Respiratory Physiology & Neurobiology 226 (2016) 81–86

Contents lists available at ScienceDirect

Respiratory Physiology & Neurobiology

journal homepage: www.elsevier.com/locate/resphysiol

Quantitative assessment of integrated phrenic nerve activity

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ARTICLE INFO

Article history:
Received 16 November 2015
Received in revised form 7 December 2015
Accepted 14 December 2015
Available online 24 December 2015

Keywords:
Respiratory motor output
Integration
Ventilatory control
Chemoreflex
ALS
Breathing

ABSTRACT

Integrated electrical activity in the phrenic nerve is commonly used to assess within-animal changes in phrenic motor output. Because of concerns regarding the consistency of nerve recordings, activity is most often expressed as a percent change from baseline values. However, absolute values of nerve activity are necessary to assess the impact of neural injury or disease on phrenic motor output. To date, no systematic evaluations of the repeatability/reliability have been made among animals when phrenic recordings are performed by an experienced investigator using standardized methods. We performed a meta-analysis of studies reporting integrated phrenic nerve activity in many rat groups by the same experienced investigator; comparisons were made during baseline and maximal chemoreceptor stimulation in 14 wild-type Harlan and 14 Taconic Sprague Dawley groups, and in 3 pre-symptomatic and 11 end-stage SOD1^{G93A} Taconic rat groups (an ALS model). Meta-analysis results indicate: (1) consistent measurements of integrated phrenic activity in each sub-strain of wild-type rats; (2) with bilateral nerve recordings, left-to-right integrated phrenic activity ratios are ~1.0; and (3) consistently reduced activity in end-stage SOD1^{G93A} rats. Thus, with appropriate precautions, integrated phrenic nerve activity enables robust, quantitative comparisons among nerves or experimental groups, including differences caused by neuromuscular disease.

1. Introduction

Since the 1970s, integrated electrical activity of the cut central end of the phrenic nerve has been routinely used as an indicator of central respiratory drive to the diaphragm (Bartoli et al., 1975; Eldridge, 1971, 1975, 1976; Schmid and Böhmer, 1984). Due to concerns about nerve electrode contact, variations among investigators, and other factors, integrated phrenic output is generally normalized to a standardized value, such as integrated activity during baseline and/or maximal chemoreflex activation. However, such standardization can introduce normalization artifacts not always appreciated by investigators (Fregosi and Mitchell, 1994), and there is often a need to directly compare absolute measures of respiratory motor output, particularly when making bilateral recordings of the same nerve in the same animal, or across animal groups with neural injury and/or disease (Kajana and Goshgarian, 2009; Lee et al., 2010; Nichols et al., 2013a).

To date, there are no thorough, quantitative assessments concerning the validity of comparing absolute values of integrated phrenic nerve activity within or across animals when precautions are taken to standardize nerve-recording conditions. In supplemental data for a previous publication (Nichols et al., 2013a), we presented preliminary evidence that the amplitude of integrated phrenic motor output can be a robust measure of respiratory motor output since simultaneous recordings of integrated left and right phrenic nerve activity yielded the same voltage (*i.e.*, their ratio was ~1.0) in both wild-type and end-stage SOD1^{G93A} rats, a rat model of ALS (Nichols et al., 2013a). These preliminary findings support the idea that integrated phrenic nerve activity can be a consistent and robust indicator of "absolute" phrenic motor output when proper precautions are taken. Here, we extended the preliminary analysis presented as Supplemental material in an earlier paper (Nichols et al., 2013a) by performing a meta-analysis of integrated phrenic burst amplitude from many experimental groups using standardized techniques performed by a single, experienced investigator (N.N.). We found that variations among group-means from many different studies of the same rat sub-strains were small

Abbreviations: O_2, Oxygen; CO_2, carbon dioxide; P_{ETCO2}, end-tidal PCO_2; PaO_2, partial pressure of arterial O_2; $PaCO_2$, partial pressure of arterial CO_2; mV, millivolts; pLTF, phrenic long-term facilitation.

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http://dx.doi.org/10.1016/j.resp.2015.12.005
1569-9048/

in comparison to differences between group means from different experimental conditions (baseline *vs.* maximal chemoreflex activation), rat sub-strains (Harlan *vs.* Taconic Sprague Dawley rats), or after the onset of motor neuron disease (end-stage $SOD1^{G93A}$ Taconic rats). Thus, with suitable precautions, absolute values of integrated phrenic nerve activity can represent a robust and reliable assessment of phrenic motor output, and may be used to detect differences caused by neural injury (*e.g.*, spinal injury) and/or the onset of neuromuscular disease (*e.g.*, ALS).

2. Materials and methods

2.1. Animals

We did a meta-analysis on 2–6.5 month old male Sprague Dawley rats, including rats from multiple colonies and vendors (Harlan 211, Houston, TX; Harlan 217 and 218a, Indianapolis, IN) and Taconic (Taconic Laboratories, Germantown, NY). Taconic rats included $SOD1^{G93A}$ mutant (MT) and age-matched wild-type (WT) littermates at different ages: pre-symptomatic (60–130 days) and end-stage (150–200 days). $SOD1^{G93A}$ mutant (MT) and age-matched wild-type (WT) littermates were bred from transgenic sires overexpressing the human $SOD1^{G93A}$ gene (bred to female WT Taconic rats). Heterozygous $SOD1^{G93A}$ progeny were identified with polymerase chain reaction (PCR) of tail DNA with primers specific for hSOD1. $SOD1^{G93A}$ rats were considered end-stage when they had lost 20% of their peak body mass, as in previous reports (Nichols et al., 2013a, 2014, 2015a,b).

Overall, we report unilateral integrated phrenic nerve activity from 14Harlan and Taconic WT rat groups, 3 groups of pre-symptomatic Taconic MT rats, and 11 groups of Taconic end-stage MT rats (see below for group details). Lastly, in 6 groups, we compared bilateral integrated phrenic nerve activity (see below for group details). All groups had at least 4–14 rats.

Rats were housed under standardized conditions, with a 12-h light/dark cycle and *ad libitum* food and water. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the School of Veterinary Medicine, the University of Wisconsin–Madison, and were in agreement with standards set forth in the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals.

2.2. In vivo *neurophysiology*

2.2.1. Experimental preparation

Experimental procedures have been described previously in multiple publications (Hoffman et al., 2012; Nichols et al., 2012, 2013a). Briefly, rats were anesthetized with isoflurane, tracheotomized, paralyzed and ventilated (Rodent Ventilator, model 683; Harvard Apparatus, Holliston, MA, USA; tidal volume ~2.5 mL, frequency ~70–80). Body temperature was assessed with a rectal thermometer (Fisher Scientific, Pittsburgh, PA, USA) and maintained ($37.5 \pm 1\,^{\circ}C$) with a heated surgical table. To monitor end-tidal PCO_2 (P_{ETCO2}), a flow-through carbon dioxide analyzer with sufficient response time to measure P_{ETCO2} in rats was used (Capnogard, Novametrix, Wallingford, CT). P_{ETCO2} was maintained at ~45 mmHg throughout the surgical preparation. Rats were bilaterally vagotomized and a polyethylene catheter (PE50 ID: 0.58 mm, OD: 0.965 mm; Intramedic, MD, USA) was inserted into the right femoral artery to monitor blood pressure (Gould Pressure Transducer, P23ID, USA) and enable blood gas analysis. The left phrenic nerves (and right for bilateral studies) were isolated (dorsal approach), cut distally, desheathed, and covered with a saline soaked cotton ball until placing the nerves on bipolar silver electrodes (see below). Some rat groups were from studies where a C_2 laminectomy had been performed to enable intrathecal drug or vehicle delivery (see below for details of laminectomy).

Isoflurane anesthesia was maintained (3.5% in 50% O_2, balance N_2) throughout surgical procedures; all rats were then slowly converted to urethane anesthesia over a 15–20 min period ($1.8\,g\,kg^{-1}$, *i.v.*,) while concurrently withdrawing isoflurane. After conversion to urethane, an intravenous infusion was initiated to maintain body fluid and acid–base balance; infusions ($1.5–6\,mL\,kg^{-1}\,h^{-1}$) consisted of a 1:2:0.13 mixture of 6% Hetastarch (in 0.9% sodium chloride), lactated Ringer's, and 8.4% sodium bicarbonate. Once rats were converted to urethane anesthesia, a minimum of 1 h was allowed before protocols commenced.

2.2.2. Nerve recordings

The left phrenic nerves (and right for bilateral studies) were submerged in mineral oil and placed on bipolar silver electrodes to record nerve activity. Neural signals were amplified (10,000 X), band-pass filtered (300–10,000 Hz, Model 1800, A-M Systems, Carlsborg, WA, USA), full-wave rectified and integrated (Paynter filter, 50 ms time constant, MA-821, CWE Inc., Ardmore, PA, USA). Integrated nerve bursts were digitized (8 kHz) and analyzed using WINDAQ data acquisition system (DATAQ Instruments, Akron, OH, USA). For animals that received intrathecal drug or vehicle delivery, a small incision was made in the dura and a soft silicone catheter (2 Fr; Access Technologies, Skokie, IL) was inserted caudally 3–4 mm until the tip rested approximately over the C_4 segment to deliver pre-treatment of drugs near the phrenic motor nucleus before protocols commenced. The catheter was attached to a 50 μL Hamilton syringe filled with drug or vehicle solutions. Rats were then paralyzed with pancuronium bromide ($2.5\,mg\,kg^{-1}$, *i.v.*).

To begin protocols, the apneic CO_2 threshold was determined by lowering P_{ETCO2} until nerve activity ceased for approximately one minute. The recruitment threshold was then determined by slowly increasing P_{ETCO2} until nerve activity resumed (Bach and Mitchell, 1996). P_{ETCO2} was raised ~2 mmHg above the recruitment threshold to establish a level of nerve activity that is stable, repeatable and is low enough that it retains substantial capacity to increase, thus minimizing the potential for "ceiling effects, and ~15–20 min were allowed to establish a stable baseline activity. Arterial blood samples were drawn during baseline, and throughout protocols; arterial CO_2 ($PaCO_2$) was maintained within ± 1.5 mmHg of baseline levels by adjusting inspired CO_2 and/or ventilator rate.

2.3. Experimental groups

The voltage of respiratory related integrated phrenic nerve activity was assessed at baseline (following intrathecal drugs if given) and the maximum hypercapnic response at the end of the protocol; this was done in all rat groups in a consistent manner (14 groups of Harlan and Taconic WT rats, 3 groups of Taconic pre-symptomatic MT rats, and 11 groups of end-stage MT rats). Data obtained from some of these groups has appeared in previous publications as percent change from baseline to study phrenic motor plasticity after various treatments, such as hypoxia and/or intrathecal drug or vehicle delivery (Devinney et al., unpublished; Hoffman et al., 2012; Nichols et al., 2012, 2013a, 2015a,b; Nichols et al., unpublished; Strey et al., 2012). However, none of these systemic and/or intrathecal treatments affected absolute phrenic nerve peak amplitude at baseline or during the maximum hypercapnia response.

In six groups, bilateral phrenic nerve recordings were made, including 2 groups of Harlan rats, and 4 groups of Taconic rats (2 WT and 2 MT age-matched groups at disease end-stage). Data from some of these groups have appeared in published studies (all studies conducted by N.N.), and are repeated here to increase the power