Phrenic Afferent Activation Modulates Cardiorespiratory Output in the Adult Rat

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Phrenic Afferent Activation Modulates Cardiorespiratory Output in the Adult Rat

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Abstract
Phrenic afferents project to brainstem areas responsible for cardiorespiratory control and the mid-cervical spinal cord containing the phrenic motor nucleus. Our purpose was to quantify the impact of small- and large-diameter phrenic afferent activation on phrenic motor output. Anesthetized and ventilated rats received unilateral phrenic nerve stimulation while contralateral phrenic motor output and blood pressure were recorded. Twelve currents of 40-Hz inspiratory-triggered stimulation were delivered (20 s on, 5 min off) to establish current response curves. Stimulation pulse width was varied to preferentially activate large-diameter phrenic afferents (narrow pulse width) and recruit small-diameter fibers (wide pulse width). Contralateral phrenic amplitude was elevated immediately poststimulation at currents above 35 µA for wide and 70 µA for narrow pulse stimulation when compared with animals not receiving stimulation (time controls). Wide pulse width stimulation also increased phrenic burst frequency at currents ≥35 µA, caused a transient decrease in mean arterial blood pressure at currents ≥50 µA, and resulted in a small change in heart rate at 300 µA. Unilateral dorsal rhizotomy attenuated stimulation-induced cardiorespiratory responses indicating that phrenic afferent activation is required. Additional analyses compared phrenic motor amplitude with output before stimulation and showed that episodic activation of phrenic afferents with narrow pulse stimulation can induce short-term plasticity. We conclude that the activation of phrenic afferents 1) enhances contralateral phrenic motor amplitude when large-diameter afferents are activated, and 2) when small-diameter fibers are recruited, the amplitude response is associated with changes in burst frequency and cardiovascular parameters.

New & Noteworthy
Acute, inspiratory-triggered stimulation of phrenic afferents increases contralateral phrenic motor amplitude in adult rats. When small-diameter afferents are recruited, the amplitude response is accompanied by an increase in phrenic burst frequency, a transient decrease in mean arterial blood pressure, and a slight increase in heart rate. Repeated episodes of large-diameter phrenic afferent activation may also be capable of inducing short-term plasticity.

Introduction
Sensory input from the diaphragm plays a role in modulating cardiorespiratory output. For example, activation of diaphragm/phrenic afferents can alter ventilation (1), influence phrenic and intercostal motor neuron excitability (2), and modify cardiovascular parameters including blood pressure and sympathetic outflow (3, 4). Afferents within the phrenic nerve include large-diameter myelinated fibers (Ia, Ib, II) and small-diameter thinly myelinated (III) and unmyelinated C fibers (IV). The composition of afferents in the diaphragm is similar to other skeletal muscles, with the exception that the diaphragm contains fewer muscle spindles (Ia fibers) (5), but their presence has been confirmed in the rat (6). Phrenic afferent input is conveyed to multiple brainstem areas involved in cardiorespiratory control including the dorsal and ventral respiratory groups (DRG and VRG, respectively), nucleus of the tractus solitarius (NTS) (7, 8), rostral ventral lateral medulla (4), and to the mid-cervical spinal cord containing the phrenic motor nucleus (9). Within the cervical spinal cord, afferent projections are primarily observed in the ipsilateral dorsal columns (10), dorsal gray matter (laminae I–IV) (9), and to a lesser extent in the intermediate gray matter (VII and X) and ventral horn (9, 11). Collectively, these reports confirm anatomical pathways between phrenic afferents and medullary centers controlling cardiorespiratory output and the phrenic neural network in the cervical spinal cord.

Activation of phrenic afferents elicits complex respiratory responses that may involve excitation and/or inhibition of phrenic motor output (for recent review, see Ref. 12). The complexity and often contradictory
nature of published results likely reflects 1) activation of different subsets of diaphragm/phrenic afferent neurons, 2) the time scale of the observed response, 3) whether spinal and/or supraspinal mechanisms were activated, and 4) if ipsilateral or contralateral phrenic motor output was considered. Indeed, activation of both large- and small-diameter phrenic afferents using single pulse stimulation triggers a short-latency (8–12 ms) inhibition of contralateral phrenic motor neurons referred to as the phrenic-to-phrenic reflex (2, 8, 13) and is likely mediated by spinal mechanisms, at least in rats (14). By contrast, repetitive activation with continuous 40-Hz stimulation to activate large- and small-diameter myelinated phrenic afferents initially decreases (several breaths) then increases respiratory parameters such as phrenic nerve activity, tidal volume, and minute ventilation (15). Overall, the literature suggests that large-diameter afferents are most often inhibitory, whereas small-diameter afferents have an excitatory effect on respiratory parameters such as diaphragm electromyogram (EMG) output, tidal volume, and frequency (1, 16, 17). Small-diameter phrenic afferents also activate sympathetic neural outflow (4, 18), increase arterial blood pressure (3), and play an important role in modulating blood flow distribution especially during exercise (3, 19). The acute impact of phrenic afferent activation on cardiorespiratory output, especially in rodent models routinely used for neural control studies, remains largely unexplored. Here, we used 40-Hz electrical stimulation to activate phrenic afferents during the inspiratory phase to determine the response of contralateral phrenic amplitude, frequency, arterial blood pressure, and heart rate. Given the variability of respiratory responses associated with fiber type, we varied the pulse width to activate large-diameter myelinated axons (narrow pulse) and recruit small-diameter myelinated and unmyelinated afferents (wide pulse) (20). We tested the hypothesis that narrow pulse width stimulation would decrease contralateral amplitude and have little effect on cardiovascular parameters, whereas wide pulse width stimulation would increase contralateral amplitude and frequency and modulate cardiovascular parameters.

Materials And Methods

Animals

Electrophysiology experiments were performed with adult, male (n = 20; 378 ± 8 g; 16 ± 0.4 wk) and female (n = 20; 249 ± 3 g; 16 ± 0.3 wk) Sprague-Dawley rats from colony 208a at ENVIGO (formerly Harlan Laboratories). Equal numbers of male (n = 5) and female (n = 5) rats were included in each of the four experimental groups discussed in section In Vivo Electrophysiology. Rats were housed in a controlled environment (12-h light/dark cycles) with access to food and water ad libitum. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Florida (Protocol No. 201807438).

In Vivo Electrophysiology

Rats were anesthetized in a closed chamber with 3% isoflurane (in 100% O2). Once anesthetized, rats were transferred to the surgical station where isoflurane anesthesia was maintained via nose cone at ~1% and a tail vein catheter was placed. Rats were converted (6 mL/h; Harvard Apparatus syringe pump) to urethane anesthesia (1.7 g/kg iv in distilled water) and isoflurane was slowly withdrawn. Core body temperature was maintained at 37 ± 0.5°C with a homeothermic heating device (CWE, model 700 TC-1000). The trachea was cannulated, and rats were mechanically ventilated (Harvard Apparatus, Rodent Ventilator 683; volume: ~2.5–3 mL; frequency: 70/min). CO2 was added (<3% FICO2/FICO2) to the inspiratory line to maintain end-tidal CO2 (ETCO2) between ~40 and 50 mmHg throughout the protocol (Capnogard; Respironics, Inc.). Lungs were periodically (~1/h) hyperinflated (2–3 breaths) during surgical procedures. A bilateral vagotomy was performed lateral to the trachea. A femoral arterial catheter was placed to monitor blood pressure and sample arterial blood gases (ABL-90). Using a dorsal approach, the left and right phrenic nerves were isolated, cut distally, and approximately half of the exposed nerve was desheathed. Animals received the neuromuscular paralytic pancuronium bromide (2.5 mg/kg iv, Hospira, Inc.) to eliminate spontaneous breathing efforts. Adequate depth
of anesthesia was verified by assessing responses to toe-pinches. Urethane supplements (0.2 mL bolus, iv) were given until toe-pinches did not cause an increase in phrenic burst amplitude, frequency, or mean arterial blood pressure. Blood pressure and fluid homeostasis was maintained by a slow infusion of a 1:4 solution (8.4% sodium bicarbonate/Lactated Ringers, iv). In animals receiving a left dorsal rhizotomy, a laminectomy was performed from C3 to C6, the dura was cut and reflected and the C3, C4, C5, C6 dorsal rootlets were identified and cut at the spinal insertion point with a micro-scissors.

Bilateral phrenic nerve output was recorded using custom-made bipolar suction electrodes filled with 0.9% saline. Compound action potentials were amplified (×10k), band-pass filtered (3 Hz to 3 kHz; A-M Systems differential AC Amplifier; model 1700), and digitized (16-bit, 25 kHz/channel; Power1401 3A, Cambridge Electronic Design, CED) in Spike2 v8 software (CED). The contralateral phrenic signal was integrated (time constant 50 ms) with a moving averager (CWE; Model MA-821/RSP) and used for the stimulation trigger paradigm (described later in this paragraph). Once phrenic nerve activity was stable, baseline output was recorded for 15 min. Animals were selected for one of four experimental groups: 1) narrow pulse, 2) wide pulse, 3) time controls, or 4) dorsal rhizotomy and wide pulse. In animals receiving stimulation, the left suction electrode was connected to a digital stimulus isolator unit (A-M Systems; Model 2300) and phrenic nerve stimulation was delivered during the inspiratory phase at 40 Hz for 20 s (Fig. 1). Narrow pulse width stimulation consisted of biphasic pulses 0.1 ms/phase or wide pulse width stimulation 1.0 ms/phase. Stimulation was delivered during the inspiratory phase using the integrated contralateral phrenic signal and the graphical sequence editor in Spike 2. Specifically, a horizontal cursor (trigger threshold) was placed on the hardware integrated contralateral signal at an amplitude that corresponded to the beginning of the phrenic burst (Fig. 1B). When the integrated signal crossed the trigger threshold, stimulation was delivered. A separate event channel was generated in real time to mark each biphasic pulse (stimulus waveform; Fig. 1B). The threshold was adjusted to maintain inspiratory-triggered stimulation. The experimental protocol consisted of 12 currents (10, 15, 20, 25, 35, 50, 70, 90, 120, 160, 220, and 300 µA) delivered for 20 s and separated by a 5-min recovery period (i.e., no stimulation). The order the currents were delivered was randomized for each animal within the experimental group and that current order was then used across the remaining experimental groups. Therefore, “animal 1” in each experimental group received the same current order. Time controls consisted of the same experimental setup, but electrical stimulation was not delivered to the animal. An arterial blood sample (~0.1 mL) was obtained via the femoral artery during the baseline, and after 4, 8, and 12 episodes of stimulation, which was roughly every 20 min. Adjustments to inspired gases or fluids were made to keep arterial Pco2 within 1.5 mmHg of baseline, arterial Po2 levels >150 mmHg, and base excess within 3 mEq/L from baseline values.

Figure 1. Experimental setup. A: schematic illustrating unilateral phrenic nerve stimulation (left nerve) and contralateral phrenic recording (right nerve). B: stimulation trigger paradigm. Example traces showing raw and integrated contralateral phrenic output used to define the inspiratory phase and deliver inspiratory-triggered stimulation (stimulus waveform) to the left phrenic nerve.
Data and Statistical Analysis
Values obtained from arterial blood gases (PaCO2, PaO2, and pH) sampled before (baseline) and after every fourth stimulation (∼20, 40, and 60 min after the first stimulation) were analyzed using separate two-way repeated-measures ANOVAs (factor one: time; factor two: experimental group) and Fisher’s least significant differences (LSD) post hoc tests were used for individual comparisons (Prism 9, RRID: SCR_005375; GraphPad Prism, RRID: SCR_002798). Mean arterial blood pressure and heart rate immediately before blood gases were analyzed using separate two-way repeated-measures ANOVAs and Fisher’s LSD post hoc tests were used for individual comparisons.

Raw phrenic nerve signals were integrated with Spike 2 software (DC remove: 0.04; rectify; smooth: 0.05 ms time constant) and used for analysis. Integrated contralateral phrenic burst amplitude and frequency were analyzed during baseline (before stimulation) and over 20-s windows before, during, and after each current or an equivalent time frame in time controls. To test our a priori hypothesis, amplitude and frequency during the 20 s after the cessation of stimulation was expressed as a percent change (amplitude) or absolute change (frequency) from data during the 20 s immediately before each current. This timepoint was selected to avoid possible contribution of stimulation artifact to amplitude. Initial analyses were performed to examine sex as a variable. A two-way repeated-measures ANOVA (using a mixed-effects model) was performed for each experimental group to detect significant differences between sexes and Fisher’s least significant differences (LSD) post hoc test for individual current comparisons. When sex was considered, resulting ANOVA values for amplitude were as follows: wide pulse: $F(1,8) = 0.6417, P = 0.4462$; narrow pulse: $F(1,8) = 1.762, P = 0.2210$; dorsal rhizotomy: $F(1,8) = 0.2676, P = 0.6189$; and time control: $F(1,8) = 0.2398, P = 0.6375$. Post hoc analysis revealed slight variations in amplitude at two currents within the narrow pulse group (70 µA, $P = 0.0361$ and 220 µA, $P = 0.0290$). Resulting ANOVA values for frequency when sex was considered: wide pulse: $F(1,8) = 0.4830, P = 0.5068$; narrow pulse: $F(1,8) = 0.7031, P = 0.4261$; dorsal rhizotomy: $F(1,8) = 0.4279, P = 0.5314$; and time control: $F(1,8) = 0.1477, P = 0.7107$. Post hoc analysis indicated some variation in frequency at 220 µA in the wide pulse group ($P = 0.0013$). Although slight differences were noted at three currents, $P$ values for sex indicated no sex differences in stimulation response curves within each experimental group and therefore, males and females were combined, and contralateral amplitude and frequency were analyzed with a two-way repeated-measures ANOVA (using a mixed-effects model), and Fisher’s LSD post hoc tests were used for individual comparisons.

Subsequent analyses were performed to examine if repeated episodes of phrenic nerve stimulation have a cumulative effect on contralateral phrenic amplitude. Amplitude during the 20-s or 5-min poststimulation was expressed as a percent change from data before the first episode of stimulation (i.e., baseline). A two-way repeated-measures ANOVA (using a mixed-effects model) was used to detect significant differences between experimental groups, and Fisher’s LSD post hoc test for individual comparisons were performed.

Results
Table 1 lists arterial blood gas values (PaCO2, PaO2, and pH), mean arterial blood pressure (MAP), and heart rate (HR) before stimulation (baseline) and for 60 min after the first episode of stimulation. No changes were detected for PaCO2 [time: $F(3,108) = 1.263, P = 0.2909$; group: $F(3,36) = 0.0467, P = 0.9864$] or pH [time: $F(3,108) = 1.805, P = 0.1506$; group: $F(3,36) = 0.2840, P = 0.8366$]. Although all groups had PaO2 well above 100 mmHg, some minor differences were noted when experimental group [$F(3,36) = 2.34, P = 0.0892$] and time [$F(3,108) = 8.47, P < 0.0001$] were considered (Table 1). When MAP and HR were considered, no changes were detected for MAP [time: $F(3,108) = 0.7222, P = 0.5409$; group: $F(3,36) = 0.6149, P = 0.6098$], but a significant interaction was observed for HR [$F(9,108) = 2.566, P = 0.0103$; Table 1].
Table 1. Arterial levels of Pco₂, Po₂, and pH and mean arterial blood pressure and heart rate within each experimental group during baseline (before stimulation) and at 20, 40, and 60 min after the first episode of stimulation

<table>
<thead>
<tr>
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<th>Pco₂, mmHg</th>
<th>Po₂, mmHg</th>
<th>pH</th>
<th>MAP, mmHg</th>
<th>HR, beats/min</th>
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<td>48.3 ± 1.2</td>
<td>338 ± 4</td>
<td>7.33 ± 0.01</td>
<td>128 ± 6</td>
<td>398 ± 6</td>
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<tr>
<td>20 min</td>
<td>48.2 ± 1.2</td>
<td>328 ± 7</td>
<td>7.33 ± 0.01</td>
<td>130 ± 7</td>
<td>396 ± 7</td>
</tr>
<tr>
<td>40 min</td>
<td>49.2 ± 1.2</td>
<td>322 ± 7</td>
<td>7.33 ± 0.01</td>
<td>131 ± 8</td>
<td>393 ± 6</td>
</tr>
<tr>
<td>60 min</td>
<td>48.2 ± 1.1</td>
<td>307 ± 10*</td>
<td>7.33 ± 0.02</td>
<td>131 ± 9</td>
<td>369 ± 6</td>
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<tr>
<td>Baseline</td>
<td>47.3 ± 1.1</td>
<td>322 ± 9</td>
<td>7.33 ± 0.01</td>
<td>134 ± 4</td>
<td>395 ± 6</td>
</tr>
<tr>
<td>20 min</td>
<td>47.7 ± 1.1</td>
<td>314 ± 10</td>
<td>7.33 ± 0.01</td>
<td>136 ± 4</td>
<td>397 ± 6</td>
</tr>
<tr>
<td>40 min</td>
<td>47.4 ± 1.1</td>
<td>306 ± 12</td>
<td>7.33 ± 0.01</td>
<td>134 ± 6</td>
<td>397 ± 7</td>
</tr>
<tr>
<td>60 min</td>
<td>47.1 ± 1.1</td>
<td>292 ± 10*</td>
<td>7.31 ± 0.01</td>
<td>132 ± 7</td>
<td>400 ± 7</td>
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<tr>
<td><strong>Dorsal rhizotomy + wide pulse</strong></td>
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<tr>
<td>Baseline</td>
<td>48.8 ± 1.0</td>
<td>335 ± 10</td>
<td>7.31 ± 0.01</td>
<td>124 ± 5</td>
<td>386 ± 5</td>
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<tr>
<td>20 min</td>
<td>48.7 ± 1.1</td>
<td>332 ± 10</td>
<td>7.31 ± 0.01</td>
<td>123 ± 8</td>
<td>379 ± 5</td>
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<tr>
<td>40 min</td>
<td>48.9 ± 0.9</td>
<td>332 ± 10</td>
<td>7.31 ± 0.01</td>
<td>121 ± 7</td>
<td>376 ± 4</td>
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<tr>
<td>60 min</td>
<td>48.8 ± 1.0</td>
<td>325 ± 11#</td>
<td>7.31 ± 0.01</td>
<td>120 ± 8</td>
<td>377 ± 5</td>
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<td><strong>Time controls</strong></td>
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<tr>
<td>Baseline</td>
<td>47.4 ± 1.6</td>
<td>310 ± 12</td>
<td>7.33 ± 0.01</td>
<td>128 ± 4</td>
<td>395 ± 10</td>
</tr>
<tr>
<td>20 min</td>
<td>47.8 ± 1.7</td>
<td>300 ± 14</td>
<td>7.33 ± 0.01</td>
<td>130 ± 6</td>
<td>395 ± 10</td>
</tr>
<tr>
<td>40 min</td>
<td>47.7 ± 1.6</td>
<td>304 ± 9</td>
<td>7.33 ± 0.01</td>
<td>129 ± 7</td>
<td>394 ± 8*</td>
</tr>
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<td>60 min</td>
<td>47.9 ± 1.5</td>
<td>293 ± 11</td>
<td>7.32 ± 0.01</td>
<td>128 ± 9</td>
<td>391 ± 8*</td>
</tr>
</tbody>
</table>

Data shown are means ± SE. Sample number (n = 10 per experimental group, balance male and female), separate two-way repeated-measures ANOVAs for each variable. HR, heart rate; MAP, mean arterial blood pressure. *Significantly different than baseline. #Significantly different than narrow pulse at 60 min. P < 0.05.

Phrenic Nerve Stimulation Increases Contralateral Phrenic Amplitude and Frequency
Representative compressed traces of recorded cardiorespiratory output before, during, and after inspiratory-triggered phrenic nerve stimulation in each experimental group are shown in Fig. 2. Representative expanded contralateral phrenic neurograms at four currents are shown in Fig. 3, A and B depicting before the first episode of stimulation (baseline) and immediately before, during, and after wide pulse width (1.0 ms) and narrow pulse width (0.1 ms) stimulation. Heat maps of average phrenic amplitude during and after wide or narrow pulse stimulation at all currents are shown in Fig. 3, C and D. Qualitative assessment indicated that wide and narrow pulse width stimulation results in an increase in contralateral phrenic motor amplitude during and after the episode of stimulation.
Figure 2. Phrenic afferent activation modulates cardiorespiratory activity. Representative compressed (left) and expanded (right) traces of integrated contralateral phrenic output (phrenic, volts), instantaneous respiratory rate (RR, breaths/min), instantaneous heart rate (HR, beats/min), arterial blood pressure (BP, mmHg), and stimulation waveform channel (stim, volts) during baseline (before stimulation), and either 12 episodes of phrenic nerve stimulation (left) or 300 µA (right) with: wide pulse width stimulation (1.0 ms; A); narrow pulse width stimulation (0.1 ms; B), wide pulse width stimulation and dorsal rhizotomy (C), or no stimulation (time controls; D). Each trace is from a representative animal (n = 4).

Figure 3. Phrenic afferent activation increases contralateral phrenic burst amplitude. A and B: representative expanded, integrated contralateral phrenic neurograms before stimulation (baseline, BL) and before, during, and for 20 s after 4 currents (20, 50, 120, and 300 µA) using a wide (1.0 ms) or narrow pulse width (0.1 ms). Stacked traces from a representative animal (n = 2). C and D: heat maps of average amplitude during and for 20 s after stimulation (% change from pre-stim) at all 12 currents using a wide (1.0 ms) or narrow pulse width (0.1 ms). Heat maps depict average data (n = 10/graph).

Expanded examples of contralateral phrenic amplitude and frequency during one episode of stimulation with wide and narrow pulse width are shown in Fig. 4A. To evaluate the response to each current and avoid the potential stimulation artifact, average contralateral phrenic amplitude immediately poststimulation was compared with amplitude before stimulation as illustrated by Fig. 4B. Average percent change in phrenic burst amplitude at each current or an equivalent point in time controls is shown in Fig. 4C. There was a significant interaction between stimulus current and experimental group on contralateral phrenic amplitude [F(33,383) = 4.842, P < 0.0001]. Post hoc evaluation revealed a difference between the wide pulse stimulation group and time controls at and above stimulus currents of 35 µA (all P ≤ 0.0023). A difference between narrow pulse stimulation and time controls was observed at and above 70 µA (all P ≤ 0.0132). In separate animals, stimulation was repeated after unilateral cervical dorsal rhizotomy to prevent afferent volleys from reaching the spinal cord.
Compared with responses after wide pulse stimulation when the dorsal roots were intact, differences were noted at currents above 35 µA (all $P \leq 0.0038$). When compared with narrow pulse stimulation, the rhizotomy group had reduced output at stimulus currents above 90 µA (all $P \leq 0.0295$). Collectively, these results indicate that wide pulse width stimulation results in a greater increase in contralateral phrenic amplitude compared with narrow pulse width stimulation, and these changes in motor output are the result of phrenic afferent activation.

![Figure 4](image)

**Figure 4.** Phrenic afferent activation increases contralateral phrenic burst amplitude and frequency. A: representative examples of wide and narrow pulse width stimulation at 160 µA depicting raw and integrated contralateral phrenic output (Raw, Int, volts), instantaneous phrenic burst frequency (Freq, breaths/min), average amplitude (Amp, volts), and stimulus waveform channel (stim, volts). RR, respiratory rate. B: schematic that illustrates data analysis to generate current response curves shown in C and D. Contralateral phrenic amplitude and frequency immediately poststimulation (initial 20 s) was compared with pre-stim values (20 s before each current). C: average change in amplitude poststimulation (% from pre-stim) at each current or equivalent period in time controls not receiving stimulation. D: average change in frequency poststimulation (absolute change from pre-stim) or an equivalent period in time controls not receiving stimulation. Data shown are means ± SE. Sample number ($n = 40$), balance male and female, two-way repeated-measures ANOVA. *Significantly different from time controls; #significantly different from dorsal rhizotomy; $significantly different than narrow pulse. $P < 0.05$.

Average change in phrenic burst frequency immediately poststimulation at each current or an equivalent point in time controls is shown in Fig. 4D. There was a significant interaction between current and experimental group for phrenic burst frequency poststimulation [$F(33,393) = 2.130, P = 0.0004$]. Post hoc evaluation revealed a
difference between wide pulse stimulation and time controls at all currents higher than 35 µA (all $P \leq 0.0438$). No differences were detected at most currents when narrow pulse stimulation was compared with time controls (all $P \geq 0.1210$) or dorsal rhizotomy (all $P \geq 0.1049$). A slight increase in frequency was noted at 50 ($P = 0.0900$), 70 ($P = 0.0947$), and 160 µA ($P = 0.0737$) when narrow pulse was compared with time controls and dorsal rhizotomy at 70 µA ($P = 0.0745$); however, this did not reach statistical significance. When dorsal rhizotomy was compared with wide pulse stimulation, differences were noted at 35, 70, and 90 µA, and currents at and above 160 µA (all $P \leq 0.0051$) and above 90 µA when compared with narrow pulse stimulation (all $P \leq 0.0295$). These results indicate that wide pulse width stimulation increases phrenic burst frequency poststimulation and requires activation of phrenic afferents.

Phrenic Afferent Activation Transiently Decreases MAP

Representative traces of before, during, and after wide or narrow pulse width stimulation at four currents are shown in Fig. 5, A and B. Heat maps illustrate average MAP during and after wide or narrow pulse stimulation at all currents are shown in Fig. 5, C and D. Qualitative assessment indicates that shortly after the onset of phrenic nerve stimulation (within the first 10 s), a brief decrease in MAP is observed and the response is greater with wide pulse stimulation.

![Figure 5: Phrenic afferent activation transiently reduces mean arterial pressure. A and B: representative traces of mean arterial pressure (mmHg) immediately prior to stimulation (PS), during, and for 20 s after 4 different currents (20, 50, 120, and 300 µA) using a wide (1.0 ms) or narrow pulse width (0.1 ms). Each data point is an average of mean arterial pressure over 2 s. Traces from a representative animal ($n = 2$). C and D: heat maps of average mean arterial pressure (absolute change from pre-stim) during and for 20 s after stimulation at all 12 currents using a wide (1.0 ms) or narrow pulse width (0.1 ms). Heat maps depict average data ($n = 10$/graph).](image)

Representative examples of contralateral phrenic amplitude, arterial blood pressure, and instantaneous heart rate during one episode of stimulation with wide and narrow pulse width are shown in Fig. 6A. To quantify the change in MAP in response to each current, average MAP during the first 10 s of stimulation was compared with values before stimulation as illustrated by Fig. 6B. Average change in MAP during each current or an equivalent point in time controls is shown in Fig. 6C. There was a significant interaction between the experimental group and current on MAP [$F(33,391) = 1.715, P = 0.0097$]. A difference between wide pulse stimulation and time controls at currents higher than 50 µA (all $P \leq 0.0259$) and from dorsal rhizotomy at currents above 25 µA (except 35 µA; all $P \leq 0.0193$) was observed. When narrow pulse stimulation was compared with time controls and dorsal rhizotomy, a difference was noted at 160 µA ($P \leq 0.0017$ and $P \leq 0.0001$, respectively). Narrow and wide pulse stimulation were different at 70, 220, and 300 µA (all $P \leq 0.0303$). These data indicate that unlike narrow pulse stimulation, wide pulse stimulation induces a transient decrease in MAP, which is mediated by phrenic afferent activation.
Figure 6. Phrenic afferent activation transiently decreases mean arterial blood pressure. A: representative examples of wide and narrow pulse width stimulation at 220 µA depicting integrated contralateral phrenic output (int, volts), instantaneous heart rate (HR, beats/min), arterial blood pressure (BP, mmHg), and stimulus waveform channel (stim, volts). B: schematic that illustrates data analysis to generate current response curves for mean arterial blood pressure (MAP) shown in C. A change in MAP was observed during the first half of stimulation and, therefore, data were analyzed during the initial 10 s of stim and compared with pre-stim values (20 s before each current). C: average change in MAP during stimulation (absolute change from pre-stim) at each current or equivalent period in time controls not receiving stimulation. D: schematic that illustrates data analysis to generate current response curves for heart rate (HR) shown in E. A change in HR was observed after stimulation and, therefore, data were analyzed post-stim (20 s) and compared with pre-stim values (20 s before each current). E: average change in HR poststimulation (absolute change from pre-stim) or an equivalent period in time controls not receiving stimulation. Data shown are means ± SE. Sample number (n = 40), balance male and female, two-way repeated-measures ANOVA. *Significantly different from time controls; #significantly different from dorsal rhizotomy. P < 0.05.

Visual inspection of the data showed a trend for heart rate (HR) to change poststimulation. To quantify the change in HR in response to each current, average HR immediately poststimulation was compared with values before stimulation as illustrated by Fig. 6D. Average change in HR after each current or an equivalent time frame in time controls is shown in Fig. 6E. There was a main effect for current \( F(11,392) = 3.106, P = 0.0005 \) but not for experimental group \( F(3,36) = 1246, P = 0.3075 \). Post hoc analysis showed wide pulse width was different than rhizotomy at 220 (\( P = 0.0251 \)) and 300 µA (\( P = 0.0006 \)) and time controls at 300 µA (\( P = 0.0042 \)). A
difference was also found between narrow pulse and rhizotomy at 220 µA ($P = 0.0142$). These results suggest that phrenic afferent stimulation with wide pulse induces a small change in HR at higher currents which is induced by phrenic afferent activation.

Repeated Episodes of Phrenic Nerve Stimulation Induces Short-Term Plasticity

Although our a priori goal was to establish current-response curves using wide and narrow pulse width stimulation, additional analysis was performed to evaluate if repeated episodes of phrenic nerve stimulation have a cumulative effect on contralateral phrenic amplitude. Contralateral phrenic amplitude immediately poststimulation was compared with amplitude before the first episode of stimulation (i.e., baseline) as shown in Fig. 7A. Average percent change in phrenic burst amplitude after each episode of stimulation or an equivalent point in time controls is shown in Fig. 7B. There was a main effect for experimental group [$F(3,36) = 7.999, P = 0.0003$] and stimulation episode [$F(11,393) = 4.625, P < 0.0001$]. Post hoc evaluation revealed a difference between wide pulse stimulation and time controls after all episodes (all $P \leq 0.0434$) and from dorsal rhizotomy at most timepoints (all $P \leq 0.0148$; except episodes 10 and 12, both $P \geq 0.0537$). Differences were detected when narrow pulse stimulation was compared with time controls (all $P \leq 0.0339$) and dorsal rhizotomy at episodes 3 and after ($P \leq 0.0274$; although episode 4 did not reach statistical significance: $P = 0.0574$). These data indicate that wide pulse stimulation does not have cumulative effect since a similar increase in contralateral phrenic amplitude was observed after all episodes. By contrast, contralateral phrenic amplitude steadily increased as additional episodes of narrow pulse stimulation were delivered.

Figure 7. Repeated phrenic afferent activation induces plasticity. Contralateral phrenic amplitude was analyzed after each episode of stimulation (i.e. 1st = first episode of stimulation) and compared with amplitude before stimulation (baseline, BL). Since currents were randomized within an experimental group, each episode (i.e. 1st, 2nd, etc.) represents a range of currents. A: schematic that illustrates data analysis to determine the initial phrenic motor response to repeated episodes of stimulation shown in B. Amplitude immediately poststimulation (initial 20 s) was compared with baseline (BL; i.e., before stimulation). B: average change in amplitude poststimulation (% change from BL) after each episode or equivalent period in time controls not receiving stimulation. C: schematic that illustrates data analysis to determine whether phrenic motor amplitude returns to baseline before the next episode of stimulation shown in D. Amplitude 5-min poststimulation was compared with baseline (BL; i.e., before stimulation). D: average change in amplitude poststimulation (% change from BL) after each episode or equivalent period in time controls not receiving stimulation. Data shown are means ± SE. Sample number ($n = 40$), balance male and female, two-way repeated-measures ANOVA. *Significantly different from time controls; #significantly different from dorsal rhizotomy. $P < 0.05$.

To determine whether amplitude remained elevated before the next episode of stimulation, contralateral phrenic amplitude 5-min poststimulation was compared with baseline (Fig. 5C). Average percent change in phrenic burst amplitude after each episode of stimulation or an equivalent point in time controls is shown in Fig. 7C. There was an interaction between experimental group and stimulation episode on contralateral phrenic amplitude poststimulation [$F(33,393) = 1.594, P = 0.0222$]. Post hoc analysis produced low $P$ values when narrow pulse was compared with time controls at episodes 4 and after (all $P \leq 0.0316$) and dorsal rhizotomy at episodes
5–10 (all \( P \leq 0.0433 \)). When wide pulse stimulation was compared with time controls, differences were observed at episodes 6–10 (all \( P \leq 0.0217 \)). These results indicate that contralateral phrenic amplitude remained elevated with repeated episodes of narrow pulse stimulation to a greater extent than wide pulse stimulation and was attenuated by the removal of cervical afferent input.

Discussion

The results of our study demonstrate that unilateral phrenic nerve stimulation used to activate phrenic afferents during the inspiratory phase alters cardiorespiratory parameters in an anesthetized, adult rat preparation. Wide pulse width stimulation increased contralateral phrenic amplitude and burst frequency at currents \( \geq 35 \) µA, induced a transient decrease in mean arterial blood pressure at currents \( \geq 50 \) µA, and caused a small increase in heart rate at 300 µA. Narrow pulse stimulation increased contralateral phrenic amplitude at and above 70 µA, did not alter phrenic burst frequency, and had little effect on cardiovascular parameters. Additional analyses revealed that repeated episodes of narrow pulse stimulation have a cumulative effect on contralateral amplitude such that after four episodes of afferent activation, output remains elevated for 5 min. Collectively, these results add to our understanding of respiratory neural control by showing that phrenic afferent activation has an excitatory effect on contralateral motor output and may be capable of inducing motor plasticity.

Activation of Phrenic Afferents

The phrenic nerve is a mixed fiber nerve, containing \( \sim 30\%–45\% \) sensory afferent fibers (21, 22). Sensory fiber types represented within the phrenic nerve include large-diameter myelinated (Ia, Ib, II) and small-diameter myelinated (III) and unmyelinated fibers (IV). During eupneic breathing, Ia fibers (muscle spindles) discharge in response to muscle lengthening and are, therefore, active primarily during expiration. Ib fibers (golgi tendon organs) show activity during inspiration, as they discharge with muscle contraction. Group III and IV fibers (free nerve endings) show irregular discharge patterns and can be active during either or both phases of breathing. Fibers within the phrenic nerve also consist of postganglionic sympathetic axons (23), preganglionic efferents (22), and pericardial afferents that discharge in phase with the cardiac rhythm (24).

In the current study, we applied electrical stimulation to the whole phrenic nerve during the inspiratory phase to activate phrenic afferent fibers. Electrical stimulation of a mixed fiber nerve can activate both motor and sensory fibers. However, few, if any, phrenic motor neuron dendrites cross the spinal midline in adult rats, and therefore antidromic motor neuron activation is expected to have no direct effect on contralateral phrenic motor output (25). This assumption is confirmed by experiments involving unilateral dorsal root rhizotomy before stimulation and suggests that contralateral phrenic and cardiovascular responses are due to the activation of ipsilateral afferents versus antidromic activation of phrenic motor neurons or sympathetic fibers.

The primary hypothesis that the activation of large-diameter afferents (groups I and II) would decrease contralateral phrenic amplitude and the activation of both large- and small-diameter afferents (recruitment of group III and IV) would increase amplitude was tested by using two pulse widths of stimulation, narrow pulse width (0.1 ms) and wide pulse width (1.0 ms), respectively. Varying pulse width during direct nerve stimulation has been established to differentially activate nerve fibers based on diameter (20). Large-diameter myelinated afferents have a low threshold of activation and can, therefore, be activated with narrow pulse width stimulation. In the adult rat, a pulse width of 0.2 ms elicits an H-reflex due to the activation of large-diameter fibers, predominantly group Ia fibers, with a possible contribution from group Ib/II (26). In contrast, small-diameter myelinated and unmyelinated afferents are high-threshold fibers and require a wider pulse width (or pulse trains) and higher stimulus currents for excitation (27–29). Indeed, C fibers (group IV) can be recruited with a pulse width \( \geq 0.5 \) ms in rats (30). In addition, selective activation of small-diameter, but not large-diameter phrenic afferents, have been shown to alter cardiovascular parameters (3, 4, 31). Our results showing differential cardiovascular responses when pulse width is varied is consistent with preferential activation of
large-diameter afferents with narrow pulse width stimulation and recruitment of small-diameter afferents with wide pulse width stimulation. Although we acknowledge the limitation of utilizing pulse width to definitively activate specific afferent fiber groups, it is a reasonable assumption based on the literature.

**Phrenic Afferent Impact on Respiratory Parameters**

Multiple reports have confirmed that phrenic afferent activation modulates phrenic motor output, intercostal motor output, and ventilation (1, 15, 16, 32). Whether the functional impact is excitatory or inhibitory to respiratory output is largely dependent on the afferent population that is activated. Small-diameter afferents (group III and IV) have been most frequently reported to have an excitatory effect on respiratory-related outcomes. For example, single pulse stimulation activates phrenic motor neurons (33), increases diaphragm EMG (32, 34), and enhances ventilation (34). In contrast, large-diameter afferents (groups Ia, Ib, and II) have been generally found to be inhibitory as they reduce phrenic motor neuron activity (2, 14, 35) and decrease diaphragm EMG output (36). Although the short-latency (∼8–12 ms) phrenic-to-phrenic inhibitory reflex has been induced by activation of both large- and small-diameter afferents (14, 35). Based on these findings, we hypothesized that, narrow pulse width stimulation would decrease contralateral amplitude, whereas wide pulse width stimulation would increase contralateral amplitude. Contrary to our original hypothesis, our results show that using these parameters, both wide and narrow pulse width stimulation increases motor output from the contralateral phrenic motor nucleus. Both the experimental conditions and stimulation parameters used in our study likely contributed to the excitatory effect on amplitude we observed. First, phrenic nerve axotomy can increase the excitability of motor neurons through increases in intracellular Ca^{2+} concentration (37). Second, phrenic nerve axotomy also eliminates baseline phrenic afferent input which has the potential to alter phrenic motor responses to subsequent perturbations (2, 38). Third, higher stimulation frequency (40 Hz) and lower current intensities may be more conducive for excitatory responses. Indeed, only low-frequency stimulation (2 Hz) induces the phrenic-to-phrenic inhibitory reflex (14). When small-diameter afferents are activated, low-intensity afferent stimulation is facilitatory, whereas high-intensity stimulation tends to be inhibitory (39). Finally, several previous studies have indicated that the timing of afferent activation with regards to breath phase or motor neuron activity is an important variable to consider. Specifically, stimulation during the inspiratory, but not expiratory phase, is required to elicit the phrenic-to-phrenic inhibitory reflex (8), and the stimulation at a set interval relative to motor neuron activation is necessary to elicit prolonged changes in respiratory frequency (40). Thus, an important component of future work will be to evaluate the specific parameters critical to produce the observed motor responses.

There are multiple anatomic pathways that could underlie the impact of phrenic nerve stimulation on respiratory responses observed in our study. Most prominently, the responses could occur due to the activation of ascending afferent projections to brainstem centers and/or via polysynaptic spinal pathways involving propriospinal networks. Previous work has shown that activation of phrenic afferents can simultaneously evoke both a brainstem mediated and a segmental spinal mediated loop (14). Phrenic afferents project to brainstem locations that can directly and indirectly impact respiratory burst frequency (7, 14). Approximately 25% of respiratory-modulated neurons in the dorsal respiratory group (DRG) and ~11% in the ventral respiratory group (VRG) are excited by phrenic nerve stimulation (41). Based on latency, Speck and Revelette concluded that small, myelinated afferents make polysynaptic connections with DRG neurons. Activation of phrenic afferents using wide pulse stimulation induces expression of the neuronal activity marker c-Fos in the rostral VRG; however, the reticular nucleus and the nucleus of the tractus solitarius (NTS) showed the highest levels of c-fos-positive staining within the brainstem following this stimulation paradigm (7). As a result, the NTS may be uniquely positioned to receive phrenic afferent input and influence motor output through connections with the VRG (42). Our data suggest that only wide pulse stimulation alters respiratory burst frequency, which is consistent with a brainstem-mediated effect.
Given the finding that narrow pulse stimulation had no discernable impact on respiratory burst frequency, we hypothesize that spinal mechanisms may impact contralateral phrenic nerve activity. Within the cervical spinal cord, phrenic afferent fibers are observed in the ipsilateral dorsal horn laminae I–III, extend into deep dorsal laminae IV–V, and infrequently are observed crossing the spinal midline and projecting to the contralateral dorsal horn (9). Projections are also observed in laminae containing interneurons including the ipsilateral intermediate gray matter (lamina VII) and near the central canal (lamina X) (9–11). Indeed, afferent stimulation readily activates the cervical interneuronal network (7, 43, 44). A small population of interneurons (i.e., prephrenic) have known synaptic connections to phrenic motor neurons (45, 46) and can provide an excitatory and inhibitory influence on phrenic motor output (47). A polysynaptic spinal-mediated pathway has been proposed to mediate afferent driven respiratory responses such as the phrenic-to-phrenic reflex, since sectioning the dorsal columns at C2 does not alter the response (14). Although it remains unknown if phrenic afferents are synaptically connected to phrenic motor neurons, dense afferent projections are observed in lamina IX in neonatal rats (48) and reach the immediate vicinity of the phrenic motor neuron pool in adult rats (9). Thus, a spinal-mediated pathway may be capable of producing the observed increases in phrenic burst amplitude.

Repeated Episodes of Phrenic Afferent Activation
The respiratory system exhibits many types of respiratory motor plasticity (for review, see Ref. 49), some of which are induced by repeated afferent activation (50, 51). Indeed, carotid afferent stimulation induces a long-lasting increase in phrenic motor output (52). To our knowledge, no study has directly investigated whether phrenic afferent activation induces motor plasticity. However, instances consistent with the expression of plasticity following afferent activation can be found in the literature. For example, Road and colleagues reported a poststimulus decrease in contralateral phrenic output following 1 min of high-intensity, 20 Hz, wide pulse width stimulation (53). In a brainstem-spinal cord preparation, intermittent stimulation of the C4 dorsal roots induced a prolonged (>1 h) increase in phrenic burst frequency (40). Finally, repeated activation of small-diameter afferents via injection of capsaicin into the phrenic artery showed blunted diaphragm EMG amplitude after the second injection (54). Although our study was designed to establish a current response curve to compare the impact of narrow and wide pulse stimulation, phrenic afferents were “repeatedly” activated. Our study indicates that the first episode of wide pulse stimulation enhances burst amplitude to a greater extent than narrow pulse. However, as additional episodes of narrow pulse stimulation are presented, amplitude poststimulation progressively increases. Amplitude remains elevated for ~5 min after four or six episodes of narrow or wide pulse stimulation, but only narrow pulse stimulation resulted in increases that were statistically different than animals receiving dorsal rhizotomy. Although our data provide additional evidence that indicates phrenic afferents may induce motor plasticity, our studies were not intended to rigorously examine the expression of plasticity, and thus, future studies are necessary to fully elucidate the potential of afferent-induced contralateral motor plasticity.

Phrenic Afferent Impact on Cardiovascular Parameters
It is established that phrenic afferents project to areas responsible for autonomic control and, as such, are known to modulate cardiovascular parameters including blood pressure, HR, and sympathetic output (7, 12). Specifically, an increase in sympathetic outflow, MAP, and HR are observed with high-intensity activation of phrenic afferents (3, 4, 16, 34, 54). Alterations in cardiovascular parameters have been attributed to activation of thin-fiber phrenic afferents including group III and IV fibers. Although wide pulse width stimulation modulated MAP in our experiments, we observed a brief decrease rather than an increase which is most frequently reported in the literature. In some of our experiments, the decrease in MAP was followed by a slight increase in pressure, but this effect was not consistent across animals and, therefore, was represented in the averaged data (Fig. 5C). Only a slight increase in HR was observed at the highest current for wide pulse width stimulation. The
observed responses may be attenuated since vagal afferent input was eliminated in our experiments and is known to have a significant impact on cardiovascular activity (55). We suspect these results may also reflect the low current intensity used for our studies. Indeed, the response required to increase MAP in anesthetized dogs was 60X twitch threshold, which is estimated to be significantly higher than the currents used for our study (34). Additional evidence is provided by the observation that HR was not modulated until a high concentration of capsaicin was delivered (54). Collectively, the parameters used for our study has an excitatory effect on contralateral burst amplitude and frequency with minimal effects on cardiovascular parameters.

Significance
To date, the cardiorespiratory impact of phrenic afferent activation has been primarily studied in dog (16, 17, 32, 34, 54, 56) and cat (2, 8, 33, 35, 36, 57). Reports in the spinal intact rat have commented on the impact of phrenic afferents with regard to interneuronal activity (44) and the ability to regulate and induce respiratory plasticity (38, 40). The majority of afferent studies in the rat have shown that phrenic afferents play an important role in regulating the neuroplastic potential of phrenic motor output following spinal cord injury (SCI) (58–60). Our data add to our understanding by showing that inspiratory-triggered activation of large- and small-diameter phrenic afferents can have an excitatory effect on contralateral phrenic motor output and may, in and of itself, induce contralateral phrenic motor plasticity in the adult, spinal intact rat. In line with these results, excitatory phrenic afferents have been shown to mediate crossed-phrenic activation post-SCI (60). Although additional studies are necessary to fully characterize afferent-induced enhancement of motor output, activation of phrenic afferents may represent an underappreciated method capable of driving phrenic motor output.

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No conflicts of interest, financial or otherwise, are declared by the authors.

Author Contributions
K.A.S. and D.D.F. conceived and designed research; K.A.S. and M.D.S. performed experiments; K.A.S. analyzed data; K.A.S. interpreted results of experiments; K.A.S. prepared figures; K.A.S. drafted manuscript; K.A.S., P.W.D., and D.D.F. edited and revised manuscript; K.A.S., M.D.S., P.W.D., and D.D.F. approved final version of manuscript.

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