings demonstrate the utility of using these analyses to quantitatively assess both functional and morphological assessment of diaphragm innervation by PhMNs in both rat and mouse models of SCI and ALS. We now include this discussion in the manuscript, as well as relevant references to our previously published studies.

**Reviewer comment:** Is their CMAP arrangement suitable for MUNE, if done using increasing stimulus intensity?

**Response:** We have never attempted to use this CMAP protocol for estimating motor unit number estimation; therefore, we did not mention this in the text because we do not have the data to back up any claims. Based on the reviewer's comment below, we have added the suggested reference for adopting the diaphragm CMAP technique to MUNE.

**Reviewer comment:** CMAP is a fairly straightforward procedure, but interpreting the size of the response correctly requires some understanding of how it works. For a methods paper, some explanation of stimulus

artifacts, how placement of the stimulating electrodes affects the response, direction and amplitude of the waveforms, etc would be important for students learning the procedure or troubleshooting. They could read about this elsewhere, but then what would the point of the article be?

**Response:** We have added these details to step 1.3.2 of the protocol.

**Reviewer comment:** There is an excellent methods paper/book chapter on CMAP/MUNE for mouse hindlimb muscles that the authors might reference for readers interested in adopting the technique to MUNE: Ngo & Bellingham "Neurophysiological Recording of the Compound Muscle Action Potential for Motor Unit Number Estimation in Mice", Stimulation and Inhibition of Neurons, Neuromethods Volume 78, 2013, pp 225-235

**Response:** As stated above, we have added the suggested reference for adopting the diaphragm CMAP technique to MUNE.

**Reviewer comment:** On section 1.3.3, the stimulations are done every 30 seconds. Since the animal is alive and breathing, should the stimulations be done during a specific phase of the animals' respiratory cycle?

**Response:** We conduct phrenic nerve stimulation during the phase of the respiratory cycle when the phrenic nerve/diaphragm is not active. We have added this to the step 1.3.3 of the protocol.

**Reviewer comment:** Not clear where abbreviations are spelled out. In 2.2.4, TBP is used but not defined until 2.2.11. A simple list of terms at the end would be good.

**Response:** We have now written out the name of all abbreviations at their first mention in the text.

**Reviewer comment:** In 2.3.9, it is not instructed what kind of statistical analysis you suggest.

**Response:** We have removed mention of statistical analysis because we feel that this is not specific to the NMJ staining protocol.

**Reviewer comment:** Sections 2.3.10 - 2.3.12 on confocal imaging are for pretty pictures but not used in your analysis, which you say uses epifluorescent scope. Since you are teaching people how to do this, you must show representative pictures that are not from the confocal so that people have an idea of what it will look like. All of figure 4 is confocal, which makes the protocol less instructive to labs that don't have one.

**Response:** We understand the reviewer's point; however, showing images taken with the epifluorescent microscope will actually provide an even less informative perspective on what the NMJ morphologies look like for analysis. The diaphragm is stained and analyzed in a whole-mount fashion; therefore, the muscle is quite thick. Consequently, most of the fluorescence staining is completely out of focus when an image is taken using regular epifluorescence microscopy. When we are analyzing the NMJs under the microscope, we actually have to constantly focus up and down through the muscle to properly identify the morphology of each of the individual NMJs. This is very difficult to illustrate in a figure using static epifluorescent images. On the other hand, the confocal images we have included, in our opinion, provide the best illustration of each of the NMJ morphology types that we describe in this protocol. Furthermore, we plan to demonstrate the technique of focusing through the muscle in the video portion of our JoVE protocol to best demonstrate this approach. For these reasons, we still show these confocal images in Figure 4. In addition, we have expanded on our description of how to identify the NMJ morphologies in the protocol text.

**Reviewer comment:** I don't understand "Representative results" comparing Fig2B (injured) to Fig2C (control), with the interpretation that the "peak CMAP was significantly reduced post SCI" since in your Figure 2C the wave looks smaller. Could these be aligned to the same axis, or labeled more in the figure?

**Response:** We mis-stated this in the text of the Representative Results section and have now corrected the statement. Panel 2C is the injured trace, while 2B is an uninjured animal.

*Minor Concerns:*

N/A

## **Reviewer #2:**

### *Manuscript Summary:*

In this manuscript, by Martin et al., the authors describe two techniques: 1) how to perform diaphragmatic CMAP recordings, and 2) anatomical assessment of diaphragmatic innervation. These are valuable tools that researchers can use in the assessment of rats and mice in both ALS and SCI paradigms. Overall this is a well written manuscript by authors who have extensively produced articles using these procedures. There are some minor points that needs to be addressed.

### *Minor Concerns:*

**Reviewer comment:** The authors write in the abstract and in the paragraph preceding the Protocol section, utilization of these techniques in mice. However, there is no real description in how to apply these approaches in the mouse (except for section 2.3.9). It is understood that the similarities between the two species makes transfer of these technique to mice easy. The authors should state this explicitly.

**Response:** We have added this suggested statement to the Discussion section.

**Reviewer comment:** Is there a preferred anesthetic to use during CMAP recordings (isoflurane or ketamine/xylazine)?

**Response:** We successfully use both anesthetic approaches, with no preference. We have added this to step 1.1.1 of the protocol.

**Reviewer comment:** How do you avoid recording from intercostal musculature vs. diaphragmatic during the CMAP recording?

**Response:** We position the recording surface electrode at a fairly caudal location. Therefore, we are likely not recording from the intercostals. Importantly, when we conduct a phrenicotomy ipsilateral to the side of stimulation/recording, we detect no subsequent CMAP response, suggesting that we are only recording phrenic nerve innervation of the diaphragm with our recording approach.

**Reviewer comment:** In the Representative Results section, the author write Figure 2B were SCI rats and 2C were laminectomy only controls. In the Figure 2 legend, it is written that panel B was laminectomy only and panel C were contused animals, which I believe is correct when looking at the images. Please correct the Representative Results section accordingly.

**Response:** We mis-stated this in the text of the Representative Results section and have now corrected the statement. Panel 2C is the injured trace, while 2B is an uninjured animal.

## **Reviewer #3:**

The article is well written and easy to follow. Steps are clear and logically make sense. The rationale and background are clearly explained.

### *Major Concerns:*

N/A

### *Minor Concerns:*

N/A