Functional connectivity in raphé-pontomedullary circuits supports active suppression of breathing during hypocapnic apnea

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Nuding SC, Segers LS, Iceman KE, O’Connor R, Dean JB, Bolser DC, Baekey DM, Dick TE, Shannon R, Morris KF, Lindsey BG. Functional connectivity in raphé-pontomedullary circuits supports active suppression of breathing during hypocapnic apnea. J Neurophysiol 114: 2162–2186, 2015. First published July 22, 2015; doi:10.1152/jn.00608.2015.—Hyperventilation is a common feature of disordered breathing. Apnea ensues if CO2 drive is sufficiently reduced. We tested the hypothesis that medullary raphé, ventral respiratory column (VRC), and pontine neurons have functional connectivity and persistent or evoked activities appropriate for roles in the suppression of drive and rhythm during hyperventilation and apnea. Phrenic nerve activity, arterial blood pressure, end-tidal CO2, and other parameters were monitored in 10 decerebrate, vagotomized, neuromuscularly-blocked, and artificially ventilated cats. Multielectrode arrays recorded spiking activity of 649 neurons. Loss and return of rhythmic activity during passive hyperventilation to apnea were identified with the S-transform. Diverse fluctuating activity patterns were recorded in the raphé-pontomedullary respiratory network during the transition to hypocapnic apnea. The firing rates of 160 neurons increased during apnea; the rates of 241 others decreased or stopped. VRC inspiratory neurons were usually the last to cease firing or lose rhythmic activity during the transition to apnea. Mayer wave-related oscillations (0.04–0.1 Hz) in firing rate were also disrupted during apnea. Four-hundred neurons (62%) were elements of pairs with at least one hyperventilation-responsive neuron and a correlational signature of interaction identified by cross-correlation or gravitational clustering. Our results support a model with distinct groups of chemoresponsive raphé neurons contributing to hypocapnic apnea through parallel processes that incorporate disfacilitation and active inhibition of inspiratory motor drive by expiratory neurons. During apnea, carotid chemoreceptors can evoke rhythm reemergence and an inspiratory shift in the balance of reciprocal inhibition via suppression of ongoing tonic expiratory neuron activity.

brainstem; network; breathing; hyperventilation; apnea

Breathing is a remarkably robust behavior that is activated at birth and continues until death, yet the brain stem neural network controlling it is even more remarkable in its malleability. For example, talking, swallowing, and coughing are motor acts that alter the breathing pattern and, rather than simply inhibiting breathing, the neural substrate for these motor acts transiently appropriates and reconfigures the respiratory pattern generator (Bolser et al. 2011, 2013; Shannon et al. 2004). However, what about conditions when breathing stops? Hyperventilation is a component of dangerous water breath-holding behaviors (Boyd et al. 2015; Craig 1961) and a common feature of disordered breathing (for discussion, see Abdala et al. 2014; Dempsey 2005; Laffey and Kavanagh 2002). If the drive from CO2 is sufficiently reduced, hypocapnic apnea, a transient cessation of breathing, ensues with its attendant and potentially adverse consequences (Bitter et al. 2011; Harper et al. 2013; Jawaheri and Dempsey 2013; Leung et al. 2012; Sankri-Tarbichi et al. 2009; Sankri-Tarbichi 2012).

During the transition from eupneic-like breathing to hyperventilatory apnea, phrenic motoneurons (Prabhakar et al. 1986) and phasic respiratory-modulated brain stem neurons either cease to discharge or assume a tonic pattern of activity (Bain ton and Kirkwood 1979; Batsel 1967; Cohen 1968; Haber et al. 1957; Nesland and Plum 1965; Orem and Vidruk 1998; St. John 1998; Sun et al. 2001, 2005). Without exception (Cohen 1968), these studies recorded neurons in the ventral respiratory column (VRC; Smith et al. 2013), one at a time. This approach precludes assessment of local connectivity within the VRC and of distributed interactions with pontine and raphé neurons of the respiratory network (Nuding et al. 2009a; Segers et al. 2008).

The circuit mechanisms for hypocapnic apnea remain poorly understood. Both reduced excitatory chemoreceptor drive and active inhibitory processes may contribute to the suspended state of the respiratory central pattern generator. Peripheral chemoreceptors of the carotid body monitor changes in arterial O2 and CO2-pH (Kumar and Prabhakar 2012), and central chemoreceptors, distributed among various brain stem sites, sense brain CO2-pH (Nattie and Li 2012). Mechanisms of their joint and separate influences on pattern-generating circuits are subjects of active research (Duffin and Mateika 2013a,b; Phillipson et al. 1981; Teppema and Smith 2013a,b; Wilson and Day 2013a,b). Central chemoreceptors and their follower neurons, collectively termed chemoresponsive, may be either functionally excited or inhibited by an increase in PaCO2 (e.g., Bochorishvili et al. 2012; Dean et al. 1989; Guyenet et al. 2010; Marina et al. 2010; Nuding et al. 2009b; Ott et al. 2011, 2012; Richerson et al. 2001).

Medullary raphé neurons have diverse responses to hypercapnia and acidosis: firing rates of serotonergic neurons increase (Brust et al. 2014; Iceman et al. 2013; Severson et al. 2003; Veasey et al. 1995; Wang et al. 1998, 2001), whereas GABAergic raphé neurons are functionally inhibited (Iceman et al. 2010).
et al. 2014). These results are consistent with the hypothesis that distinct populations of chemoresponsive raphé neurons produce an additive “push-pull” enhancement of breathing via excitation and disinhibition, respectively (Richerson et al. 2001), a notion similar to that proposed for baroreceptor-evoked modulation of breathing via raphé-mediated excitation and disinhibition of ventral respiratory column inspiratory neurons (Lindsey et al. 1998). The distinct chemoresponsive profiles of different raphé neuron populations led us to conjecture that cells effectively excited during hypercapnia would exhibit decreased firing rates during hypocapnia and vice versa for neurons inhibited during hypercapnia. This possibility and gaps in our knowledge of network interactions motivated us to test the hypothesis that medullary raphé, VRC, and pontine neurons have functional connectivity as well as persistent and evoked activities appropriate for roles in the suppression of respiratory drive and rhythm during hyperventilation and hypocapnic apnea.

Sears et al. (1982) demonstrated reciprocal tonic activation of inspiratory and expiratory motor neurons during hypocapnic apnea. A PaCO2 drive below the apneic threshold may promote expiratory activity and functionally suppress inspiration. Hypoxia associated with apnea evokes increased peripheral chemoreceptor activity, enhances or elicits tonic inspiratory motor neuron activities, and can reestablish respiratory rhythmogenesis. Fluctuations in this peripheral chemoreceptor-mediated “inspiratory shift,” operating through unknown circuit mechanisms, may contribute to periodic breathing in heart failure and central sleep apnea (Lovering et al. 2012).

Our multielectrode arrays allow concurrent single-unit recordings from multiple brain stem nuclei that generate and modulate breathing. This approach is well suited for testing our hypothesis and assessment of the activity patterns of many neurons under the same conditions. Thus we recorded changes in firing rates during different chemoreceptor-evoked perturbations of breathing and evaluated spike trains for correlation features indicative of functional connectivity. Preliminary accounts of this work have been presented (Lindsey et al. 2014; Nuding et al. 2005, 2013).

METHODS

All experiments were performed according to protocols approved by the University of South Florida’s Institutional Animal Care and Use Committee with strict adherence to all American Association for Accreditation of Laboratory Animal Care International, National Institutes of Health and National Research Council guidelines. Descriptions of methods that were recently published (Segers et al. 2015) are briefly reviewed.

Surgical protocols. Data were obtained from 10 adult cats (2.8–5.4 kg) of either sex. Animals were initially anesthetized with 5% isoflurane mixed with air or with ketamine hydrochloride (5.5 mg/kg im) followed by isoflurane; all were maintained with 0.5–3.0% isoflurane until decerebration (Kirsten and St. John 1978). Arterial blood pressure, end-tidal CO2, and tracheal pressure were monitored continuously; arterial PO2, PCO2, and pH were measured periodically. Animals were neuromuscularly blocked, bilaterally vagotomized, and ventilated with either air (n = 8) or 100% O2 (n = 2). At the end of each experiment, animals were euthanized [Beuthanasia (0.97 mg/kg; Schering-Plough Animal Health) or sodium pentobarbital (28 mg/kg) followed by a saturated solution of KCl in water].

Changes in chemical drive. The transition from eupneic-like baseline breathing to hypocapnic apnea was induced by increasing the ventilation rate and tidal volume. End-tidal CO2 immediately began to decrease and hyperventilation was additionally confirmed by measurement of PaCO2 and pH during six trials in five animals. A hypercapnic ventilatory stimulus was presented by switching from compressed air to a hypercapnic mixture of 5% CO2 in air for 30 s. Peripheral chemoreceptors were selectively stimulated by infusion of CO2-saturated 0.9% saline solution (pH 5.0–5.2; 1.0 ml) delivered via the carotid artery over a period of 30 s. In two experiments, carotid chemoreceptors were selectively stimulated during hypcapnic apnea to determine if the respiratory rhythm could be reactivated. Three to five stimulus trials were performed; each succeeding trial occurred at least 3 min after the previous one. We monitored blood pressure continuously and actively supported it during hyperventilation in six cats by inflating an embolectomy catheter in the descending aorta to maintain brain perfusion during hypcapnia. Two cats were ventilated with a FIO2 of 100%; six other cats were ventilated with room air supplemented with O2 during hyperventilation to limit the potential consequences of ischemia.

Data acquisition and analysis. Efferent phrenic nerve activity was monitored and used to identify the phases of breathing and as an indicator of respiratory drive and stimulus effectiveness. In some cats, the lumbar nerve was also recorded. Extracellular neuronal activity was acquired on three or four multielectrode arrays with individually adjustable (submicrometer steps) high impedance tungsten microelectrodes (1-μm tip diameter; 10–12 MΩ). Electrode placement was guided by anatomical landmarks (obex, brain stem midline), appropriate stereotaxic coordinates derived from Berman (1968), and the results of previous studies (Connelly et al. 1992; Nuding et al. 2009b; Ott et al. 2012; Schwarzacher et al. 1995; Segers et al. 2008, 2015). Neurons in the vicinity of the medullary raphé nuclei, the ventral respiratory column, and the pontine respiratory group were recorded. Signals from single neurons were isolated using one of two interactive spike sorting software packages (Datawave Technologies) (O’Connor et al. 2005). Stereotaxic coordinates of recording sites were mapped into the three-dimensional space of a computer-based brain stem atlas derived from The Brain Stem of the Cat: A Cytoarchitectonic Atlas with Stereotaxic Coordinates (Berman 1968) with permission of the University of Wisconsin Press, as described in Segers et al. (2008).

Respiratory cycle-triggered histograms (CTHs) were constructed for each neuron from a 30-min baseline period recording before any stimulus protocols. The average firing rate represented in the standard CTH (Cohen 1968) may be “blurred,” particularly at phase transitions, because cycle durations varied. Therefore, cycle- and phase-normalized histograms were also calculated (Segers et al. 2015). Neurons were classified as respiratory modulated if either of two complementary statistical tests rejected the null hypothesis (P < 0.05) (Morris et al. 1996; Netick and Orem 1981; Orem and Netick 1982). Respiratory-modulated neurons were classified as inspiratory (I) or expiratory (E) according to the part of the cycle during which the cell was most active (Cohen 1968). If the peak firing rate occurred during the first or second half of the phase, I and E cells were further classified as decrementing (Dec) or augmenting (Aug), respectively. Neurons were additionally designated as phasic (P) if, as estimated by the CTH, their firing probability was zero during any part of the respiratory cycle or tonic (T) otherwise (Morris et al. 1996). Cells without a preferred phase of maximum activity as assessed by both tests were designated nonrespiratory modulated.

The S-transform and central apnea. To visualize changes in frequency and identify the loss and reappearance of rhythmic activity with hyperventilation, a joint time-frequency representation was generated for each spike train using an implementation of the S-transform (Stockwell 2007; Stockwell et al. 1996) modified to provide a variable time-frequency resolution tradeoff. The
S-transform was calculated by numerical integration directly from its continuous-time definition:

$$S(\tau, f) = \int_{-\infty}^{\infty} h(t) \frac{1}{\sigma \sqrt{2\pi}} e^{-\frac{(t-\alpha\tau)^2}{2\sigma^2}} e^{-2\pi i f t} dt$$

The spike train signal $h(t)$ was treated as a train of unit impulses (Dirac delta functions), thereby turning the integral into a summation:

We set $\sigma = t_{ref}/\sqrt{2\pi f}$, this choice made $t_{ref}$ approximately equal to the half-amplitude width of the Gaussian in cycles and made the product of $t_{ref}$ in cycles and the frequency resolution (as a fraction of the frequency) equal to one. Unless otherwise noted, we used a constant relative time resolution (fraction of the frequency) equal to one. Unless otherwise noted, we made the product of $t_{ref}$ in cycles and frequency resolution equal to $1/4$.

The frequency range used to generate S-transforms for a spike train extended at least one order of magnitude below and above the control respiratory frequency range as measured from the phrenic nerve signal before the onset of hyperventilation. The S-transform is displayed as a heat map with luminance proportional to the displayed quantity; the extended beyond the injection or stimulus period to ensure that activity within a 60-s response window was surveyed for a response during the immediately preceding 90-s “control” period; similarly, neuronal firing rate during the immediately preceding 90-s “control” period fell below the significance threshold.

Stimulus effectiveness and neural responses. The effectiveness of each stimulus protocol was confirmed by measures of the peak amplitude of the integrated phrenic nerve signal; effective reflexes were identified by a change $>2$ SD in the peak integrated phrenic nerve amplitude from the mean of prestimulus values (Nuding et al. 2009b). For hypercapnic stimulation, neuronal firing rates in respiratory cycles during a 90-s “response” window (30-s stimulus duration plus a 60-s poststimulus period) were compared with rates in cycles during the immediately preceding 90-s “control” period; similarly, activity within a 60-s response window was surveyed for a response to peripheral chemoreceptor stimulation. Response window durations extended beyond the injection or stimulus period to ensure that responses with varying time lags would be detected. Results reported for hypercapnic and chemoreceptor stimuli are averages of three to five trials.

To identify hyperventilation-evoked changes in neuronal firing rates, the firing rate of each neuron during a control period just before the start of hyperventilation was compared with the rate during a “preapneic” interval (“A,” between the start of hyperventilation and the onset of the central apneic period) and during the central apnea (“B”). The response analysis algorithm calculates multiple measures (e.g., mean and peak firing rates) for each interval within the control and response windows; analysis of the variation in those values over the intervals determines whether a significant response is present. Typically, the respiratory cycles and phases are the intervals used. However, for analysis of response during apnea, when respiratory cycles are absent, we defined intervals that were equal to the mean duration of the respiratory cycles occurring from the start of the control period until the beginning of central apnea. This approach, and dividing each interval in half, enabled rate comparisons.

Significant responses in each of the stimulus protocols were identified using a bootstrap-based statistical method (as described in Nuding et al. 2009b); the $P$-value threshold (significance level) was set by controlling the false discovery rate to a level of 0.05 (Benjamini and Hochberg 1995). Responses of individual cells were classified according to the observed change in neuronal firing rate. Neurons could exhibit an increase (↑), a decrease (↓), or no change in rate (→). Some neurons ceased activity during central apnea (“OFF”), whereas activity was evoked in others that were “silent” during the control interval (“ON”). Neurons were also assessed for significant changes in the depth of breathing modulation [i.e., “rate ratio” ($)]; the rate ratio is a measure of cross-phase modulation. A change in rate ratio indicates that the ratio of per-cycle maximum to minimum firing rates became more (or less) pronounced upon stimulation (Ott et al. 2012). A neuron’s response was classified as $\downarrow$ if its change in rate ratio was not accompanied by a significant change in neuronal firing rate as defined above.

We evaluated spike-timing relationships with two methods. Cross-correlation histograms (CCHs) were calculated for all pairs of simultaneously monitored neurons. Peak or trough features were identified with a Monte Carlo test using surrogate spike trains (Pauluis and Baker 2000) with gamma-distributed interspike intervals (Miura et al. 2006). The false discovery rate was controlled to be <5%. A detectability index (equal to the ratio of the maximum amplitude of feature departure from background activity to the standard deviation of the correlogram noise) was calculated for each identified feature; values $\geq 3$ indicated a significant correlogram feature (Aertsen and Gerstein 1985; Mølgaard and Epping 1987). All offset-feature data are presented with a positive time lag. Correlation feature maps for groups of simultaneously monitored neurons were generated to graphically represent sets of detected cross-correlogram features (Segers et al. 2008).

Gravitational clustering was used to identify functional associations among some neurons during apnea. This method represented each of $N$ simultaneously monitored neurons as a particle in $N$-space with time-varying charges that were a filtered version of the corresponding spike train. Charge-associated forces resulted in particle movements (Gerstein 2010). Three-dimensional plots of trajectories of the interparticle distance and the distance each particle moved from its original location in the $N$-space were used to parse the types of interactions (Lindsey and Gerstein 2006).

RESULTS

This work was part of a larger study on raphé-pontomedullary respiratory network chemoreceptor reflex circuits. Complementary results on functional connections for shaping the chemoreceptor-evoked responses of neurons have been reported (Nuding et al. 2009a; Ott et al. 2011, 2012; Segers et al. 2015).

The data presented were acquired during 12 hyperventilation-to-apnea attempts (11 recording sessions with 10 cats). In these sessions, apnea was achieved in all but one attempt; mean end-tidal $CO_2$ values dropped from $29.0 \pm 2.5$ to $13.0 \pm 4.1$ mmHg (Table 1). For six trials, analysis of blood gases confirmed alkalosis during hyperventilation, with $pH$ values ranging from 7.6 to >7.9. Using several electrode arrays, spike trains of up to 116 neurons were monitored simultaneously in 3 different regions of the brain stem. Overall, data were acquired from 358 neurons in the VRC (2.6 mm caudal to 8.6 mm rostral to the obex, 3.5 to 4.7 mm lateral to the midline, and 2.3 to 5.4 mm below the dorsal surface of the medulla),
157 cells in the midline raphé nuclei (0.2 mm caudal to 9.1 mm rostral to the obex, 0.2 to 1.8 mm lateral, and depths between 0.1 to 5.6 mm), and 134 neurons in the pons (0.0 to 1.0 mm caudal to the caudal edge of the inferior colliculus, 3.3 to 6.1 mm lateral, and at depths from 1.0 to 5.2 mm).

From a representative experiment, we mapped recording sites onto a dorsal view of the brain stem (Fig. 1A) and included examples of the recorded signals (Fig. 1B). Following the onset of mechanical hyperventilation, phrenic nerve activity decreased until cycling could no longer be discerned by eye from the integrated trace or by ear from the audio signal and all simultaneously monitored neurons were either silent or exhibited no significant rhythmic activity within the frequency band of interest. The presence of respiratory rhythm in neurons was verified with the S-transform test. Two cats are excluded from this table; see text. ET, end tidal.

Table 1.  

<table>
<thead>
<tr>
<th>Animal</th>
<th>Control ETCO₂, mmHg</th>
<th>Loss of phrenic cycling</th>
<th>Beginning of central apnea (A)</th>
<th>Duration of Central Apnea (B)</th>
<th>End of central apnea</th>
<th>Return of phrenic cycling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time, s</td>
<td>ETCO₂, mmHg</td>
<td>Time, s</td>
<td>Time, s</td>
<td>Time, s</td>
<td>Time, s</td>
</tr>
<tr>
<td>1</td>
<td>28</td>
<td>207.1</td>
<td>14</td>
<td>207.1</td>
<td>47.7</td>
<td>16.0</td>
</tr>
<tr>
<td>2</td>
<td>27</td>
<td>111.9</td>
<td>11</td>
<td>329.2</td>
<td>183.7</td>
<td>190.1</td>
</tr>
<tr>
<td>3</td>
<td>34</td>
<td>117.7</td>
<td>23</td>
<td>164.0</td>
<td>512.4</td>
<td>191.8</td>
</tr>
<tr>
<td>5</td>
<td>28</td>
<td>181.5</td>
<td>8</td>
<td>286.1</td>
<td>93.7</td>
<td>130.0</td>
</tr>
<tr>
<td>6</td>
<td>31</td>
<td>140.2</td>
<td>12</td>
<td>239.6</td>
<td>345.4</td>
<td>211.7</td>
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<tr>
<td>7</td>
<td>30</td>
<td>63.9</td>
<td>11</td>
<td>63.9</td>
<td>460.0</td>
<td>249.3</td>
</tr>
<tr>
<td>8</td>
<td>28</td>
<td>613.5</td>
<td>11</td>
<td>706.2</td>
<td>237.2</td>
<td>104.6</td>
</tr>
<tr>
<td>9</td>
<td>30</td>
<td>162.6</td>
<td>11</td>
<td>277.2</td>
<td>334.9</td>
<td>231.8</td>
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<td>8</td>
<td>194.8</td>
<td>1,104.0</td>
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<td>Means</td>
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<td>199.2</td>
<td>13.0</td>
<td>274.2</td>
<td>368.8</td>
<td>178.2</td>
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<tr>
<td>SD</td>
<td>2.5</td>
<td>152.5</td>
<td>4.1</td>
<td>169.4</td>
<td>298.4</td>
<td>76.2</td>
</tr>
</tbody>
</table>

“Central apnea” was operationally defined as the period during which phrenic cycling could no longer be discerned by eye from the integrated trace or by ear from the audio signal and all simultaneously monitored neurons were either silent or exhibited no significant rhythmic activity within the frequency band of interest. The presence of respiratory rhythm in neurons was verified with the S-transform test. Two cats are excluded from this table; see text. ET, end tidal.

To expedite the identification of rate changes during hyperventilation, we constructed a heat map and individually scaled
Fig. 1. Firing rate histograms and S-transforms from animal 1. A: dorsal view of color-coded spheres [red: pons; blue: midline raphé; and green: ventral respiratory column (VRC)] marking recording sites in 1 animal mapped in the coordinate space of a cat brain stem atlas. B: examples of signals from 3 electrode arrays together with phrenic and lumbar activities during control. C: firing rate histograms for 7 of the neurons during the hyperventilation-to-apnea trial together with integrated phrenic and lumbar nerve activities, arterial blood pressure, and end-tidal CO₂. For this and subsequent firing rate histograms, each neuron’s location, respiratory-modulated discharge pattern, and ID code are at left. Preapneic interval (between the beginning of mechanical hyperventilation and the start of central apnea): gray block labeled “A”; central apnea: pink block labeled “B.” Red asterisk denotes beginning of blood pressure support. D: S-transforms computed for the 5 VRC neurons represented in C. The S-transform is displayed as a heat map with a luminance proportional to the displayed quantity (and therefore has a natural perceptual ordering that is preserved when converted to gray scale). The range of S-transform magnitudes is represented by the colored scale above the plots; each S-transform is scaled individually from 0 to its maximum magnitude value (right). S-transforms were computed using a frequency range of 0.01–2.0 Hz. Frequency is represented by a log scale and the respiratory frequency and its harmonics appear as multiple yellow horizontal bands. False discovery rate (FDR) for each S-transform: 805: 0.0063; 813: 0.0051; 815: 0.0041; 834: 0.014; and 884: 0.010. E: significance plot for the S-transforms shown in D indicating times of significant rhythmic activity (P < 0.0005); red dashed lines denote periods when a significant respiratory rhythm was absent from each cell’s discharge activity. I, inspiratory; E, expiratory; T, tonic; P, phasic; DEC, decrementing; AUG, augmenting.
the firing rates of neurons recorded simultaneously in the three sampled brain stem regions of animal 1 (Fig. 3; the cell IDs of 944 and 969 are in red). This method revealed that many cells with diverse respiratory-related discharge patterns (Fig. 3, labels at left) increased their firing rates as respiratory drive was reduced (blue rectangles). Others exhibited declines in rate (red rectangles). Extreme changes during central apnea were apparent: 15 cells ceased spiking (“OFF”) and 3 “silent” neurons were activated (“ON”). The change in a cell’s firing rate during the transition to central apnea (preapneic period, square A) was not always indicative of its activity during apnea (square B). After the DEC and OFF responses were combined and the INC and ON were grouped together, only about half of the cells (n = 35 of 67) responded in the same way during the preapneic period and central apnea in this animal. A similar proportion was found for all neurons in the study evaluated under both conditions (335 of 592 cells).

Following reemergence of the respiratory rhythm, the amplitude of integrated phrenic nerve activity declined and rhythm generation ceased (Fig. 4A, periods A and B). The labeled respiratory-modulated discharge patterns were determined from baseline, phase-normalized respiratory CTHs (Fig. 4B). An asterisk next to a label indicates that the corresponding neuron (n = 6) had a reduced firing rate during hypercapnia. Included among these six neurons were four that significantly increased their firing rates during the hypocapnic A or B interval (914, 969, 807, and 853; see color-coded rectangles) and two that did not (842 and 931).

The arrows to the right of the firing rate histograms (Fig. 4A, right) represent functional excitatory (blue) and inhibitory (red) relationships inferred from short-time scale correlations of the spike trains (Fig. 4C). The CCH triggered by tonic inspiratory raphé neuron 914, one of several cells with an increased firing rate during the transition from eupnea to apnea, revealed a time-locked transient decline in the firing probability of VRC tonic inspiratory neuron 842 indicative of an inhibitory action (Fig. 4C, i). In turn, neuron 842 triggered CCHs for five different raphé target neurons, all featuring offset troughs (e.g., Fig. 4C, 2–4). One of these raphé neurons (931) triggered CCHs with features suggestive of excitatory actions upon both VRC tonic inspiratory neuron 807 and tonic inspiratory pontine neuron 533 (Fig. 4C, 5–6). The functional relationships detected among all of the represented neurons are

Fig. 2. Response plots for 2 raphé neurons during hypocapnia and hypercapnia. Average firing rates per cycle for the hyperventilation trial (left; red traces on gray backgrounds) and the 5 stimulus response periods for hypercapnia (right; red traces on white backgrounds) are shown together with a summary of paired control data (black traces; green horizontal lines indicate means ± 1 SD). Note that responses during hypocapnia were analyzed using intervals equivalent to mean control cycles; see METHODS.
Firing rate heat map

Key
- Increase
- Decrease
- Change in rate ratio
- No change

<table>
<thead>
<tr>
<th>Animal</th>
<th>Phrenic n.</th>
<th>Lumbar n.</th>
<th>BP</th>
<th>ETCO2</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>B</td>
<td></td>
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<tr>
<td>2</td>
<td>A</td>
<td>B</td>
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Change in mechanical ventilation

Blood pressure supported

Animal 1 700 s

I-AUG-T
I-DEC-T
I-AUG-P
I-DEC-P
E-AUG-T
E-DEC-T
E-AUG-P
E-DEC-P

NRM

VRC RAPHÉ PONS
summarized in the correlation feature map shown in Fig. 4D. Each small rectangle is labeled with the identification number of the corresponding neuron and its respiratory modulated discharge pattern. The large rectangles represent the three monitored brain stem regions. Such maps aid development of abbreviated model circuits that can replicate the experimentally observed correlation features (Aertsen et al. 1989; Moore et al. 1970; see Discussion).

Overall, 400 of the 649 neurons studied (62%) were elements of pairs with at least one hyperventilation-responsive neuron and a correlational signature of interaction. Tables 3 and 4 show results for the preapnic and apnic intervals, respectively. The number of pairs in each table differs because the apnic period had more pairs with responders. For CCHs with features indicative of paucisynaptic interactions, there were about three times more peaks than troughs under each condition. Further characterizing the features, the numbers of offset and central peaks were similar, whereas the number of offset troughs was twice that of central ones.

To facilitate subsequent discussion, pairs with at least one responsive neuron and a correlogram feature will be interpreted simply as evidence for one of four functional actions: excitation, inhibition, disfacilitation, or disinhibition. These actions may affect firing rate in the target cell and are marked with symbols (see legends for Tables 3 and 4). When considering responses during the transition to apnea (Table 3), 123 of 390 offset features indicated that the response of the trigger neuron may have contributed to the change in the target cell’s activity. The prevalent effects were excitation (39%) and disfacilitation (33%). During central apnea, 145 of 425 offset features were evidence for the influence of the rate change in the trigger cell upon the target’s activity during the hyperventilatory response, with disfacilitation as the dominant mechanism (63%; Table 4). Additional correlation feature sets derived from groups of simultaneously monitored responsive neurons are considered subsequently.

**Loss of respiratory modulation at the transition to apnea.** Results from another experiment (animal 2) provided evidence that changes in the firing rates of raphé neurons affected VRC neuron activity during the transition to apnea (Fig. 5A). The corresponding S-transforms (Fig. 5B) showed the staggered times when significant rhythms within the respiratory fre-

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**Table 2. Responses of neurons to hyperventilatory hypocapnia during the transition to apnea (period A) and during central apnea (period B) in all trials**

<table>
<thead>
<tr>
<th></th>
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Arrows indicate direction of significant change in neuronal firing rate over the entire respiratory cycle: ↑, increase; ↓, decrease; →, no significant change. ↓, significant change in the depth of respiratory modulation not accompanied by a significant overall change in neuronal firing rate. The responses of 592 cells were evaluated under both conditions. These data include 56 neurons from 1 trial in which central apnea was not achieved; those neurons were evaluated only during the transition to central apnea. I, inspiratory; E, expiratory; N, nonrespiratory modulated neuron; VRC, ventral respiratory column.

Fig. 3. Firing rate heat map (with a luminance proportional to the displayed quantity) for 67 cells during hyperventilation and hypocapnia, the immediately preceding control interval, and a recovery period together with integrated phrenic and lumbar nerve activities, arterial blood pressure (BP), and end-tidal (ET) CO₂. The firing rates of neurons from animal 1 during hyperventilation are individually scaled (see range of channel normalized rates above map); brain stem region, respiratory modulation, and ID numbers are to the left of the traces. Responses of cells highlighted in red are also presented in Fig. 2. For this and subsequent plots, the 2 colored rectangles to the left of each row of bins indicate the direction of a significant change in rate relative to preceding normocapnic control conditions (see key) during the transition to central apnea (A; gray panel) and within it (B; pink panel). Red dashed line in blood pressure trace denotes blood pressure range seen during preceding control interval. The amplitude of integrated phrenic nerve activity returned to control values when the catheter supporting blood pressure was deflated, a result consistent with known baroreceptor influences on inspiratory drive (Lindsey et al. 1998).
Selective stimulation of peripheral chemoreceptors, a result breathing did not return in neurons 102.

Significant rhythmic activity within the frequency band of apparent (Fig. 5, see B). Considerable variability in recovery onset times was also persistent respiratory modulation in VRC inspiratory inspiration began; another 308.6 s passed until the loss of the most isolated VRC solution of rhythmic activity occurred first in expiratory modulation in the respective neurons. The dissipation frequency range were lost in the respective neurons. The dissolution of rhythmic activity occurred first in expiratory modulated VRC neuron 102, 20.6 s after mechanical hyperventilation began; another 308.6 s passed until the loss of the most persistent respiratory modulation in VRC inspiratory neuron 133. Considerable variability in recovery onset times was also apparent (Fig. 5B, see right dashed lines in S-transforms). Significant rhythmic activity within the frequency band of breathing did not return in neurons 102, 104, and 125 until selective stimulation of peripheral chemoreceptors, a result presumably attributable to a change in drive or threshold. The signal from raphé inspiratory neuron 912 was lost when the carotid chemoreceptors were stimulated.

Two “dual-feature” cross-correlograms characterized the relationships between VRC expiratory neuron 114 and raphé target neurons 912 and 906 (Fig. 5C, 1-2). Positive time lag offset trough features indicated a transient decline in firing probabilities in both raphé neurons after the VRC cell spikes.

Peaks with negative and zero time lags relative to the correlogram origin were consistent with simple interpretations of reciprocal excitation and unobserved shared influences, respectively. Spikes in a second VRC neuron, respectively. Spikes in a second VRC neuron, another 308.6 s passed until the loss of the most persistent respiratory modulation in VRC inspiratory inspiration began; another 308.6 s passed until the loss of the most isolated VRC solution of rhythmic activity occurred first in expiratory modulation in the respective neurons. The dissipation frequency range were lost in the respective neurons. The dissolution of rhythmic activity occurred first in expiratory modulated VRC neuron 102, 20.6 s after mechanical hyperventilation began; another 308.6 s passed until the loss of the most persistent respiratory modulation in VRC inspiratory neuron 133. Considerable variability in recovery onset times was also apparent (Fig. 5B, see right dashed lines in S-transforms). Significant rhythmic activity within the frequency band of breathing did not return in neurons 102, 104, and 125 until selective stimulation of peripheral chemoreceptors, a result presumably attributable to a change in drive or threshold. The signal from raphé inspiratory neuron 912 was lost when the carotid chemoreceptors were stimulated.

Two “dual-feature” cross-correlograms characterized the relationships between VRC expiratory neuron 114 and raphé target neurons 912 and 906 (Fig. 5C, 1-2). Positive time lag offset trough features indicated a transient decline in firing probabilities in both raphé neurons after the VRC cell spikes. Peaks with negative and zero time lags relative to the correlogram origin were consistent with simple interpretations of reciprocal excitation and unobserved shared influences, respectively. Spikes in a second VRC neuron, 106, were also fol-
lowed by a reduced firing probability in raphé neuron 906 (Fig. 5C, 3), a result that, when considered together with the central peak in the 106–114 correlogram (Fig. 5C, 4), suggests a coordinated convergent functional inhibition of raphé neuron 906 by VRC cells 114 and 106. VRC cell 114 also triggered a correlogram with VRC inspiratory neuron 160 that included an offset trough and central peak (Fig. 5C, 5), features indicative of inhibition of the inspiratory neuron and additional inputs.

Table 3. Correlogram features detected among 15,852 neuron pairs in which the firing rate of at least 1 cell of the pair changed during transition to apnea

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Offset (n = 390) and central (n = 356) correlogram features detected among 15,852 neuron pairs grouped according to brain stem location and response to hyperventilation during the transition to apnea (arrows). Each neuron pair included at least 1 cell that responded with an altered firing rate. Trigger (left) and target (top) neurons are organized so that offset features have positive time lags. Numbers of offset and central peaks (Pk) and troughs (Tr) are reported; numbers in “Total” rows are the total number of pairs composed of neurons with the locations and stimulus responses indicated by the row and column labels. Table cells that would contain information for 2 neurons with no response are marked with an “X.” Central features are reported only at the top right because the designation of trigger and target neuron is irrelevant for central feature correlations; the central feature will still be present after switching the 2 spike trains. Similarly, total numbers of specific pairings are found only at the top right. For example, a total of 108 cell pairs composed of a Pons ↑ and a Pons ↓ neuron were analyzed. Correlograms calculated for 8 of these pairs contained an offset feature: 5 were suggestive of a Pons ↑ to Pons ↓ influence (2 peaks, 3 troughs) and 3 of a Pons ↓ to Pons ↑ effect (2 peaks, 1 trough). In addition, 3 central features were detected (2 peaks, 1 trough) for pairs of this type. Symbols indicate the pair types (and numbers of such pairs) whose offset correlogram feature and neuronal responses are simply interpreted as functional actions that promote changes in target cell activity during transition to apnea: *excitation (feature, response of trigger and target cell: peak, ↑↑); †inhibition (trough, ↓↓); **disfacilitation (peak, ↓↓); ‡disinhibition (trough, ↓↑).
shared by the two cells. Features in correlograms triggered by VRC inspiratory neuron 129 included offset troughs, suggesting other local inhibitory actions on both a VRC expiratory neuron (132, Fig. 5C, 7) and another inspiratory neuron (133, Fig. 5C, 6); a central peak was also apparent in the 129-133 pair correlation. The inter- and intraregion relationships identified among neurons in this data set, graphically represented in a correlation linkage map (Fig. 5D), provide additional evidence for raphé-VRC interactions and for reciprocal inhibitory interactions between inspiratory and expiratory populations.
within the VRC. Although the respective baseline inspiratory and expiratory discharge patterns of neurons 129 and 132 (CTHs, Fig. 5E, top) were absent during the apneic interval, a cross-correlogram trough indicated a persistent interaction (Fig. 5E, bottom).

With the use of the S-transform significance test, an inspiratory neuron was the final neuron to lose its respiratory-modulated discharge pattern before apnea in nine of ten hyperventilation-to-apnea trials. (One recording contained two trials; the sequential loss of rhythmic activity was assessed in the first trial only.) The persistence of inspiratory activity was significant ($P = 0.022$) given the null hypothesis that the probability of an inspiratory cell being the last to lose rhythm in each recording is equal to the proportion of inspiratory cells within that recording.

All animals were bilaterally vagotomized to remove vagal afferent feedback from pulmonary stretch receptors. In two animals, however, we performed hyperventilation to apnea trials both before and after vagotomy within the same recording. A total of 10 VRC cells were recorded during both the pre-
and postvagotomy periods. After vagotomy, these cells retained their respiratory rhythm longer subsequent to the start of mechanical hyperventilation (means ± SD = 165.2 s ± 95.4) than before (66.5 s ± 98.9; P = 0.002, Wilcoxon signed rank test). Of the same 10 VRC neurons, 9 recovered their respiratory rhythm more quickly after (132.8 s ± 70.0) than before vagotomy (229.5 s ± 81.3; P = 0.00391); 1 cell failed to resume a respiratory rhythm following apnea. Both of these animals were ventilated with 100% O2.

Selective stimulation of carotid chemoreceptors during apnea evoked the respiratory rhythm. In two animals, peripheral chemoreceptor stimulation (PCS) was applied during both normocapnic control conditions and hypocapnic apnea. Sixty-one neurons responded under both conditions with a change in firing rate; for 40 of these cells, the direction of firing rate change was the same. A comparison of the discharge patterns evoked by stimulation of peripheral chemoreceptors before (Fig. 6A) and during (Fig. 6B) hyperventilatory hypocapnia in animal 3 confirmed that respiratory rhythmogenesis could be reinitiated under the experimental conditions associated with mechanical hyperventilation. The arrows to the left of the firing rate histograms indicate the neuron’s average response to PCS trials before (n = 5) and during (n = 3) hypocapnia compared with immediately preceding control periods (see METHODS). Five neurons that responded differently in Fig. 6 (red arrows) were E cells. Three of these changed from a phasic to a tonic discharge pattern during hypocapnia; the remaining phasic cell became silent. Each of these four cells reverted to a phasic firing pattern during PCS (Fig. 6B); the loss of hypcapnia-induced tonic activity during inspiration in response to PCS in cells 860 and 868 accounts for their opposite response. This decline of tonic activity (seen also in VRC E cells 857, 841, 655, and 807) in response to PCS during hypcapnia was coincident with increased activity in a number of simultaneously monitored inspiratory neurons.

Some simultaneously monitored VRC neurons expressed evoked rhythmic activity only during and immediately following transient PCS. However, oscillations identified by the S-transform persisted in other neurons well beyond the stimulus interval (Fig. 6C). For example, evoked activity in inspiratory neuron 644 included a prolonged initial inspiratory “ramp” followed by successively shorter bursts of smaller amplitude, each aligned with a corresponding interval of reduced activity in simultaneously monitored VRC expiratory neuron 816.

Gravitational clustering analysis confined to the apneic interval before carotid chemoreceptor perturbations (Fig. 7A) revealed spike timing relationships indicative of ongoing interactions among neurons that had inspiratory or expiratory discharge patterns during prior baseline conditions. With the sign of the interparticle force set to allow aggregation for excess asynchronous firing as would be caused by inhibition, the trajectories of three particles representing neurons 644, 816, and 841 clustered in the projection from N-space to a plane (Fig. 7B). Because of loss of information in such a projection, the distance between particles of each pair as a function of time was evaluated for significant aggregation using 1,000 surrogate spike trains in a Monte Carlo significance test. Particles for pairs 841-816 and 816-644 condensed more than those for all their surrogate pairs. Particles for 841-644 transiently achieved an interparticle distance equal to the smallest corresponding surrogate value.

Gravity parameters were set to be sensitive to firing sequence; each particle carried both “acceptor” and “effector” charges. Acceptor charge kernels decayed backward and effector charges forward in time, so that a particular particle would move as if it represented a postsynaptic neuron, i.e., toward a relatively stationary particle corresponding to a presynaptic neuron. Another view of clustering in N-space was generated in a three-dimensional projection in which each point corresponded to the interparticle distance for each pair over time and the distance each particle moved from its original location (Fig. 7C). This perspective revealed a trajectory for pair 841-816 that remained near the left back wall (particle 841, as “particle 1,” remained near its starting position) with a small final interparticle distance close to the floor of the cube. This final position was outside the “cloud” of trajectory end points generated from surrogate spike trains (Fig. 7D). The trajectory for pair 841-644 descended toward the floor down a path closer to the principal diagonal, indicating that both particles moved from their starting positions (Fig. 7C). The trajectory for pair 816-644 showed that those particles closed to a distance similar to that of pair 841-644, with particle 816 moving more from its starting position than did particle 644. These three major trajectories suggested the presence of at least two pairs of asynchronously discharging neurons with a common presynaptic element. This conclusion was corroborated by significant offset and asymmetrical central troughs in CCHs for pairs 841-816 and 841-644, respectively, generated from the same data (Fig. 7E).

Comparison of firing rate histograms for a group of VRC neurons also in animal 3 during the transition to apnea and the recovery after the chemoreceptor stimulation trials (Fig. 8A) revealed an asymmetry: although the declining peak burst frequencies and rhythm loss were similar to those seen during transitions in other recordings, the reemergence of respiratory rhythm across these cells was synchronous and abrupt, with, in many cases, greater peak firing rate than those occurring in the control period. Such a sudden reemergence was not apparent in any other animal or in a prior hyperventilation-to-apnea trial in animal 3 without peripheral chemoreceptor stimulation during the apnea. The return to control ventila-

Fig. 6. Transient activation of the respiratory central pattern generator by peripheral chemoreceptor stimulation during hypocapnic apnea: firing rate histograms and S-transforms during peripheral chemoreceptor stimulation (PCS) trials in animal 3. Discharge patterns evoked by PCS before (A; in normocapnia) and during (B) hyperventilatory hypocapnia. Arrows to the left of the firing rate histograms for each cell indicate the neuron’s average response to PCS challenge compared with average activity during immediately preceding control periods; note that the control period for B is under steady-state hypocapnic conditions. Red arrows denote cells with different responses during normocapnia and hypocapnia. Heart symbol indicates time of blood collection for blood gas analysis; the blue asterisk indicates the same PCS trial examined in C. C: firing rate histograms and S-transforms (frequency range = 0.05–1.0 Hz; log scale) for 2 of the neurons together with integrated phrenic nerve activity during 2 PCS trials under hypocapnia. White arrows in the transforms indicate the end of significant rhythmic respiratory cycling; note that oscillatory activity persists well beyond the 30-s stimulus. FDR for each S-transform: 644: 0.0068; and 686: 0.014.
**Figure 1**

(A) Response to PCS during normocapnia and hypocapnia for different stimuli. 

(B) Response to PCS during hypocapnia for different stimuli. 

(C) PCS during hypocapnia for different stimuli.

**Legend**

- **E**: Excitatory
- **I**: Inhibitory
- **DEC**: Decerebrate
- **AUG**: Augmented
- **NRM**: Nucleus raphe magnus
- **VRC**: Ventral respiratory center

**Parameters**

- **ETCO2**: End-tidal CO2
- **BP**: Blood pressure
- **pH**: Hydrogen ion concentration
- **PaCO2**: Partial pressure of carbon dioxide

**Animal 3**

- **Peripheral chemoreceptor stimulation**
- **Response to PCS**

**Statistical Analysis**

- Max. spikes/s
- Max. S-transform magnitude

**Notes**

- **Increased BP**: 165 mmHg
- **Decreased BP**: 158.4 mmHg

**References**

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coordinated onset of inspiratory bursts in neurons 616, 820, 677, and neurons 841. The reemergence of spiking in firing rates of the monitored expiratory neurons, including VRC neurons together with integrated phrenic nerve activity, arterial blood pressure, and end-tidal CO$_2$ during the transition to apnea (represented in A with integrated phrenic nerve activity, arterial blood pressure, and end-tidal CO$_2$ during apnea.

Fig. 7. Firing rate histograms and correlation dynamics among 5 VRC neurons during apnea. A: firing rate histograms for a group of 5 VRC neurons together with integrated phrenic nerve activity, arterial blood pressure, and end-tidal CO$_2$ during apnea. B: projection of gravity particle trajectories for spike train data represented in A. Aggregation of particles for neurons 644, 816, and 841 indicates firing asynchrony, as would be caused by inhibition. Gravity parameters: acceptor charges backward and effector charges forward; charge increment time constants were set to 3.5 ms. Number of spikes for each cell: 644: 1,342; 655: 4,845; 686: 421; 816: 1,672; and 841: 7,320. C: 3-dimensional projection of trajectories for points representing each pair shown in B. Coordinate axes: interparticle distance (initially 100 arbitrary units) and the distance each particle in the pair moved from its original location in the N-space over time. D: image of the 3-dimensional trajectory path for neurons 841 and 816 with a “cloud” of trajectory end points for 100 of the 1,000 pairs generated from surrogate spike trains. E: cross-correlograms for indicated pairs from spike train data recorded during apnea and used in the gravity calculation.

Fig. 8. Loss of respiratory modulation during hyperventilation and abrupt reemergence of rhythm following PCS in animal 3. A: firing rate histograms for a group of VRC neurons together with integrated phrenic nerve activity, arterial blood pressure, and end-tidal CO$_2$ during the transition to apnea (left) and the recovery after the chemoreceptor stimulation trials (right). B: cross-correlation feature set calculated from pairs of neurons represented in A. The detectability index and bin width (in ms) for each CCH are as follows: 1: 4.3, 5.5; 2: 4.2, 5.5; 3: 3.8, 5.5; 4: 3.5, 2.5; 5: 8.0, 2.5; 6: 4.3, 5.5; 7: 13.1, 5.5; and 8: 8.8, 2.5. Number of spikes for each cell: 603: 85,335; 644: 201,037; 655: 109,890; 686: 52,680; 816: 133,080; 841: 198,262; and 872: 48,547. C: correlation linkage map for a subset of the neurons shown in A; relationships discussed in text are highlighted by the gray rectangle. VRC neurons with ID codes in the 600s were recorded to the left, whereas those in the 800s were recorded to the right. We note here that the spike waveforms of neurons 841 and 861 recorded during PCS under control eupneic drive have been documented previously, together with other results, including trough and peak features in respective spike triggered averages of phrenic nerve signals (Figs. 3B and 4B in Segers et al. 2015).
Firing rate histograms

Max. spikes/s

BP

ETCO₂

PC stimulus injection

Change in mechanical ventilation

4243.2 s

Animal 4

PCS during hypcapnia (trial 2)

Firing rate histograms

Max. spikes/s

BP

PC stimulus injection

180 s

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Additional short-time scale correlations identified in animal 3 provided evidence for interactions among neurons that responded both to hyperventilation and to concurrent peripheral chemoreceptor challenges (Fig. 8B). These relationships included both local functional connectivity and linkages between bilateral VRC microcircuits (Fig. 8C). The correlograms for expiratory modulated trigger neuron 841 included an offset peak with expiratory neuron 655 (Fig. 8B, 1) and an offset trough with inspiratory modulated target neuron 603 (Fig. 8B, 2). Conversely, inspiratory neuron 861 triggered correlograms that included an offset peak with inspiratory target 603 (Fig. 8B, 3) and an offset trough with expiratory neuron 655 (Fig. 8B, 4). The correlogram feature for this pair of trigger neurons was a central trough (Fig. 8B, 5). These relationships are highlighted by the gray rectangle in the correlation linkage map (Fig. 8C). Other detected features included offset peaks in correlograms triggered by neurons 861 and 644, each with target neuron 872 (Fig. 8B, 6–7) and a trough for expiratory neurons 841 and 816 (Fig. 8B, 8), extending evidence for their interaction beyond the period of hypocapnic apnea documented in Fig. 7.

In another experiment (animal 4), peripheral chemoreceptor stimulation during hypocapnic apnea evoked rhythmic activity in a subset of the simultaneously monitored neurons that persisted after the PCS and throughout the remaining hyperventilatory period, thus cutting short the apnea. Other neurons in this animal, as well all the neurons recorded in animal 3, exhibited only transient activity until the next stimulus in the series or until recovery following the return of control ventilation parameters (Fig. 9A). S-transforms were calculated for 10 VRC cells recorded during 5 PCS trials under hypocapnic conditions (Fig. 9A). In some trials, two neurons (817 and 820) exhibited significant rhythmic activity (indicated by green bars) only during and immediately following the infusion of CO₂-saturated saline, but other neurons (806, 815, and 861) continued firing with sustained cycling throughout the remaining trials. Details of their firing patterns during the second PCS trial (Fig. 9B) included an early initial enhancement of inspiratory activity during the concurrent increased blood pressure (yellow block), indicating that this selective perturbation of peripheral chemoreceptor drive was sufficient to counterbalance the known suppressive influence of baroreceptor stimulation on inspiratory effort (e.g., Fig. 3). Subsequently, expiratory neuron firing rates increased (orange block) and inspiratory neuron impulse activity declined (e.g., 806 and 813) or ceased (e.g., 815).

**Concurrent loss of slower network rhythms.** S-transforms of neural activity during hypocapnic apnea revealed that neurons with oscillations in firing rate with the same frequency as Mayer waves were also suppressed during apnea. Firing rate histograms were plotted for pontine, raphé, and VRC neurons monitored simultaneously during hyperventilation and apnea from a representative recording (animal 5, Fig. 10). The phrenic signal had a cycling frequency of ~0.21 Hz, as did many of the phasic cells in this recording (e.g., VRC neuron 821, Fig. 10B). The rhythmic modulation of discharge rates in other neurons had a lower frequency of about 0.1 Hz (e.g., raphé cell 910, Fig. 10C). Activity at both frequencies was disrupted during the apnea. Similar results were found in two other cats with low frequency oscillations in neural activity.

**DISCUSSION**

During the transition to hypocapnic apnea, diverse fluctuating activity patterns were recorded in the raphé-pontomedullary respiratory network. These rate modulations and those observed during other chemoreceptor challenges, together with the identified correlational signatures of neuronal interactions, support the hypothesis that distinct groups of chemoresponsive raphé neurons contribute to hypocapnic apnea through parallel and serial processes that incorporate disfacilitation and active inhibitory suppression of inspiratory motor drive and central pattern generation. The results also provide a neurophysiologic basis for prior observations on reciprocal tonic activation of inspiratory and expiratory motor neurons under conditions of reduced chemical drive and the inspiratory shift and rhythm reemergence with suppression of ongoing tonic expiratory neuron activity by peripheral chemoreceptors (Sears et al. 1982).

We observed that VRC inspiratory neurons were usually the last to “drop out” or to lose rhythmic activity during the transition to apnea. This result and the association of inspiratory neuron bursts with rhythm reemergence, including oscillatory activity evoked by carotid chemoreceptor stimulation, are consistent with upstream drivers in the inspiratory neuron chain having a primary role in the reinitiation of breathing. Because we sampled a relatively small number of neurons at once, the sources of inspiratory burst initiation within or following apnea remain unresolved, as does the potential role of the immediately preceding increase in tonic expiratory activity. Candidate populations for this inspiratory “driver” function include pre-Bötzinginger inspiratory neurons (Richter and Smith 2014) or a recently described rostral group of postinspiratory neurons (Ramirez et al. 2014).

In the course of this work, we noted that network oscillations slower than the breathing rhythm were also disrupted during hypocapnic apnea. Neurons in the brain stem respiratory network may express 0.04–0.1 Hz Mayer wave-related oscillations (MWROs) in firing rate. These oscillations are often phase locked to 0.1-Hz oscillations of arterial pressure, are typically slower than the breathing frequency (Julien 2006), and can occur in the absence of discernible oscillations of arterial pressure (Morris et al. 2010). Functional interactions among raphé and pontine neurons with MWRO discharge patterns have suggested that elements of the brain stem respi-
ratory network participate in the dissemination or generation of these slower rhythms and the coordination of respiratory and vasomotor oscillations (Morris et al. 2010). Most, but not all, stimuli that increase sympathetic nerve activity also increase the amplitudes of Mayer and Traube-Hering waves (Julien 2006). The observed disruption of the slow rhythm suggests that the opposite is also a property of the network in that the decrease of a potent sympathetic stimulus, chemical drive, decreases or eliminates MWROs and Mayer waves.

Advantages and limitations of the approach. Our multiarray approach affords three significant advantages over prior single electrode studies. First, monitoring neuronal activity that persists during apnea and the correspondence of each recorded group’s discharge patterns both before and after apnea provide useful indicators of recording stability. Second, responses and comparisons of the times of loss and recovery of rhythmic activity for neurons of each group are measured under the same experimental conditions (blood pressure, PaCO₂). Furthermore,
the S-transform provides an objective criterion for identifying the dissolution and reemergence of respiratory modulated firing rates; its property of constant relative time resolution for all frequencies of interest also facilitates assessment of the dynamics of the slower MWROs. Third, although the approach precludes the identification of neurotransmitters in the monitored neurons, as done in other complementary work (e.g., Iceman et al. 2013, 2014), our correlational analyses of the parallel recordings allow identification of multiple functional interactions within and among the various brain regions and provide a model framework of candidate circuit modules through which hypocapnia contributes to the generation of apnea induced by assisted ventilation. Other strengths and limitations of our approach have been published (Ott et al. 2012; Segers et al. 2008). The sensitivity of the gravity method and its efficacy under conditions of nonstationary firing rates permit screening for and detection of spike timing relationships indicative of synaptic interactions during relatively brief apneic intervals.

Hyperventilation-induced apnea via mechanical ventilation is only one route to apnea; apnea is also associated with panic attacks (Stein et al. 1995), opioid overdoses (Howard and Sears 1990; Lee-Iannotti and Parish 2014), and central sleep apnea (Momomura 2012). Changes in neuron firing may not be the same during hyperventilation produced by assisted ventilation and during breathing driven by the physiological control system. In conscious animals, learning as well as hypocapnia may play a role in the cessation of breathing during assisted ventilation (Orem and Vidruk 1998). In intact spontaneously breathing unanesthetized adult cats, Lovering et al. (2012) demonstrated shifts in tonic apneic and phasic hyperpneic respiratory neuron activity under conditions of periodic breathing similar to the transient rhythmic patterns evoked by carotid chemoreceptor stimulation during hypocapnia in the present work.

The peripheral chemoreceptor-evoked rhythmogenesis demonstrated that central pattern generating mechanisms for breathing were capable of operation under the experimental conditions of hypocapnia, which may include brain stem vasoconstriction in response to a reduction of $P_aCO_2$ (Ainslie and Duffin 2009) even though medullary blood flow is preferentially maintained during hypocapnia compared with other brain regions (Sato et al. 2012; Skow et al. 2013; Willie et al. 2012). As noted, two protocols were used as necessary to mitigate the potential consequences of hypotension and ischemia associated with hypocapnia. First, blood pressure was continuously monitored and actively supported by inflation of an embolectomy catheter in the descending aorta, a nonpharmacological manipulation. Second, ventilation was supplemented transiently with an increased FIO$_2$ during hyperventilation. In two experiments, cats were ventilated with 100% O$_2$ throughout the recording period; similar results were obtained from animals ventilated with air or oxygen. Ventilation with 100% O$_2$ was otherwise not used during the experiments so as to avoid possible brain hyperoxia and the potential confound of paradoxical stimulation of central chemoreceptors by free radicals (Dean et al. 2004; Matott et al. 2010). While FIO$_2$s greater than found in air could potentially depress peripheral chemoreceptor drive, we note that robust network responses to carotid chemoreceptor stimulation were measured in these animals, reflecting the efficacy of CO$_2$-saturated saline as a peripheral chemoreceptor stimulus.

Animals were usually bilaterally vagotomized before the start of the recording to remove the potential confounds of vagal afferent feedback from pulmonary stretch receptors during passive hyperventilation with the attendant changes in tidal volume. In two experiments, the hyperventilation protocol was performed before and after vagotomy. Neurons lost rhythmic activity earlier and recovered later in the prevagotomy trials, a result consistent with an influence of afferent feedback on the respiratory central pattern-generating circuits.

**Inferred connectivity.** Simple interpretations of the primary correlogram features (Aertsen and Gerstein 1985; Kirkwood 1979; Moore et al. 1970) and changes in neuronal firing rates during hyperventilation suggest circuit mechanisms for the modulation and suppression of breathing during hypocapnic apnea (Fig. 11). An offset trough suggests inhibition, operationally defined as a synaptic relationship that reduces target cell firing probability following trigger neuron spikes. A peak offset in time relative to the trigger event origin suggests excitation of the target neuron or an “unobserved” input that influences both cells with different delays.

We detected neurons (highlighted with red circles, Fig. 11, A and C) with putative synaptic actions of opposite sign upon different target cells. Two such cells, raphé neuron 914 and pontine neuron 511, exhibited short-time scale spike synchrony (Fig. 11A, square 1) and both increased their activity during the transition to hypocapnic apnea. Both of these neurons triggered correlograms with VRC target neurons 842 and 853, suggesting highly coordinated actions within the VRC from distributed regions of the network (Fig. 11A, 2–3). For example, the putative inhibitory action of neuron 914 on VRC inspiratory neuron 842 is consistent with disinhibitory operations of a raphé-VRC-raphé loop for promoting the observed enhancement of raphé and pontine activity during the preapneic transition period during hyperventilation. In this model, shared target cell 842 inhibited several raphé neurons and VRC inspiratory neuron 853 (Fig. 11A, 4). Correlograms with offset peaks, also triggered by the same raphé-pontine neuron pair (914 and 511), suggested convergent excitation of raphé target neuron 969 (Fig. 11A, 5). Simple interpretations of additional correlogram peaks found for this set of simultaneously monitored neurons included convergent excitation of VRC expiratory neuron 807 by raphé cells 931 and 915.

Reciprocal interactions of opposite sign identified between raphé (912) and VRC (114) neurons in animal 2 (Fig. 11B, 6) suggest an equilibrium-seeking relationship for stabilizing that neuronal pair’s firing rates: more activity in neuron 912 promotes its inhibition by cell 114. Similar relationships between raphé and VRC neurons have been identified previously (Lindsey et al. 1994) and implicated in circuits for baroreceptor modulation of breathing (Lindsey et al. 1998). The set point defined by this relationship is changed by modulatory influences that alter impulse activity in either neuron. In this regard, the diminished firing rates of neurons 114 and 160 during apnea and their central correlogram peak (Fig. 11B, 7), a signature of shared input, are consistent with a reduction in excitatory chemoreceptor drive, a consequence of reduced $P_aCO_2$ and the accompanying alkalosis (Basting et al. 2015).

VRC inspiratory neuron 861 and expiratory neuron 841 also triggered correlograms with offset peak and offset trough features in different VRC target cells (Fig. 11C, 8–9). Responses during the transition to apnea and the correlogram peaks were consistent with the transient hypothalamic rhythmogenesis reported in the caudal ventrolateral medulla. Beyond these examples, we observed several other relationships, each with the expected sign and time course. For example, in animal 3, the VRC inspiratory neuron 981 triggered correlograms with offset trough and offset peak features in a phrenic nucleus neuron 924 (Fig. 11B, 8).
with an excitatory action of 841 on expiratory neuron 655 and a disfacilitatory influence of 861 on inspiratory target neurons 872 and 603; Conversely, responses and troughs provided evidence for inhibition of 605 by neuron 841 and a disinhibitory action of 861 on 655 during hyperventilation.

The model circuits in Fig. 11D enumerate simple alternative mechanisms for generation of peak and trough features in different target neurons by the same trigger cell. A particular neuron may evoke opposite actions at different targets (Fig. 11D, 1) by release of distinct excitatory and inhibitory transmitters (e.g., El Mestikawy et al. 2011; Seal and Edwards 2006; Stornetta et al. 2005), 2) because of ionic plasticity associated with ion transporters (Kaila et al. 2014), or 3) through different postsynaptic receptors (Hartzell et al. 1977). These considerations highlight the possibility that separate neuronal populations are not necessarily required to evoke all “direct” excitatory and inhibitory interactions within the VRC network and have implications for contemporary models (Smith et al. 2013) and future research. Other alternative mechanisms include intervening “sign-inverting” interneurons (Fig. 11D, 2); influences shared by the trigger neuron and other unobserved neurons, which then affect the target cells with opposite synaptic actions (Fig. 11D, 3); and correlated influences with opposite effects and different time delays acting upon the trigger neuron and one of the target cells (Fig. 11D, 4).

The correlational features of pairs of neurons and associated responses to hyperventilation found in this study (tallied in Fig. 11E) provide experimental support for parallel and serial operations between cells located in different brain stem regions as well as within all three areas sampled. Abstracted summaries include roles for disfacilitation and inhibition in the transition to (Fig. 11F) and maintenance of (Fig. 11G) apnea, processes that represent candidate mechanisms for generation of the altered balance of tonic reciprocal inhibition between expiratory and inspiratory motor and premotor drives during hypoxia (e.g., Basting et al. 2015; Eldridge 1973; Sears et al. 1982).

**Relationship to other prior work and future directions.** We confirmed the common conclusion of previous studies, made under decerebrate or anesthetized conditions (St. John 1998; Sun et al. 2001, 2005) and extended to different sleep states (Orem and Vidruk 1998), that VRC neurons with phasic discharge patterns during eupneic-like conditions of CO2 drive either cease to discharge during apnea or lose respiratory modulation of their firing rates (Bainton and Kirkwood 1979; Batsel 1967; Cohen 1968; Haber et al. 1957; Nesland and Plum...
1965). Given current knowledge, circuit mechanisms that underlie the transition from eupnea to hypocapnic apnea (Fig. 12, square A) likely include reduced chemoreceptor drive, mediated in part by a disfacilitatory reduction in glutamatergic excitation from retrotrapezoid nucleus/parafacial region (RTN-pF) chemoresponsive neurons (Guyenet 2014), as well as other operations between the RTN-pF and more caudal domains of the VRC (Ott et al. 2011; Ott et al. 2012). Altered modulation of the VRC by changes in serotonergic influences on central pattern generation, for example, are also likely and consistent with the present results (Corcoran et al. 2014; Ramirez et al. 2012). Moreover, our observation that raphé neurons operationally inhibited by hypercapnia increase their activity during hypocapnia suggests a GABAergic apnea-promoting role for that brain region (Iceman et al. 2014).

Although rhythmic activity that ceased during apnea was reinitiated in some neurons as PaCO2 values returned to control levels, other cells did not regain respiratory modulation until a higher level of PaCO2 was present or the peripheral chemoreceptors were stimulated (i.e., their breathing threshold changed; Dempsey 2005). Leevers et al. (1993) have hypothesized that persistent phrenic apnea illustrates an inherent “inertia” characteristic of the respiratory control system: a certain PaCO2, exceeding the “normal” eupneic PaCO2, must be reached before the rhythm resumes, regardless of the specific mechanism that initiated the apnea. Conditions contributing to this apparent asymmetry or hysteresis (Bainton and Kirkwood 1979; Bainton et al. 1978; Satoh et al. 2001) remain unknown but could include altered medullary blood flow (Ainslie and Duffin 2009), modulatory inputs, plasticity, and local alkalinity or other changes in the microenvironment of the neurons or associated glia (Funk et al. 2015). As noted, serotonin is thought to modulate respiratory neuron discharge to finely tune the respiratory pattern in a state-dependent fashion (for review, see Hilaire et al. 2010). In the cat, both hyperventilatory apnea and application of 5-HT1A receptor agonist 8-OH-DPAT similarly depressed or eliminated phrenic nerve activity, hyperpolarized late-E premotor neurons, and decreased neuronal input resistance, effectively depressing neuronal excitability (Lalley et al. 1994). The transient expression of rhythmogenesis or its persistent reemergence with peripheral chemoreceptor stimulation during apnea (Fig. 12, square B) presumably reflected the influence of several factors, including the relationship between the apneic threshold and PaCO2 and pH, as well as facilitatory processes (Mahamed et al. 2011), including those involving serotonin (Deviney et al. 2013) and medullary raphé circuits (Morris et al. 2001), which may have been masked by low peripheral and central chemoreceptor drives during apnea.

In heart failure patients, as well as in patients suffering from neurological diseases, hypocapnia and respiratory instability during sleep are common and can lead to central sleep apnea (Bitter et al. 2011; Carnevale et al. 2011). During sleep disordered breathing, Cheyne-Stokes ventilation patterns with hypocapnic central apneas occur (Leung et al. 2012). Chapman et al. (1988) showed that oscillations in ventilation are common during sleep and occur more frequently in periodic breathers with hypopnea, suggesting that these oscillations can be converted to periodic breathing with apnea when loop gain is increased. Periodic breathing can also be evoked in vertebrates by artificially holding blood gases constant while preventing chest wall movement and is likely a property intrinsic to the vertebrate respiratory control system (Fong et al. 2009). Subsequent addition of tonic respiratory drive (such as increased CO2) will restore a normal respiratory rhythm. A better understanding of the mechanisms and consequences of these conditions remains an important goal for the future.

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