QUANTITATIVE ASSESSMENT OF INTEGRATED PHRENIC NERVE ACTIVITY

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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.

Keywords

respiratory motor output; integration; ventilatory control; chemoreflex; ALS; breathing

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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 in comparison to differences between group means from different experimental conditions (baseline *versus* maximal chemoreflex activation), rat sub-strains (Harlan *versus* 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 to 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; Nichols et al., 2014; Nichols et al., 2015a).

Overall, we report unilateral integrated phrenic nerve activity from 14 Harlan 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 to 14 rats.

Rats were housed under standardized conditions, with a 12-hour 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; Nichols et al., 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 ($37.5 \pm 1°C$) with a heated surgical table. To monitor end-tidal P_{CO_2} (P_{ETCO_2}), a flow-through carbon dioxide analyzer with sufficient response time to measure P_{ETCO_2} in rats was used (Capnogard, Novametrix, Wallingford, CT). P_{ETCO_2} was maintained at ~45 mmHg throughout the surgical preparation. Rats were bilaterally vagotomized and a polyethylene catheter (PE50 ID: 0.58mm, OD: 0.965mm; 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 minute 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} hr^{-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 hour 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_{ETCO_2} until nerve activity ceased for approximately one minute. The recruitment threshold was then determined by slowly increasing P_{ETCO_2} until nerve activity resumed (Bach and Mitchell, 1996). P_{ETCO_2} 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 (Pa_{CO_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; 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 of our meta-analysis and enable comparisons among rat sub-strains (Nichols et al., 2013a; 2013b; 2015b).

2.4 Data and Statistical analyses

Integrated phrenic nerve burst peak amplitude was averaged over 1 min bins at each time point assessed during baseline and maximum chemoreflex activation. Statistical comparisons among (integrated peak burst amplitude or the right/left ratio) were made *via* one way ANOVA. When significant ANOVA differences were detected, individual

comparisons were made with Fisher's least significant difference *post hoc* test (Sigma Plot version 12.0; Systat Software Inc., San Jose, CA, USA). Differences between the groups were considered significant if p < 0.05; all values are expressed as means ± 1 S.E.M.

3. Results

3.1 Integrated phrenic nerve activity is consistent across studies in Harlan rats

Representative traces of an unprocessed and an integrated phrenic neurogram during baseline and with maximal chemoreflex activation are shown in Figure 1. Mean values of integrated phrenic activity assessed using common methods and equipment in 14 studies on Harlan rats (data obtained from the following studies: Devinney et al., unpublished; Hoffman et al., 2012; Nichols et al., 2012; Strey et al., 2012) during baseline and maximum chemoreflex activation are shown in Figure 2 (A & B). Mean integrated phrenic burst peak amplitude showed no significant variations across studies when comparisons were made during baseline or during maximal chemoreflex activation (p>0.05; Figures 2A & B). In Harlan rats, mean baseline integrated phrenic burst peak amplitude was 1.7 ± 0.09 mV (each group n = 4 or greater; 75 rats total; 95% confidence interval = 1.53 to 1.88) and increased to 3.7 ± 0.15 mV during maximal chemoreflex activation as expected (p<0.05 *versus* baseline; 95% confidence interval = 3.40 to 4.01). Thus, in Harlan Sprague Dawley rats, measurements of integrated phrenic burst amplitude are repeatable when performed in comparable conditions by an experienced investigator.

3.2 Integrated phrenic nerve activity is lower in Taconic wild-type versus Harlan rats

Mean values of integrated phrenic activity assessed using the same methods in 14 studies on Taconic wild-type rats (data obtained from the following studies: Nichols et al., 2013a; 2015a; Nichols et al., unpublished) during baseline and maximum chemoreflex activation are shown in Figure 2 (C & D). These values were consistently lower in Taconic *versus* Harlan Sprague Dawley rats during both baseline and chemoreflex activation (p<0.05), indicating that there are systematic differences in integrated phrenic nerve activity among rat sub-strains derived from different colonies/vendors. In these studies on Taconic wild-type rats, mean integrated phrenic burst peak amplitude during baseline conditions was 1.3 ± 0.06 mV (each group n = 5 or greater; 113 rats total; 95% confidence interval = 1.15 to 1.38) and increased to 2.9 ± 0.10 mV during maximal chemoreflex activation (p<0.05 *versus* baseline; 95% confidence interval = 2.67 to 3.07). Thus, in Taconic Sprague Dawley rats, we confirm that measurements of integrated phrenic burst peak amplitude are repeatable when performed in similar animals under comparable conditions by an experienced investigator, and can reveal subtle differences among groups such as sub-strain variations.

3.3 Integrated phrenic nerve activity is decreased by motor neuron disease

Phrenic burst peak amplitude was also assessed under baseline and maximum conditions in pre-symptomatic and end-stage mutant rats (Taconic Sprague Dawley rats; data obtained from the following studies: Nichols et al., 2013a; 2015a; Nichols et al., unpublished). In 3 studies on pre-symptomatic mutant rats, mean integrated phrenic burst peak amplitude was 1.1 ± 0.11 mV for baseline and 2.5 ± 0.22 mV during chemoreflex activation (each n = 6 or more; 23 rats total; 95% confidence intervals: 0.88 to 1.33 for baseline; 2.01 to 2.93 for

maximum). These values in pre-symptomatic rats were not significantly different from wild-type Taconic rats (p>0.05).

In 11 groups of end-stage mutant rats, integrated phrenic burst peak amplitude was assessed during baseline conditions and with maximal chemoreflex activation (Figure 3A & B). Mean integrated phrenic burst peak amplitude was 0.5 ± 0.03 mV during baseline and 1.4 ± 0.06 mV during chemoreflex activation (each n = 4 or more; 71 rats total; 95% confidence intervals: 0.47 to 0.60 for baseline and 1.24 to 1.49 for chemoreflex activation). Although there were minor fluctuations in these 11 group means from end-stage mutant rats in different generations, there were no significant differences among groups at baseline or during chemoreflex activation (p>0.05). On the other hand, all 11 group means were below the 95% confidence interval in either wild-type or pre-symptomatic mutant Taconic rats. Thus, assessment of integrated phrenic burst activity is a robust indicator of diminished phrenic motor output when comparing wild-type or pre-symptomatic mutant rats with mutant rats at disease end-stage.

Our results are summarized in Figure 3C, where mean values of peak integrated phrenic burst peak amplitude are compared between Harlan, Taconic wild-type, Taconic pre-symptomatic mutant and Taconic end-stage mutant rats during baseline conditions and maximal chemoreflex activation. Using the methods described here, we are able to distinguish differences in phrenic motor output between baseline and chemoreflex activation (even when paired comparisons are not used), rat sub-strains (Harlan *versus* Taconic) and with motor neuron disease progression.

3.4 Bilateral phrenic nerve recordings are comparable, regardless of rat group

Bilateral phrenic nerve recordings were made at baseline and during chemoreflex activation in multiple groups, including Harlan rats, wild-type Taconic rats, and end-stage Taconic mutant rats (data obtained from the following studies: Nichols et al., 2013a; 2013b; 2015b). In 6 groups, mean values of integrated phrenic nerve burst peak amplitude were not significantly different between the left and right sides in any group at baseline or during chemoreflex activation (all p>0.05). When expressed as a left-to-right ratio (Figures 4A & B), values were not significantly different from 1.0, regardless of group or condition.

4. Discussion

4.1 Integrated phrenic nerve activity is a robust measure of phrenic motor output

This meta-analysis of studies reporting integrated phrenic nerve burst amplitude using comparable techniques performed by a single experienced investigator demonstrates that integrated phrenic nerve activity can be a robust indicator of phrenic motor output. This finding validates the use of integrated phrenic nerve activity to investigate the impact of neural injury or disease on phrenic motor output since such questions require comparisons across nerves and/or across animals (*e.g.* Kajana and Goshgarian, 2009; Lee et al., 2010; Nichols et al., 2013a).

The quantitative similarity of mean integrated phrenic nerve activity across experimental groups from the same condition and rat sub-strains exemplifies the repeatability and

reliability of this technique to monitor phrenic motor output across animals. For example, during baseline or maximal chemoreflex activation, mean values from 14 different studies are within a tight range in either Harlan (Figures 2A & B) or Taconic wild-type Sprague Dawley rats (Figures 2C & D). The same conclusion is reached when comparing experimental groups with active motor neuron disease (Figures 3A & B), or when comparing bilateral phrenic recordings regardless of the rat sub-strain or animal condition (Figure 4).

However, to achieve such robust, quantitative assessment of phrenic motor output, similar methods must be employed by an experienced investigator, and comparisons must be made under comparable conditions. Relevant methods standardization should include (but is not limited to): 1) similar anesthetic protocols (here, all were isoflurane induction followed by urethane maintenance); 2) similar regulation of fluid and acid-base balance with active monitoring and intervention; 3) similar ventilator settings and endtidal P_{CO_2} regulation during surgical preparations and experimental protocols; 4) active body temperature maintenance; 5) avoidance of seemingly inconsequential stimuli during experimental preparations that may trigger plasticity and render comparisons invalid (*e.g.* avoid even brief ventilator apneas to trigger nerve activity during surgical/recording preparations); and 6) similar nerve isolation and recording techniques, including electrode placement and signal processing.

4.2 Integrated phrenic nerve activity differs across rat sub-strains

In this meta-analysis, we detected somewhat surprising differences between sub-strains (colonies/vendors) of outbred Sprague Dawley rats (Figure 3C). There are well known differences in ventilatory control among inbred strains of rats (Dwinell et al., 2005; Strohl et al., 1997; Strohl and Thomas, 2001) and mice (Tankersley et al., 1994; 2000), although it is less clear how these differences will be reflected in an outbred strain such as Sprague Dawley rats. On the other hand, there are clear strain (Baker-Herman et al., 2010; Golder et al., 2008; Wilkerson and Mitchell, 2009) and sub-strain (Fuller et al., 2000; 2001; Streeter and Baker-Herman, 2014) differences in the expression of phrenic motor plasticity. How these previous reports of strain and sub-strain differences relate to diminished baseline and maximal chemoreceptor driven phrenic activity is uncertain.

4.3 Integrated phrenic nerve activity decreases with motor neuron disease

Not surprisingly, we detected consistent decreases in integrated phrenic nerve activity under comparable conditions (baseline and chemoreflex activation) in rats with motor neuron disease (Figure 3A & B). This is an important finding since across rat comparisons are at the heart of many studies concerning the impact of spinal cord injury (Alilain et al., 2008; 2011; Doperalski and Fuller, 2006; El-Bohy and Goshgarian, 1999; Fuller et al., 2003; 2006; Golder and Mitchell, 2005; Golder et al., 2003; Kajana and Goshgarian, 2009; Lee et al., 2010; 2013; Liou and Goshgarian, 1994; White et al., 2010) and ALS (Nichols et al., 2013a; Nichols et al., 2015a) on respiratory motor output. With this meta-analysis, we add that the onset of motor neuron disease can be detected since integrated phrenic nerve activity in pre-symptomatic SOD1^{G93A} rats had not yet decreased in comparison with wild-type littermates (Figure 3C).

4.4 Conclusion: Measuring and comparing phrenic motor output

A major key to making comparisons of absolute phrenic nerve activity across multiple studies is to have a highly experienced investigator perform nerve recordings with standardized methods on all rats under investigation. Criteria required to make absolute phrenic nerve activity comparisons across studies are: 1) the same electrodes with a fixed distance between electrodes; 2) consistent signal processing and integration techniques; 3) similar anesthesia; 4) active monitoring and regulation of fluid and acidbase balance, body temperature, respiratory gases, etc.; and 5) avoidance of confounding protocol variations, such as extraneous ventilator apneas. Absolute comparisons of integrated phrenic nerve activity are critical to investigate the impact of neural injury or disease on respiratory motor output.

Acknowledgements

Abbreviations

O_2	Oxygen
CO_2	carbon dioxide
P_{ETCO_2}	end-tidal P_{CO_2}
PaO_2	partial pressure of arterial O_2
$PaCO_2$	partial pressure of arterial CO_2
mV	millivolts
pLTF	phrenic long-term facilitation

Reference

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Highlights

1. Reproducibility of integrated phrenic nerve activity was assessed.

2. Integrated phrenic nerve activity consistently reflects condition across rat groups.

3. However, phrenic nerve activity varies among rodent sub-strains.

4. Integrated phrenic nerve activity reveals pathology during motor neuron disease.
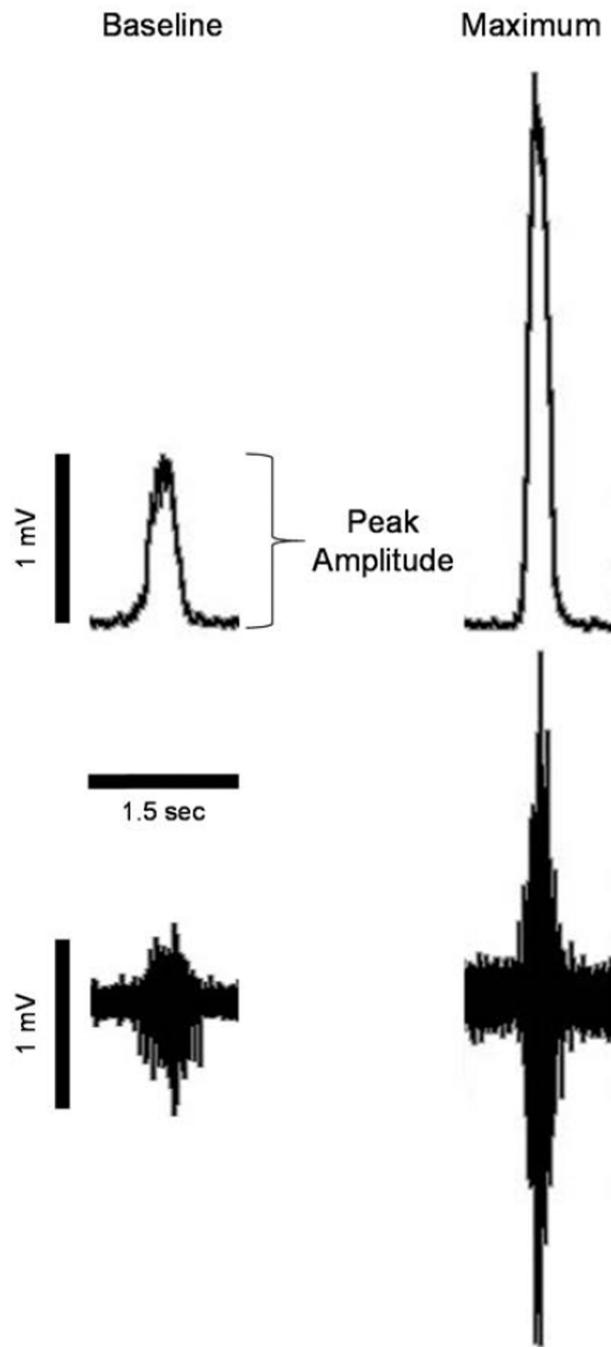
Baseline   **Maximum**

Figure 1.
Representative traces of raw and integrated phrenic nerve activity for single breaths are shown for integrated (top) and raw (bottom) phrenic motor output under baseline conditions (left) and during maximal chemoreflex activation (right).
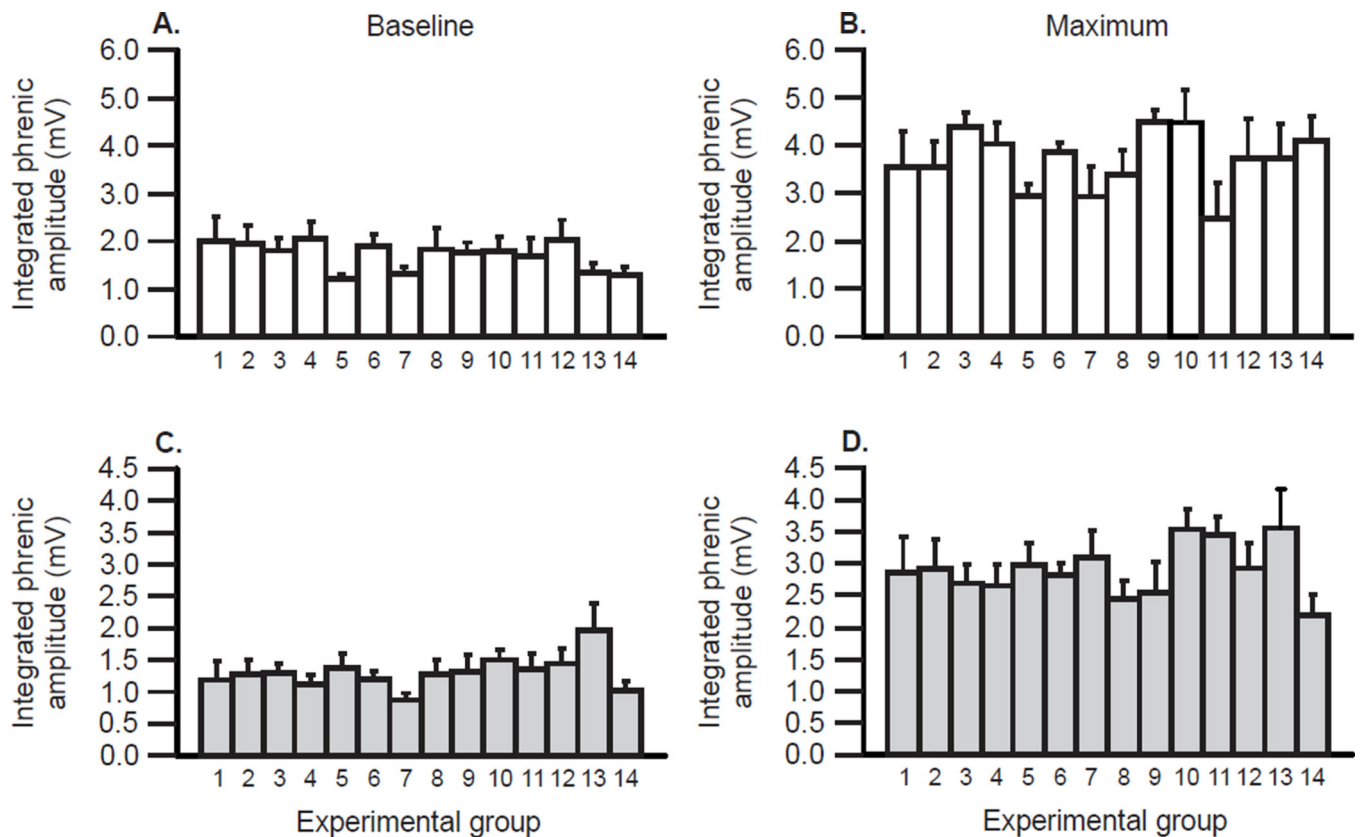
Figure 2.
Mean integrated phrenic nerve activity is consistent in 14 comparable studies on Harlan Sprague Dawley rats (white bars; A & B) under baseline conditions (A) and with maximal chemoreflex activation (B). There were no significant differences among studies ($p > 0.05$). In the lower panels (C & D), a similar analysis is presented for 14 groups of wild-type Taconic Sprague Dawley rats (light gray bars) under baseline conditions (C) and with maximum chemoreflex activation (D). Although integrated phrenic nerve activity was not significantly different in any study of Taconic rats during baseline or chemoreflex activation ($p > 0.05$), the average activity was significantly lower in Taconic *versus* Harlan rats, regardless of condition ($p < 0.05$).
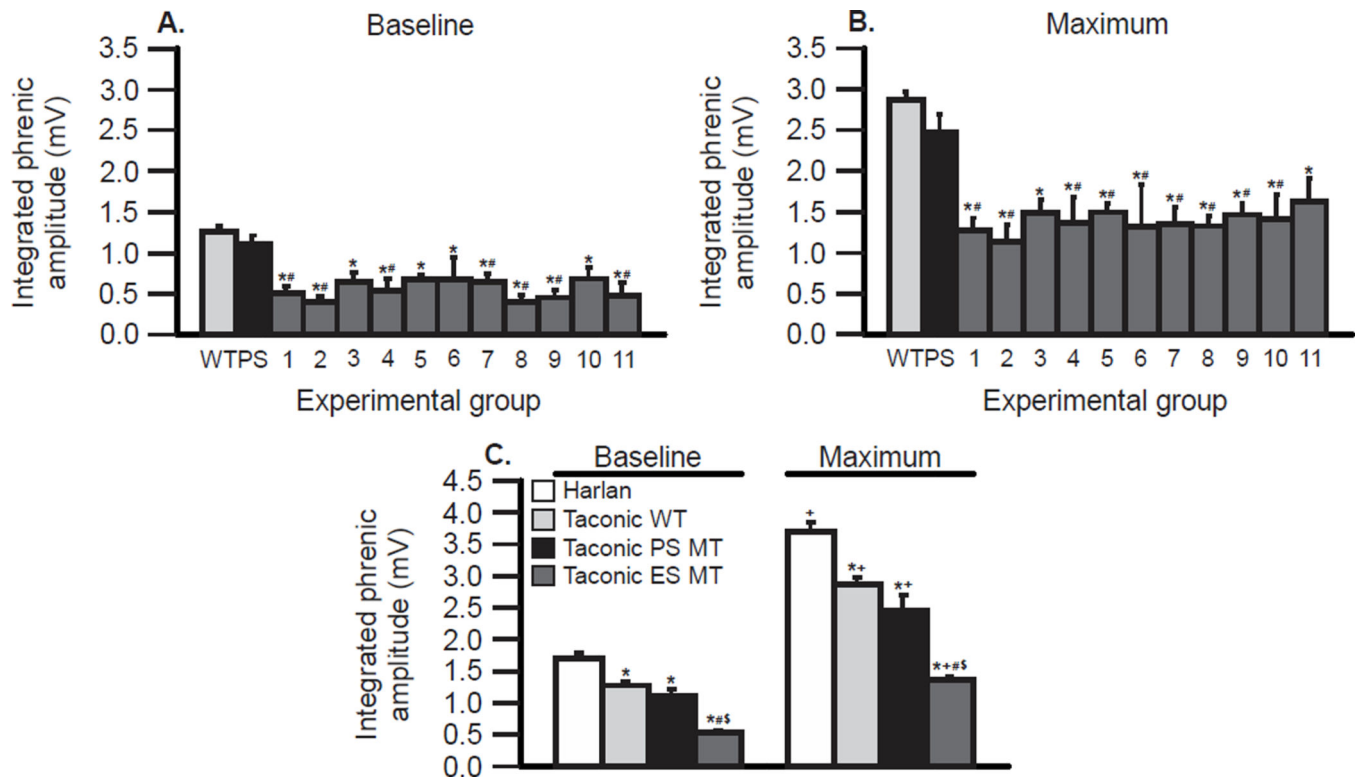
Figure 3.

Integrated phrenic nerve activity is consistent across 11 groups of end-stage Taconic SOD1^{G93A} (ES MT) rats (dark gray bars; A & B), but is decreased in comparison with wild-type (WT) and pre-symptomatic Taconic rats (PS MT; grand means represented as the light gray and black bars, respectively). In C, grand means are compared between Harlan and Taconic WT rats, and PS MT and ES MT rats during baseline conditions (left) and with maximal chemoreflex activation (right). In A & B, integrated phrenic nerve activity from 11 individual ES MT rat groups (dark gray bars) is compared with grand means from Taconic WT rats in all studies (light gray bars; 113 rats total) and PS MT (black bars; 23 rats total) during baseline (A) and maximum chemoreflex activation (B). There were no significant differences among ES MT groups ($p > 0.05$), but integrated activity in all ES MT groups was lower than WT (*; $p < 0.05$) and PS MT rats (#; $p < 0.05$). In C, integrated grand means of phrenic activity are compared between all rat groups presented in this meta-analysis, including ES MT (dark gray bar; 71 rats total), Harlan (white bar; 75 rats total), Taconic WT (light gray bar; 113 rats), and PS MT rats (black bar; 23 rats) under baseline conditions and with maximum chemoreflex activation. Phrenic activity is significantly greater in Harlan *versus* all other groups (*; $p < 0.05$). Integrated phrenic activity in ES MT rats is significantly lower *versus* all other groups (# *vs.* WT rats, $ *vs.* PS MT rats; all $p < 0.05$). Maximum phrenic activity was significantly increased *versus* baseline in all groups (+; $p < 0.05$).
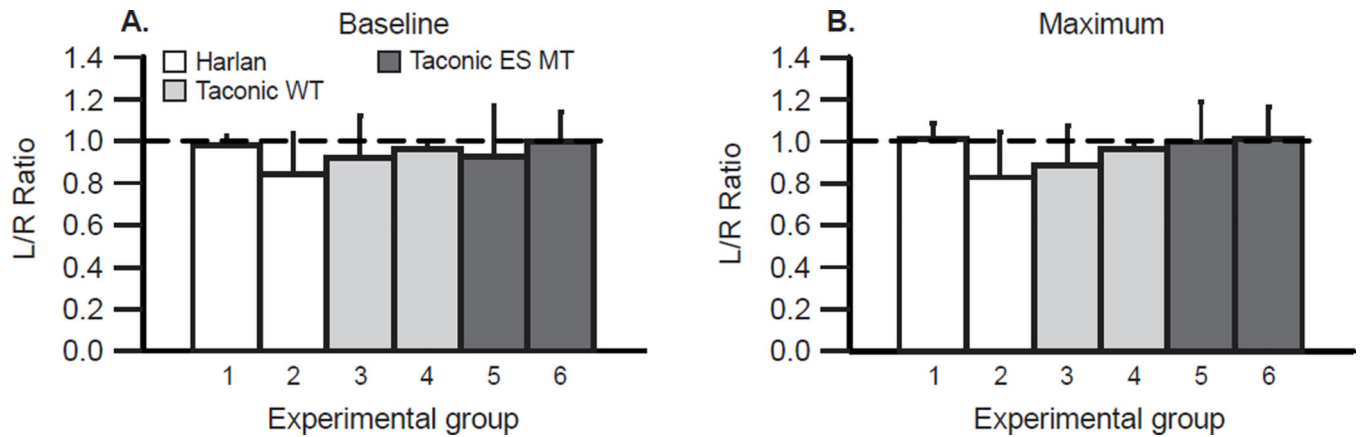
Figure 4.
With bilateral phrenic recordings, the mean left-to-right ratio of integrated phrenic activity is 1.0, regardless of animal group. The ratio of integrated phrenic nerve activity (left/right) was assessed in 2 Harlan (white bars), 2 wild-type (WT) Taconic (light gray bars), and 2 Taconic end-stage SOD1^{G93A} mutant (ES MT) rat groups (dark gray bars) under baseline conditions (A) and during maximum chemoreflex activation (B). In all studies, the left/right ratio was not significantly different from 1.0, and did not vary significantly among groups ($p>0.05$). Bilateral phrenic nerve recordings are the most effective way to assure that recordings are made under comparable conditions. Thus, a ratio of 1.0 demonstrates consistent recording outcomes.