Peripheral chemoreceptors tune inspiratory drive via tonic expiratory neuron hubs in the medullary ventral respiratory column network

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ABSTRACT

Models of brainstem ventral respiratory column (VRC) circuits typically emphasize populations of neurons, each active during a particular phase of the respiratory cycle. We have proposed that “tonic” peri-columnar expiratory (t-E) neurons tune breathing during baroreceptor-evoked reductions and central chemoreceptor-evoked enhancements of inspiratory (I) drive. The aims of this study were to further characterize the coordinated activity of t-E neurons and test the hypothesis that peripheral chemoreceptors also modulate drive via inhibition of t-E neurons and disinhibition of their inspiratory neuron targets. Spike trains of 828 VRC neurons were acquired by multi-electrode arrays along with phrenic nerve signals from 22 decerebrate, vagotomized, neuromuscularly blocked, artificially ventilated adult cats. Forty-eight of 191 t-E neurons fired synchronously with another t-E neuron as indicated by cross-correlogram central peaks; 32 of the 39 synchronous pairs were elements of groups with mutual pair-wise correlations. Gravitational clustering identified fluctuations in t-E neuron synchrony. A network model supported the prediction that inhibitory populations with spike synchrony reduce target neuron firing probabilities, resulting in offset or central correlogram troughs. In 5 animals, stimulation of carotid chemoreceptors evoked changes in the firing rates of 179 of 240 neurons. Thirty-two neuron pairs had correlogram troughs consistent with convergent and divergent t-E inhibition of I cells and disinhibitory enhancement of drive. Four of 10 t-E neurons that responded to sequential stimulation of peripheral and central chemoreceptors triggered 25 cross-correlograms with offset features. The results support the hypothesis that multiple afferent systems dynamically tune inspiratory drive in part via coordinated t-E neurons.
INTRODUCTION

Understanding the connectivity and functions of oscillating networks in the brain is a major goal in neuroscience (e.g., Akam and Kullmann 2014). Brainstem circuit mechanisms that control the drive to breathe are of particular interest not only because of their vital functions in maintaining ventilation and cardiovascular coupling (Nicholls and Paton 2009). The brainstem respiratory network can concurrently express multiple rhythms, in addition to the basic breathing rhythm, with frequencies spanning several orders of magnitude (Dick et al. 2005; Funk and Parkis 2002; Morris et al. 2010). These circuits for breathing have remarkable adaptive and emergent properties (Devinney et al. 2013; Lindsey et al. 1992, 2012; Ramirez et al. 2012) and participate in the generation of numerous other behaviors (Bartlett and Leiter 2012), including vocal communication (McLean et al. 2013), coughing and swallowing, and their coordinated expression as a meta-behavioral response to aspiration (Pitts et al. 2013; Shannon et al. 2000). Breathing may also bind orofacial sensations (Kleinfeld et al. 2014) and be involved in hippocampal processes for learning and memory (Lockmann and Belchior 2014).

Core circuits for generating the respiratory motor pattern are found in the ventral respiratory column (VRC; Richter and Smith 2014) and are embedded in a larger pontomedullary respiratory network (Nuding et al. 2009a; Segers et al. 2008). Models of the VRC typically emphasize interactions among populations of neurons, each active only during a particular phase of the respiratory cycle or at a phase transition (Lindsey et al. 2012). However, peri-columnar “tonic” neurons with or without respiratory modulated discharge patterns have also been identified and proposed to serve as intermediaries in the regulation of breathing by sensory (e.g., baroreceptor, chemoreceptor) systems and by internal state-dependent drives (Orem 1989). We have proposed that the VRC includes an excitatory inspiratory (I) neuron chain tuned by feed-forward and recurrent inhibition from other I neurons and by the inspiratory-phase inhibitory actions of tonically firing expiratory (t-E) neurons, i.e., cells that are active predominantly during the expiratory (E) phase but that also discharge during the I phase under eupneic-like normocapnic conditions (O’Connor et al. 2012; Segers et al. 1987).

Several observations support the hypothesis that t-E neurons serve as a downstream network “hub” (van den Heuvel and Sporns 2013) where signals from multiple afferent systems functionally converge for modulation of inspiratory drive. First, baroreceptor-evoked reductions in inspiratory drive are associated...
with increased firing rates in t-E neurons that have inhibitory functional connections with inspiratory
premotor or motor neurons (Lindsey et al. 1998). Second, central chemoreceptor-evoked enhancement of
inspiratory drive occurs during suppressed inspiratory-phase activity in t-E neurons (Ott et al. 2012).
Third, such disinhibitory amplification of inspiratory drive also occurs during a bout of coughing (Segers et
al. 2012).

In the course of these prior studies, we identified cross-correlation feature sets indicative of
distributed functional inhibitory connectivity of t-E neurons within the column (Ott et al. 2012; Segers et al.
2012) and noted that some pairs of t-E neurons exhibited short-time scale spike synchrony. One aim of
the present work was to confirm and extend that result. Such coordinated firing would be consistent with
the hub concept and cooperative behavior among t-E neurons that could influence the efficacy or duration
of their collective actions on common targets.

Peripheral chemoreceptors of the carotid body rapidly sense changes in arterial O$_2$ and CO$_2$-pH and
also modulate the drive to breathe (Kumar and Prabhakar 2012), although the functional connections
through which they act upon VRC circuits remain incompletely understood (Nuding et al. 2009b; Spyer
and Gourine 2009). Having previously identified differential peripheral chemoreceptor modulation of
inspiratory neurons in the pre-Bötzing region and more caudal medullary domains (Morris et al. 1996,
2001), our second objective was to test the hypothesis that carotid chemoreceptors enhance inspiratory
drive via downstream disinhibitory actions of t-E neurons upon caudal columnar inspiratory neurons.

Numerous disorders of breathing and cardio-respiratory coupling are associated with dysfunctional
chemoreceptor drive mechanisms (Dempsey and Smith 2014; Garcia et al. 2013; Perez and Keens 2013;
Plataki et al. 2013). Brain mechanisms that mediate the separate and joint actions of central
chemoreceptors, sensors of brain CO$_2$ and pH, and the peripheral chemoreceptors are topics of active
research and debate (Duffin and Mateika 2013; Nuding et al. 2009b; Teppema and Smith 2013; Wilson
and Day 2013). Thus, a related third aim was to determine if t-E neurons are dually modulated by both
central and peripheral chemoreceptor influences.

We employed electrode arrays with individual sub-micrometer electrode depth adjustments to monitor
VRC neurons at multiple sites simultaneously. Acquired data sets were screened for short-time scale
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correlations indicative of paucisynaptic functional connectivity and altered firing rates during chemoreceptor perturbations (Lindsey et al. 2013; Segers et al. 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 (AAALAC), National Institutes of Health and National Research Council guidelines.

Data were obtained from 22 adult cats (2.8 – 5.6 kg) of either sex and descriptions of most methods have been published previously (Nuding et al. 2009b; Ott et al. 2011). Animals were initially anesthetized with isoflurane (3-5%) or with ketamine hydrochloride (5.5 mg kg\(^{-1}\), IM) followed by isoflurane; all were maintained with 0.5 – 3.0% isoflurane until decerebration (Kirsten and St. John 1978). Cats were artificially ventilated, neuromuscularly blocked (pancuronium bromide; initial bolus 0.1 mg kg\(^{-1}\) followed by 0.2 mg kg\(^{-1}\) hr\(^{-1}\), IV) and monitored. Arterial blood pressure, end-tidal CO\(_2\), and tracheal pressure were monitored continuously; arterial PO\(_2\), PCO\(_2\), and pH were measured periodically. Animals were bilaterally vagotomized to remove vagal afferent feedback from pulmonary stretch receptors. In some animals, a concentric catheter was inserted into the right external carotid artery and advanced to a point immediately caudal to the carotid sinus (Arita et al. 1988; Li et al. 1999). An additional concentric catheter was inserted into the left axillary artery and advanced to the bifurcation of the vertebral artery (Nuding et al. 2009b); preceding branches of the axillary artery were ligated (Kuwana and Natsui 1987). Heparinized saline was slowly infused from the outer catheter to prevent clotting within the inner catheter. At the end of each experiment, animals were euthanized (Beuthanasia [0.97 mg kg\(^{-1}\); Schering-Plough Animal Health] or sodium pentobarbital [28 mg kg\(^{-1}\)] followed by a saturated solution of KCl in water).

Extracellular neuronal activity was acquired with multi-electrode arrays with individual electrode (1 \(\mu\)m tip diameter; 10-12 M\(\Omega\)) sub-micrometer depth adjustments along with signals from phrenic and vagus nerves. Signals from single neurons were isolated using one of two interactive spike sorting software packages [Datawave Technologies; O'Connor, 2005 #4763]. Coordinates of recording sites were mapped into the three-dimensional space of a computer-based brainstem atlas (Segers et al. 1987).
To stimulate the carotid (peripheral) chemoreceptors, 1 mL of CO₂-saturated saline was injected over a period of 30 seconds into the right carotid artery via a concentric catheter. Subsequent trials occurred at least 5 minutes after the beginning of the previous injection; a minimum of five stimulus trials were performed during each recording. In 4 recordings from 3 animals, the central chemoreceptors were similarly stimulated via a concentric catheter inserted within the left vertebral artery (Nuding et al. 2009b). Stimulus effectiveness was assessed by measures of the peak amplitude of the integrated phrenic nerve signal; effective trials were identified by a change greater than 2 standard deviations from the mean of pre-stimulus control values. Changes in firing rates of single neurons during effective stimuli were identified over the entire respiratory cycle as well as during just the I and E phases using a bootstrap-based statistical method with the false discovery rate controlled to be less than 5% (Benjamini and Hochberg 1995) and classified into one of several response categories: increase (↑), decrease (↓), biphasic [increase-decrease (↑↓) or decrease-increase (↓↑)], or no change (→) (Nuding et al. 2009b). Neurons were also assessed for significant changes in the depth of respiratory modulation (i.e., “rate ratio” (↕): the ratio of the maximum to the minimum mean firing rate; see Ott et al. 2012). The numbers used to calculate the rate ratio were obtained by dividing each respiratory cycle within a response evaluation period and its corresponding pre-stimulus control period into 20 slices and measuring the mean firing rate in a 7-slice-wide window for each of the 20 possible starting locations of the window, yielding 20 numbers for each cycle. The rate ratio is the ratio of the maximum to the minimum of the 20 mean firing rates. A change in rate ratio indicates that the ratio of maximum to minimum firing rates became more (or less) pronounced upon stimulation. A neuron’s response was classified as ↑ if its change in rate ratio was not accompanied by a significant change in neuronal firing rate as defined above.

Standard firing rate histograms were calculated to illustrate neuronal activity; maximum firing rates were calculated by dividing the spike count in the highest bin shown by the binwidth. Respiratory cycle-triggered histograms (CTHs) were constructed for each neuron from activity recorded during a control period consisting of the first 30 minutes of the recording, prior to any stimulus protocols. The average firing rate represented in the standard CTH may be “blurred,” particularly at phase transitions, because cycle durations vary. Therefore, cycle- and phase-normalized histograms were also calculated. To
compute the cycle-normalized CTHs, the duration of each cycle was shortened or lengthened to match
the average cycle length and the time of each spike was changed to keep the spike at the same
proportional location within its cycle before calculating the CTH. The spike count in each bin of each cycle
was divided by the unnormalized bin width for that cycle, and the resulting rates averaged across cycles,
producing the firing rate for that histogram bin. The same method was applied separately to neuronal
activity during the I and E phases to calculate phase-normalized CTHs.

Neurons were classified as respiratory modulated if either of two complementary statistical tests
rejected the null hypothesis ($p < 0.05$). The first test partitioned 50 consecutive respiratory cycles into 20
equal time segments. A subjects-by-treatments analysis of variance was performed using the respiratory
cycles as subjects and the segments as treatments (Netick and Orem 1981; Orem and Netick 1982). A
second nonparametric sign test divided each respiratory cycle into 20 partitions. The first 50 cycles were
used to develop a hypothesis as to the half of the respiratory cycle (or group of 10 consecutive partitions)
in which the cell was more active. Cycles were divided so as to give the greatest total difference between
the halves. All remaining respiratory cycles were then halved accordingly and inspected to determine if
the hypothesis held true significantly more often than chance (Morris et al. 1996).

Respiratory-modulated neurons were classified as inspiratory (I), expiratory (E), or phase-spanning
(IE or EI) according to the part of the cycle during which the cell was most active, and phasic (P) if their
firing probability was essentially zero during any part of the control respiratory cycles or tonic (T)
otherwise as judged from the averaged rate histograms. Thus, for example, t-E neurons were active
during the inspiratory phase of at least some control cycles. Neurons were further described as
decrementing (Dec) or augmenting (Aug) if the average peak firing rate occurred during the first or
second half of the phase, respectively. Cells without a preferred phase of maximum activity were
designated non-respiratory modulated (NRM).

Cross-correlation histograms (CCHs) were calculated using the entire recording for all pairs of
simultaneously monitored neurons. Short-time scale peak or trough features with half-widths ranging from
0.5 msec to 100 msec (consistent with synaptic mechanisms in the VRC; Ott et al. 2012) were identified
using cycle-shifted surrogates (Ott et al. 2012) or with a Monte-Carlo test using surrogate spike trains
(Pauluis and Baker 2000) with gamma–distributed inter-spike intervals. The shape parameter of the
gamma distribution was estimated from the data (Miura et al. 2006). The false discovery rate was controlled to be less than 5%. All offset-feature data are presented with a positive time lag. A detectability index (equal to the maximum amplitude of feature departure from background activity divided by the standard deviation of the correlogram noise) greater than 3.0 indicated a significant correlogram feature (Aertsen and Gerstein 1985; Melssen and Epping 1987). Correlation linkage maps were generated to graphically represent sets of detected cross-correlogram features among simultaneously monitored neurons (Segers et al. 2008). Significant features in spike-triggered averages of full-wave rectified phrenic signals were identified (method adapted from Poliakov and Schieber 1998) using a two-sided Wilcoxon signed-rank test with Bonferroni correction; \( p < 0.05 \) (for details, see Ott et al. 2012).

Gravitational clustering (Gerstein 2010) was used to assess dynamic associations among neurons during stimulus trials. In this approach, each neuron is represented as a particle in N-space with a time-varying charge that is a filtered version of the corresponding spike train. Aggregation of particle pairs reflects neuronal timing relationships; during any interval, a particle pair’s aggregation velocity is related to the product of particle charges and the spike synchrony of the corresponding neurons. Significance of particle aggregation was evaluated using a Monte-Carlo test with surrogate spike trains generated as noted above. In addition to standard gravity, we also evaluated each pair-wise correlation in 1-dimensional space using only that pair’s charge product to define the forces acting on the particles (Lindsey and Gerstein 2006).

Computational models and simulations were run on 64-bit Intel multiprocessor-based computers using a previously described software package (Rybak et al. 2008). Briefly, at each integration step (0.5 msec), the simulator updates state variables for membrane potential, spike generation threshold, post action potential potassium conductance and synaptic conductances for each neuron of each population and each synaptic type. When the membrane potential of any cell exceeds threshold, an “action potential” is generated. Simultaneously, input conductances are activated and all target cells receive synaptic currents defined by a synaptic strength parameter, the number of terminals, and the type of synapse. Model parameters are detailed in the RESULTS.
RESULTS

This work was part of ongoing studies on ventral respiratory column network organization and chemoreceptor reflex circuits. Complementary results on the central chemoreceptor-evoked responses of a subset of neurons described herein have been reported (Ott et al. 2012).

Coordinated clusters of peri-columnar tonic expiratory neurons. The spike trains of 828 VRC neurons were monitored in 22 animals; Table 1 presents the neuronal respiratory-modulated discharge patterns as assessed during the 30-min control period. One hundred ninety-one neurons were classified as tonically active expiratory (t-E) neurons (65% of the E cells sampled). Average firing patterns illustrative of t-E activity are shown in cycle-triggered histograms (CTHs) calculated for two simultaneously recorded t-E neurons (Fig. 1A). The brainstem atlas illustrations in Fig. 1B show that the stereotaxic coordinates of the recording sites of t-E neurons were interspersed among the coordinates of phasic I and E neurons identified in the same data set (t-E neuron recording locations are indicated in red; some recording sites are obscured due to the angle of presentation or because more than one cell was recorded at the same coordinates).

Overall, 25% (48 of 191) of the t-E cells we recorded fired synchronously with another t-E neuron as indicated by central peaks straddling the origin as the primary correlogram feature (e.g., Fig. 1C). The spike synchrony represented by the central peaks was not necessarily confined to a particular phase of the respiratory cycle. Significant central peaks were found in both inspiratory and expiratory phase-segmented data from 6 of 7 pairs of t-E neurons evaluated; results from one such pair are shown in Fig. 1D (Animal C). The mean distance between recording sites for the t-E neurons of 39 synchronously firing pairs identified (1.69 mm ± 1.37 SD) was less than the mean distance (3.16 mm ± 2.17 SD) for the pairs (n = 846) without a significant correlogram feature (p = 0.00003, 2-tailed t-test). Thirty-two of the synchronous pairs of t-E neurons were elements of groups with mutual pair-wise correlations (Fig. 1E).

Correlations in a model circuit in which synchronous neurons inhibit a target population. A goal of cross-correlation analysis is to define simple models of neuronal circuits that can reproduce the observed features (Aertsen et al. 1989). Central peaks are indicative of shared influences of like sign or effective cross-connectivity, whereas peaks and troughs offset with respect to the correlogram origin are commonly

To aid identification of correlogram features predicted under the inhibitory t-E neuron hub hypothesis, we modeled a circuit composed of three “layers” of integrate-and-fire neuron populations (Fig. 2A; Table 2). Mapping of connectivity among the three layers was generated according to a sequence of pseudo-random numbers calculated from a unique seed number for each source-to-target connection. Targets were chosen with replacement (i.e., the same target could be selected more than once). Connectivity parameters are detailed in Table 3. Shared excitatory inputs from neurons in population 1 caused excess synchrony between pairs of inhibitory population 2 neurons, as illustrated in the large correlogram central peak for neurons 9 and 12 (Fig. 2B).

Each member of the inhibitory group (population 2) made connections with randomly selected population 3 target neurons. Cross-correlograms for two pairs composed of the inhibitory trigger neuron 9 and two different population 3 target neurons, 20 and 21 (Fig. 2C), had different trough onset times relative to the origin. Trough onset times were defined as the first bin with a count (normalized to firing rate) less than the smallest value found in all correlograms constructed using surrogate “control” spike trains, as assessed at the maximum temporal resolution of the simulation (0.5 msec). The trough in the left correlogram had a negative onset time. There were no direct connections between the trigger and target neurons; the trough was due exclusively to the effects of presynaptic synchrony within the group of inhibitory population 2 neurons. The trough in the right correlogram had a positive onset time; the 7 terminals in the direct connection between neurons 9 and 21 had a more dominant influence in the interaction than did the effect of the presynaptic synchrony. The distribution of trough onset times for all 100 distinct pairs composed of population 2 trigger neurons and population 3 targets is shown in Fig. 2D.

These model-based results support the inference that an inhibitory population with spike synchrony similar to that observed between t-E neurons can generate reductions in target neuron firing probabilities that appear as either offset or central correlogram troughs. These observations on CCH features are reminiscent of earlier predictions from simulations (Balas et al. 1994) and remain an inherent property of spike timing relationships as measured with cross-correlation and related spike-triggered averaging methods (Davies et al. 1985). Details of feature onset and shape are likely to vary with different model
parameters for divergence, convergence, and synaptic scaling (MacGregor and Tajchman 1988), properties which remain unresolved for the VRC network.

**Responses to selective stimulation of carotid chemoreceptors.** The hypothesis that t-E neurons exhibit reduced inspiratory-phase firing rates during carotid chemoreceptor stimulation-evoked increases in inspiratory drive was tested with data from a subset of 6 recordings (5 animals). The PaCO₂ in these animals ranged from 25 to 36 mmHg, similar to the values observed in cats during eupneic breathing (Herbert and Mitchell 1971). Data from 240 VRC neurons were acquired in a brainstem region extending from 1.5 caudal to 7.8 mm rostral to the obex, 3.0 to 4.6 mm lateral to the midline, and 2.3 to 5.8 mm below the dorsal surface of the medulla.

Table 4 gives a summary of the neuronal responses to peripheral chemoreceptor. Overall, 179 of the 240 (75%) tested neurons exhibited changes in firing rate over the course of respiratory cycles in the assessed stimulus intervals compared to firing rates during the immediately preceding control periods. Of the 179, 64% responded with an increased firing rate, whereas the rates of 26% initially decreased. For 18 neurons, the primary response to peripheral chemoreceptor stimulation was a change in the depth of respiratory modulation. We note that the I-phase firing rates of 34 of the 58 t-E neurons in this data set changed during carotid chemoreceptor stimulation; the ratio of cells exhibiting a decrease vs. an increase in rate was roughly 2:1 (n = 23 vs. n = 11).

A subset of the recording site coordinates from one animal (designated A), in which two series of 5 stimuli were applied, are shown mapped onto a brainstem atlas (Fig. 3A, sagittal view); signals acquired during one stimulus trial, spike waveforms, and associated projections of sorted waveform clusters are also shown (Fig. 3B). Respiratory cycle-triggered histograms document the cells’ average discharge patterns (Fig. 3C). Firing rate histograms from another trial show typical responses to peripheral chemoreceptor stimulation (Fig. 3D). These plots from eight simultaneously recorded VRC neurons (together with integrated phrenic nerve activity and arterial blood pressure) are labeled with each neuron’s (i) respiratory-modulated discharge pattern, (ii) response as assessed from inspiratory phase rate measurements (e.g., Fig 3E), and (iii) identification number. Detailed response plots for each of 5 stimulus trials show the average firing rates in each inspiratory phase during the 60-s response evaluation periods (red traces) for t-E neuron 841 and I neuron 846 (Fig. 3E). These two neurons responded to
stimulation of peripheral chemoreceptors with a significant decrease and increase, respectively, in I-phase firing rates compared to preceding control cycles.

Short-time scale correlations and linkage maps. The number of VRC neurons recorded and tested for response in each carotid chemoreceptor stimulation series ranged from 16 to 80, yielding spike train data from 5,728 distinct cell pairs for cross-correlation analysis. Overall, the cross-correlograms of 473 pairs contained central (n = 266) or offset (n = 207) features. Table 5 is a summary of identified correlogram features grouped according to the respiratory modulations of the constituent neurons and their overall responses to peripheral chemoreceptor stimulation; only those pairs in which both neurons were tested for response to peripheral stimulation are included. This table is specifically intended to complement previously reported results on VRC neuron responses to central chemoreceptor stimulation and identified correlations among those cells (Ott et al. 2012). Two hundred and nine of the 3,626 pairs containing at least one E cell were correlated; in 88% (n = 183) of these cases, the E cell discharged tonically. We identified 32 cell pairs with an offset or central cross-correlogram trough consistent with t-E neuron inhibition of I neurons and disinhibitory enhancement of inspiratory drive during response to peripheral chemoreceptor stimulation. In each of these pairs, the I-phase activity of the t-E cell decreased and the I neuron’s firing rate increased in response to stimulation. Both offset (n = 26) and central (n = 6) troughs are included in this total because, as demonstrated by the data from the simulated network (Fig. 2), spike synchrony among the cells of an inhibitory presynaptic population (t-E neurons, in this case) can result in both offset and central correlogram troughs that reflect the reduction in firing probability of their target (I) neurons. Similar to the model results, the troughs for these 32 pairs more often had a positive onset time (n = 22 vs. n = 11 pairs with negative onset times).

Cross-correlograms for the three inspiratory neurons (644, 861, and 846) represented in Fig. 3 included asymmetrical peaks starting at or near the origin (Fig. 4A, 1–3), features consistent with excitatory actions of the trigger neurons upon the targets and pre-synaptic synchrony effects or unobserved shared influences. The offset peak in the average of full wave rectified contralateral phrenic motor neuron activity triggered by spikes in neuron 861 support a functional excitatory influence of cell 861 upon inspiratory drive (Fig. 4B, a). The offset trough in another correlogram triggered by I neuron 861 is consistent with a paucisynaptic inhibitory action upon t-E neuron 655 (Fig. 4A, 4).
Most of the primary features in correlograms for pairs composed of t-E triggers and I neuron targets within this subset were central or offset troughs (Fig. 4A, 5–7), results consistent with paucisynaptic functional inhibitory actions upon the various I neuron targets, as was the dip in the phrenic signal average triggered by spikes in neuron 841 (Fig. 4B, b). Other results found in correlograms triggered by t-E neuron 841 with t-E target cells 816 and 655 included an offset trough (Fig. 4A, 8) and an offset peak (Fig. 4A, 9), respectively. Another offset peak was noted in a correlogram triggered by t-E neuron 857 with I neuron target 861 (Fig. 4A, 10).

The correlation linkage map (Fig. 4C) summarizes these short-time scale correlations. Each neuron is represented by a "sphere" labeled with its identification number, respiratory modulation pattern, and peripheral chemoreceptor-evoked response during the inspiratory phase (arrow; see legend, Table 4). Small white and black circles at the ends of the black lines represent offset peaks and troughs, respectively; gray curved lines with full or half-full circles at both ends indicate central correlogram peaks and troughs. Numbers in yellow circles correspond to correlograms that document the indicated features; lower case letters in pink circles correspond to features in spike-triggered averages of phrenic nerve activity. Correlogram features and peripheral chemoreceptor-evoked changes in I-phase firing rate provided evidence for an inhibition-mediated reduction in the I-phase activity of a t-E neuron (pair 861–655) and disinhibitory enhancement of I neuron activity (841–846). Responses and short-time scale correlations among three neurons recorded in another animal (designated Animal B) were also consistent with disinhibitory amplification of inspiratory neuron activity (Fig. 4D). Offset troughs were identified in two correlograms triggered by neuron t-E 808, each with a different inspiratory target cell (Figs. 4D, 11 and 12).

We applied gravitational clustering methods to assess the dynamics of t-E neuron firing synchrony and t-E–inspiratory neuron interactions during individual peripheral chemoreceptor stimulation trials. Each of 10 trials in Animal A were evaluated; these included spikes of an additional t-E neuron, 807 (Fig 5A, top), recorded simultaneously with t-E neuron 841 and I cell 846. Both t-E neurons discharged during the inspiratory phase, although the firing probability of neuron 807 was lower as seen in the firing rate histograms from the trial illustrated (Fig 5A, bottom).
Trajectories projected from N-space during standard gravity analysis revealed aggregation of particles corresponding to t-E neurons 807 and 841 and repulsion of the particle for I neuron 846 (Fig. 5B). The results were consistent with the peak and trough cross-correlation features, respectively, from the same data subset (insets, Fig. 5B). We note that particle aggregation is caused by appropriate force rules and the product of (nearly) coincident positive transient spike-induced charges on both particles, whereas repulsion reflects a slightly negative charge on one particle during the absence of spikes, as occurs with an inhibitory interaction.

Because of the loss of information inherent in the projection from higher dimensional space, particle distance as a function of time (PDFT) plots were generated for each pair. For these plots, the associated gravity calculation incorporated an optional enhancement that removed the influence of the remaining third particle. The PDFT plot for t-E neurons 807 and 841 revealed significant aggregation; for most of the gravity run the inter-particle distance was less than the distance found in gravity analysis of 1,000 surrogate data sets (Fig. 5C, top, shadowed line). Red line color in the plot marks periods of aggregation velocity (and thus firing synchrony) greater than found in any surrogate trial during corresponding time intervals. We note that particles representing this neuron pair also aggregated significantly during gravity analysis of each of the other 9 stimulus trials. High aggregation velocities were intermittently present during all 10 gravity runs; high velocities occurred during the stimulus delivery period in 3 of the 10 trials. The repulsion indicative of inhibition was confirmed in the PDFT plot for neuron pair 841–846 (Fig 5C, bottom) for this and four other trials. Significant repulsion consistent with the trough in the correlogram for t-E neuron 807 and I neuron 846 was also identified in PDFT plots from two trials.

Cross-correlograms for concatenated spike data acquired during the 10 stimulation intervals from a total of 38 respiratory cycles were calculated and compared with data from the same total number of cycles from the 10 control intervals preceding the trials (Fig. 5D). Cycles from one trial are indicated by the highlighted segments of the firing rate histogram in Fig 5A. Overall, significant firing synchrony of t-E neurons 807 and 841 was identified during both concatenated control and stimulus cycles (Fig. 5D, top). For the same correlogram bin values, a significant trough was found in the control correlogram for the t-E–I neuron pair 841–846, but not for the stimulus trials.
Functional convergence of peripheral and central chemoreceptor influences on VRC t-E neurons.

Data from 4 of the 6 recordings evaluated for interactions among neurons responsive to stimulation of peripheral chemoreceptors were from 3 animals in which central chemoreceptors were also selectively stimulated. Inspiratory-phase responses of 26 t-E neurons were tested with both protocols (Table 6) to address the hypothesis that the two sensory systems share t-E neurons as network hubs for inspiratory drive modulation. Ten neurons responded to both peripheral and central chemoreceptor stimulation (i.e., “dual responders”); all exhibited the same direction of firing rate change following peripheral and central stimulation. Two hundred and twenty-one cell pairs, each pair containing at least one t-E dual responder, were evaluated; 25 correlations were detected, involving 7 of the dual responders. Four dual responders triggered CCHs characterized by an offset peak or trough, correlational signatures of excitation or inhibition, with more than one target neuron, a result consistent with functional divergence. Two dual responders were target neurons in offset-feature correlograms; in each case, the dual responder was correlated with more than one trigger neuron.

The responses of 9 inspiratory and 3 expiratory neurons in Animal C during a peripheral chemoreceptor stimulation trial (Fig.6A; arrows in columns labeled “PC”) included the recruitment of decrementing expiratory (E-Dec) neuron 860 and I neuron 857 in addition to the modulation of ongoing activity in other members of the group. The arrows in the columns labeled “CC” indicate the corresponding directions of significant changes in respiratory cycle and inspiratory phase firing rates for each neuron following central chemoreceptor stimulation. A correlation feature map generated automatically from the analysis results for this group (Fig. 6B) illustrates the divergent functional inhibitory influence of t-E neuron 847 upon 9 target I cells (offset troughs in Fig. 6C, 13–21). Additional offset troughs were detected in two correlograms triggered by t-E neuron 879 with inspiratory target neurons 862 (Fig. 6D, 22) and 890, suggesting convergent functional inhibition by both t-E cells upon these targets. The sole offset feature in correlograms triggered by phasic E-Dec neuron 860 was an offset peak with inspiratory target neuron 862 (Fig. 6D, 24), which triggered a correlogram with target I neuron 857 that also featured an offset peak (Fig. 6D, 23). Central peak correlogram features indicated by the dashed blue lines in the map include the peak documented previously for t-E neurons 879 and 847 (Fig. 1C, A). Neuron 879 also had a central peak feature in its correlogram with E cell 860 (Fig. 6D, 25).
The firing synchrony of t-E neuron pair 847–879 was confirmed with gravitational analysis of different segments of the recording. Firing rate histograms and PDFT plots from data acquired during peripheral (Fig. 7A) and central (Fig. 7B) chemoreceptor stimulus trials show responses and evidence of fluctuations in coordinated activities during the respective stimulations. For each case, intervals with high particle aggregation rates included, but were not confined to, the stimulus period.

**DISCUSSION**

This work supports a novel hypothesis on circuit mechanisms for peripheral and central chemoreceptor reflex pathway interactions and the regulation of ventilatory motor drive. Short-time scale correlations of t-E neuron spike trains identified with cross-correlation confirmed the presence of coordinated clusters of t-E neurons within the ventral respiratory column. Complementary gravity clustering methods revealed fluctuations in firing synchrony that presumably reflect actions of shared influences, including functional connections identified in this study and previously (Lindsey et al. 1998; Nuding et al. 2009a, 2009b; Ott et al. 2012). Both methods also detected evidence of inhibitory actions of chemoresponsive t-E neurons upon inspiratory neurons, results consistent with roles in gain modulation circuits for adjustment of inspiratory drive.

Strengths and limitations of these approaches have been considered previously (Ott et al. 2012; Segers et al. 2008). We note that 25% of the t-E cells recorded were synchronous with another t-E neuron. Of the 301 pairs composed of a t-E neuron with decreased activity during carotid chemoreceptor stimulation and an inspiratory neuron with increased activity, we identified 32 cell pairs (10.6%) with an offset or central cross-correlogram trough consistent with t-E neuron inhibition of I neurons and disinhibitory enhancement of inspiratory drive. These results are consistent with previous studies in the VRC and other regions of the brainstem respiratory network done with a similar approach, and presumably reflect underlying network organization, synaptic properties, sample acquisition with multi-electrode arrays, and the sensitivity of the methods (Nuding et al. 2009a; Ott et al. 2012; Segers et al. 2008). Given the complexity of the mammalian central nervous system, these percentages are probably underestimates because we certainly did not record all of the t-E neurons nor all of their inputs and targets.
Most instances of pair-wise t-E synchrony detected in our data were associated with groups of 3 to 6 mutually correlated pairs. Spike synchrony among presynaptic neurons provides a greater impact upon the firing probability of their common target neuron(s) than do independently-timed inputs (Abeles 1982; Lindsey 1982; Moreno et al. 2002; Salinas and Sejnowski 2000). A neuronal population is sensitive to changes in input correlation (Tetzlaff et al. 2008); synchronized inputs may signal the occurrence of large events in input drive, communicate information with temporal reliability across a population of neurons (Stevens and Zador 1998), and control the strength and spread of the event to downstream neurons by regulating the gain of the postsynaptic cells (Salinas and Sejnowski 2001). Reduced I-phase activity within a hub of synchronously firing t-E neurons (whose inhibitory influences diverge among as well as converge upon their target cells) in response to a stimulus event would promote quick, coordinated dissemination of that information within the network, producing a controlled and metabolically efficient response to the need for increased inspiratory drive. Different clusters of t-E neurons could serve to “tune” activity along the inspiratory chain under conditions of high excitatory drive, with their spike synchrony providing a mechanism for amplification of inhibition at common targets.

The responses and identified correlation feature sets in our data support a network model (Fig. 8A) in which peripheral chemoreceptor stimulation evokes feed-forward and recurrent operations within the ventral respiratory column. Increased activity in the excitatory inspiratory neuron chain (Fig. 8A, 1) drives other inspiratory neurons, which recurrently inhibit chain elements (Fig. 8A, 2) as well as expiratory neurons, including t-E neurons (Fig. 8A, 3). These t-E neurons have divergent functional inhibitory actions upon the inspiratory neuron circuit (Fig. 8A, 4 and 5) and upon other t-E neurons (Fig. 8A, 6). Chemoreceptor-evoked reductions in the inspiratory-phase firing rates of t-E neurons further enhance inspiratory drive via disinhibition (although such disinhibitory enhancement would be constrained to the complete suppression of t-E discharge during the I phase). Concurrent opposed rate changes in t-E neurons and their I cell targets in response to chemoreceptor (and other sources of) stimulation could mitigate local metabolic demand associated with increased inspiratory drive. The parallel excitatory actions of some t-E neurons upon inspiratory neurons (Fig. 8A, 7) may reflect a counterbalancing disfacilitatory homeostatic process or a transient shift of inspiratory drive sources to less excitation and more disinhibition.
The present results document the functional convergence of peripheral and central chemoreceptors on the same t-E neurons and support the hypothesis that tonic VRC expiratory neurons constitute a hub for converging afferent systems that regulate inspiratory drive intensity and tidal volume. This joint influence is represented in Figure 8B in a circuit abstracted from other models (O'Connor et al. 2012; Ott et al. 2012) with multiple sources of inspiratory drive. “I-Driver” neurons (Segers et al. 1987), a class of pre-Bötzinger complex “pre-inspiratory” neurons (Lindsey et al. 2012), provide one source of drive (Fig. 8B, 1). During peripheral chemoreceptor stimulation, the firing rates of some downstream inspiratory neurons are enhanced (Morris et al. 1996, 2001), as they are during central chemoreceptor stimulation and cough (Fig. 8B, 2). Disinhibitory enhancement of inspiratory drive (Fig. 8B, 3 and 4) during cough is a previously described model-derived prediction (O'Connor et al. 2012) supported by in vivo data (Segers et al. 2012). The decreased activity of t-E neurons during a cough may contribute to the enhanced inspiratory drive characteristic of that motor pattern. More generally, decreased activity of t-E neurons during inspiration may operate to counter-balance the suppressive influence of increased arterial blood pressure upon inspiratory drive due to excitation and disinhibition of t-E activity (Fig. 8B, 5) via raphé circuits during chemoreceptor- and cough-evoked increases in blood pressure (Lindsey et al. 1998; Poliaček et al. 2011).

These results support previous assertions of Orem (1989) that tonic E and other neurons with low respiratory modulation integrate respiratory and non-respiratory inputs to form a negative feedback system that modulates inspiration, for instance during deliberate cessation of breathing, and have implications for ongoing efforts to model network changes associated with airway defensive behaviors such as cough and swallow (Bolser et al. 2013; Pitts et al. 2014). Computational models that include mechanisms to produce both voluntary and involuntary behaviors serve to focus future in vivo experimental aims.

The present results also provide information on important routes of feedback from peripheral and central chemoreceptors to the respiratory network. Previous work has elucidated and simulated inputs resulting in increased inspiratory drive such as that from chemoreceptors (O'Connor et al. 2012) and feedback from the cardiovascular system via baroreceptors (Arata et al. 2000; Dick and Morris 2004; Dick et al. 2005; Poliaček et al. 2011). Conversely, influences from the respiratory system are reflected in...
elements of the cardiovascular system: cardio-respiratory coupling is noticeable in increased heart rate
during inspiration (respiratory sinus arrhythmia) and in changes in blood pressure and its respiratory
modulation in response to chemoreceptor challenge (Dick et al. 2009; Koshiya et al. 1993). Further,
breathing, heart rate, and blood pressure show rhythmic interaction on multiple time scales: heart beat to
heart beat, breath to breath, and in slow wave oscillations (i.e., Mayer waves) (Dick et al. 2014; Morris et
al. 2010). Efficient coordination of the respiratory and cardiovascular systems lessens the physiological
work load; for example, a recent computational study (Ben-Tal et al. 2012) indicates that respiratory sinus
arrhythmia minimizes the work done by the heart while maintaining healthy blood gas levels. Current
simulations are accomplished with a hybrid stochastic/mechanistic model: networks of integrate-and-fire
neurons with connectivity derived from the study of multi-neuron recordings generate output that drives a
biomechanical model of the respiratory muscles, airway, and lungs developed using published measures
from human subjects (O'Connor et al. 2012). The additions of brainstem cardiovascular control networks,
a deterministic biomechanical model of gas exchange, and cardio-respiratory coupling to our current
model simulations will "close the loop" and aid investigation of the advantages of matching breathing with
blood pressure and heart rate.

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DISCLOSURES

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


TONIC EXPIRATORY NEURONS MODULATE INSPIRATORY DRIVE


FIGURE LEGENDS

Fig. 1. Coordinates of recording sites and examples of central peak cross-correlogram features. A: respiratory cycle-triggered histograms (CTHs) for two t-E neurons recorded in Animal C. Yellow histograms show the average pattern of each cell’s discharge throughout the respiratory cycle. Efferent phrenic nerve activity (indicative of the inspiratory phase) is shown in gray; 1824 respiratory cycles averaged. B: sagittal (left) and dorsal (right) views of color-coded spheres marking recording sites within 22 animals mapped in the coordinate space of a cat brainstem atlas. Some recording sites are obscured due to the angle of presentation. If more than one neuron was recorded at the same coordinates, spheres were vertically offset in the sagittal projection to allow representation of each cell’s discharge modulation. Square indicates area shown in panel E. C: cross-correlation histogram (CCH) with a central peak indicative of spike synchrony in the t-E neuron pair shown in panel A. This CCH, labeled with an “A” encircled in yellow, is included in the data set presented subsequently in Fig. 6B. D: a significant peak was identified in both inspiratory and expiratory phase-segmented data sets. The detectability index, bin width (in msec.), and numbers of trigger neuron and target neuron spikes for each CCH are as follows: C: 20.3, 0.5, 237,059 and 137,069; D (E phase; top): 15.0, 0.5, 145,597 and 110,829; D (I phase; bottom): 16.1, 0.5, 91,462 and 26,780. E: linked spheres mark coordinates of 39 t-E neuron pairs with firing synchrony identified by central correlogram peaks (mean detectability index = 9.93 ± 8.24 SD; mean half-width = 19.16 msec ± 18.64). Mutually correlated t-E neuron pairs are indicated by matching colors; spheres of more than one color indicate separate neurons recorded at locations with the same A/P and R/L coordinates.

Fig. 2. Diagram of simulated network used to aid identification of correlogram features predicted under the inhibitory neuron hub hypothesis and results from cross-correlation analysis of the model’s spike outputs. A: circuit composed of three layers of integrate-and-fire neuron populations. In this simulated circuit, population 1 “neurons” excite population 2 cells, which inhibit population 3 neurons. Simulated neurons are represented as large circles; the number inside each circle is the cell’s identification code. A line between two neurons indicates an “axon” projection from one to the other. The darker lines indicate projections to cells involved in cross-correlograms shown in panels B and C. An excitatory or inhibitory
“synapse” is shown as a small white or black circle, respectively, at the end of a line; the arrangement of
the synapse circles reflects the left-to-right order of the trigger cell circles. The number inside a synapse
circle is the number of terminals associated with that synapse; a blank circle indicates that these two cells
were not directly connected within the simulated network. Cross-correlograms of spike outputs of some
neurons are shown in panels B and C. B: the central correlogram peak for neurons 9 and 12 is due to
shared excitatory inputs from population 1. C: cross-correlograms for trigger neuron 9 and two different
population 3 target neurons linked either by presynaptic synchrony alone (pair 9–20) or linked directly as
well as by presynaptic synchrony (pair 9–21) exhibited distinct trough onset times relative to the origin.
The red dashed line indicates the mean bin values of all surrogate correlograms calculated for that pair;
surrogate CCHs are generated until there are enough to keep the false discovery rate below 5%. The
surrogate control confidence limits (solid red lines) are the maximum and minimum values of the
surrogate CCHs for each bin. Bins within the data CCH which exceed the confidence limits are
considered significant. D: distribution of trough feature onset times for neuron pairs composed of
population 2 trigger neurons and population 3 targets. These circuit simulations illustrate that when a
member of a synchronously firing inhibitory population triggers a CCH with a neuron in the target
population, the resulting trough feature may have an onset time on either side of the correlogram origin.
See text for further details.

Fig. 3. Changes in firing rates of simultaneously recorded expiratory and inspiratory VRC neurons
monitored in Animal A during selective stimulation of peripheral chemoreceptors. A: sagittal view of
recording site coordinates and respiratory modulation. Tonic E neurons are shown in yellow; see key in
panel C. Note that the depths of neurons 807 and 861 were estimated due to a malfunction of the
electrode array controller depth display. The recording coordinates of cells 816 and 846 are marked with
a single dot because they were recorded on the same electrode; note that signals from both cells are
apparent in the third trace in panel B. B. examples of signals from an electrode array recorded before and
during a 30-s injection of 1 mL CO$_2$-saturated saline into the carotid artery; the third trial in the first series
of 5 stimulus trials is shown. Sets of 100 superimposed waveforms derived from spike sorting of the
recorded segment and examples of waveform clusters and noise projected from 64-dimensional space
are shown to the right of the raw data traces. This spike sorting method automatically determined the number of discriminable spike waveforms (to be used as templates) using a mean shift clustering algorithm (Georgescu et al. 2003) on a whitened unreduced feature space of 64-sample waveforms with sub-sample alignment of waveforms by sinc interpolation followed by spike detection and classification by least-squares template matching to potential spike waveforms (O’Connor et al. 2005). An additional signal from this trial (neuron 807) is shown subsequently in Fig. 5. Asterisks indicate neurons also represented in subsequent panels. C: respiratory cycle-triggered histograms for neurons shown in panel A; 285 cycles averaged and 10.6 s time scale for all CTHs except 857 (1120 cycles and 16.0 s). Here and subsequently, colors (see Key) indicate categories of respiratory modulation and correspond to representations of the same neurons in recording site coordinate projections and correlation linkage maps. D: firing rate histograms for 8 of the neurons during the second series of 5 stimulus trials together with integrated phrenic nerve activity and arterial blood pressure, labeled with each neuron’s respiratory-modulated discharge pattern, direction of significant change in firing rate during the I phase (arrows), and ID code. E: response plots for t-E neuron 841 (decreased firing rates) and I cell 846 (increased firing rates) during the first series of trials. Average firing rates per inspiratory phase for the 5 stimulus response periods (red traces on gray backgrounds) are shown together with a summary of paired control data (solid green and dotted black horizontal lines indicate mean ± 1 SD); see Methods. Abbreviations: I, inspiratory; E, expiratory; t, tonic; p, phasic; Aug, augmenting; BP, arterial blood pressure.

Fig. 4. Cross-correlation features and spike triggered averages. A: Cross-correlograms from pairs of neurons represented in Fig. 3, grouped by respiratory modulated discharge patterns of trigger and target neurons; numbers in yellow circles indicate a corresponding functional connection represented in the correlation linkage map (panel C). Minimum and maximum bin values, normalized to spikes/sec/trigger event, are shown for each correlogram. The detectability index and bin width (in msec.) for each CCH are as follows: 1: 6.5, 1.5; 2: 13.1, 1.5; 3: 6.3, 1.5; 4: 3.5, 5.5; 5: 8.0, 1.5; 6: 13.9, 1.5; 7: 5.6, 1.5; 8: 8.8, 2.5; 9: 4.3, 5.5; 10: 6.4, 0.5. Number of spikes for each cell: 644: 201,037; 655: 109,890; 816: 52,680; 841: 133,080; 846: 68,479; 857: 45,504; 860: 95,875; 861: 198,262. B: spike-triggered averages of full wave rectified phrenic motor neuron activity triggered by spikes in inspiratory neuron 861 (a) and tonic.
expiratory neuron 841 (b). C: correlation linkage map shows each neuron represented by a “sphere” labeled with identification number, chemoreceptor-evoked I-phase response (arrow), and respiratory modulation pattern (see Key). White and black circles at the ends of the lines represent peak and trough features offset relative to the origin, respectively; gray curved lines with full or half-full circles at both ends indicate central correlogram peaks and troughs. Small black circles indicate a link branch point that represents a putative functionally divergent interaction. In this and subsequent maps, numbers in yellow circles correspond to respective correlograms that document the indicated features; lower case letters in pink circles correspond to features in spike-triggered averages of phrenic nerve activity. D: examples of cycle triggered histograms, cross-correlograms, and a correlation linkage map from responsive neurons in another animal, designated Animal B. Detectability index and bin width (msec.) for each CCH: 11: 10.0, 0.5; 12: 7.4, 0.5. Number of spikes for each cell: 802: 125,604; 808: 106,496; 839: 192,496. See text for details.

Fig. 5. Responses to carotid chemoreceptor stimulation and correlation dynamics of a neuron trio from Animal A during a single trial and effective connectivity over 10 trials compared to control cycles. A: top: signal from neuron 807 during the same interval shown in Fig. 3B together with 100 superimposed spike waveforms and the spike cluster and noise projected from 64-dimensional space. Bottom: Firing rate histograms together with integrated phrenic nerve activity and arterial blood pressure before, during, and after the stimulus interval. The raw data trace from neuron 807 corresponds to the period marked by the red rectangle in the corresponding firing rate histogram (arrow). B: projection of gravity particle trajectories for spike train data represented in panel A. Aggregation of particles for neurons 807 and 841 indicates firing synchrony. The trajectory (repulsion) of the particle for neuron 846 is consistent with anti-correlated activity. Cross-correlogram features for the three pairs (insets) for the same data subset are consistent with the gravity result. Gravity parameters: acceptor and effector charges forward; charge increment time constants were set to 2.5 msec. Numbers of spikes: 807: 3,374; 841: 5,340; 846: 2,312. C: particle distance as a function of time (PDFT) plots for neuron pairs 807–841 and 841–846 (thick lines in each plot) document significant aggregation and repulsion, respectively (i.e. the final particle distance is less than or greater than the null hypothesis represented by the empirical Monte Carlo confidence limit...
“cone”; periods of significant aggregation or repulsion are marked by a shadow). The cones show the minimum, maximum, and mean particle distances (thin black lines) found at each plotted time step in PDFTs calculated using 1000 surrogate spike trains derived from the original data applying the approach of Pauluis and Baker (2000) with the shape parameter of the gamma distribution estimated from the data (Miura et al. 2006). Gravity parameters are as in panel B, except that charges from the third neuron did not influence aggregation velocities of the respective pairs (1-dimension option; see Lindsey and Gerstein 2006). The red portions in the top PDFT mark periods of time during which the mean velocity of aggregation exceeded that found for the corresponding interval in any of the surrogate trials and indicate fluctuations in synchrony. Note the presence of these high aggregation periods before, during (vertical gray band), and after the stimulus interval. D: cross-correlograms for pairs 807–841 (top) and 841–846 (bottom) calculated from spike train data concatenated into two sets composed of 38 control respiratory cycles occurring immediately before each stimulus onset (left; control cycles for the stimulus trial shown in panel A are enclosed within a gold rectangle) or 38 cycles from all 10 trials of carotid chemoreceptor stimulation (right; gray rectangle in panel A). Numbers of spikes: control cycles: 807: 4,383; 841: 5,379; 846: 1,834; stimulus cycles: 807: 3,759; 841: 4,006; 846: 6,955. See text for further discussion.

**Fig. 6.** Functional convergence of peripheral and central chemoreceptor influences on a ventral respiratory column circuit and evidence for divergent inhibitory actions of a t-E neuron. A: firing rate histograms show responses of 9 inspiratory and 3 expiratory neurons in Animal C during a peripheral carotid chemoreceptor stimulation trial; arrows in columns labeled “PC” and “CC” respectively indicate significant changes in rate during the whole respiratory cycle and the I phase for each neuron following selective peripheral and central chemoreceptor stimulation. B: correlation feature map automatically generated from the analysis results for this group of neurons using the open source graph visualization tool Graphviz (Gansner and North 2000); see Key for correlogram features and stimulus responses. Each neuron’s respiratory cycle and I-phase responses to peripheral and central chemoreceptor stimulation are depicted as colored rectangles above the cell’s ID and respiratory type (see Key). Line thickness reflects the value of the detectability index for that neuron pair. C, D: cross-correlograms; circled numbers correspond to labeled map features in panel B. Detectability index and bin width (msec.) for each CCH:
Fig. 7. Comparison of t-E neuron responses and correlation dynamics during single trials of peripheral and central chemoreceptor stimulation. A: top, firing rate histograms, integrated phrenic nerve activity, and arterial blood pressure before, during, and after peripheral chemoreceptor stimulation; bottom, corresponding PDFT plot for neurons 847 and 879 shows significant particle aggregation with final particle distances less than the null hypothesis represented by the empirical Monte Carlo confidence limits. High velocity aggregation (red portions of plot) was identified during and immediately following the stimulus interval (gray vertical band) as described in the legend of Fig. 5. Numbers of spikes: 847: 9,879; 879: 1,949. B: top, firing rate histograms, integrated phrenic nerve activity, and arterial blood pressure before, during, and after central chemoreceptor stimulation; bottom, PDFT plot for neurons 847 and 879, again showing evidence of fluctuating spike synchrony during the stimulus period. Numbers of spikes: 847: 9,637; 879: 6,200. Gravity parameters: acceptor and effector charges forward; charge increment time constants were set to 2.5 msec. See text for further discussion.

Fig. 8. A: summary of functional connectivity inferred from correlation linkages among VRC neurons identified in this study. Represented neuron populations are indicated by circles with color-coded respiratory cycle-triggered histograms. Changes in firing rate in response to peripheral chemoreceptor stimulation are indicated by the direction of arrows. Inferred connections are indicated by the small circles at the ends of the connecting lines (see Key). B: model of circuit for modulation of t-E neurons and inspiratory drive by multiple afferent systems inferred from present results and related prior work. See text for further discussion.
**A** Cycle-triggered histograms

- Neuron ID code
- Phrenic nerve activity
- Max. rate (spikes sec$^{-1}$)

- Neuron 847
  - 37.0 spikes sec$^{-1}$
  - 7.05 s
- Neuron 879
  - 34.3 spikes sec$^{-1}$

**B** Cross-correlogram

- A / P
- Depth
- R / L
- Level of obex

**Key**

- **t-E neurons** (n = 191)
- **E-Phasic cells** (n = 103)
- **I-Phasic cells** (n = 212)

**C** Cross-correlogram

- 847 > 879
  - Spikes sec$^{-1}$ (min; max)
  - 847 > 879 (18.3; 27.7)
  - Entire recording

**D** Phase-segmented cross-correlograms

- 847 > 879
  - E phase
    - (25.5; 36.8)
  - I phase
    - (6.5; 14.3)
**A**

Diagram of a neural network with populations 1, 2, and 3. Nodes are labeled from 1 to 25, with excitatory and inhibitory connections indicated. Nodes 9 and 12 are highlighted.

**B**

Graph showing spikes per second with a range of (124.0; 217.5) and a trough onset lag of 25 msec.

**C**

Statistics for negative and positive lags:
- Negative lag: 0 terminals, range (6.6; 15.5).
- Positive lag: 7 terminals, range (3.3; 14.0).

**D**

Bar chart showing the number of occurrences for trough onset lag relative to origin:
- **-**: 8 occurrences
- **0**: 35 occurrences
- **+**: 57 occurrences
**Figure A**

Animal A

- Depth
- A/P
- Rostral
- Caudal
- Level of obex

**Figure B**

Examples of recordings (trial 3 of first stimulus series)

- 860
- 655
- 816, 846
- 841
- 644
- 861

Start peripheral chemoreceptor stimulus injection

20.9 s

Projection from 64-D space

 Spike waveforms
 Noise Waveform cluster

**Figure C**

Cycle-triggered histograms

- 644
- 857
- 816
- 861
- 860
- 655
- 841
- 846

**Figure D**

Firing rate histograms (trial 2 of second stimulus series)

- Resp. mod.
- PC
- Cell ID
- p-E → 860
- t-E → 857
- t-E → 655
- t-E → 816
- t-E → 841
- I → 846
- I → 644
- I → 861

Maximum spikes sec⁻¹

**Figure E**

Inspiratory phase responses

- t-E neuron 841
- I neuron 846

Firing rate per I phase (spikes sec⁻¹)

Maximum I-phase rate of preceding control cycles

Mean I-phase rate of preceding control cycles

I-phase rates in each cycle

Trial 1
Trial 2
Trial 3
Trial 4
Trial 5
A

Animal A
Cross-correlograms

1. 644 > 861
   (41.1; 48.5)

2. 644 > 846
   (13.5; 15.7)

3. 861 > 846
   (14.6; 16.8)

4. 861 > 655
   (9.7; 10.7)

B

Spike-triggered averages of phrenic nerve signal

5. 841 > 861
   (10.7; 14.1)

6. 841 > 846
   (1.9; 5.0)

7. 841 > 644
   (13.6; 16.2)

8. 841 > 816
   (5.19; 7.33)

9. 841 > 655
   (15.7; 16.9)

10. 857 > 861
    (2.9; 8.1)

C

Correlation linkage map

Key

- Offset peak
- Offset trough
- Central peak
- Central trough
- Branch point

D

Animal B
Correlation linkage map

11. 808 > 802
    (2.9; 8.1)

12. 808 > 839
    (7.5; 13.4)

Animal A
Correlation linkage map

1. 644 > 861
   (41.1; 48.5)

2. 644 > 846
   (13.5; 15.7)

3. 861 > 846
   (14.6; 16.8)

4. 861 > 655
   (9.7; 10.7)

Animal B
Correlation linkage map

11. 808 > 802
    (2.9; 8.1)

12. 808 > 839
    (7.5; 13.4)
A

Animal A

Trial 3 of first series

B

Cross correlation

Gravity particle trajectories

Aggregation

Repulsion

D

38 Control cycles

38 Stimulus cycles

807 > 841

841 > 846
Response to PC stimulation
- Increased firing rate
- Decreased firing rate

Inferred connectivity:
- Excitation
- Inhibition
- Branch point

Premotor

A

B

Response to PC stimulation
- Increased firing rate
- Decreased firing rate

PC
- CC
- Cough

Excitation
- Inhibition
- Disinhibition

Blood pressure

Premotor
Table 1. Control discharge patterns of 828 VRC neurons

<table>
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<tr>
<th>Respiratory modulation</th>
<th>Phasic</th>
<th>Tonic</th>
<th>Total</th>
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<tr>
<td>I</td>
<td>212</td>
<td>127</td>
<td>339</td>
</tr>
<tr>
<td>IE</td>
<td>4</td>
<td>5</td>
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<tr>
<td>E</td>
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<tr>
<td>NRM</td>
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<td>185</td>
<td>185</td>
</tr>
<tr>
<td>Total</td>
<td>320</td>
<td>508</td>
<td>828</td>
</tr>
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</table>

I, inspiratory; E, expiratory; IE and EI, phase-spanning; NRM, not respiratory modulated.
Table 2. Population parameters for simulated circuit shown in Fig. 2

<table>
<thead>
<tr>
<th>Neuron parameter</th>
<th>Population</th>
<th>Parameter value</th>
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<tr>
<td>Number of neurons in each population, N</td>
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<td>Resting threshold, THO (mV)</td>
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<td>Adaptation threshold increase, C</td>
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<td>Adaptation time constant, TTH (ms)</td>
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<tr>
<td>Noise amplitude</td>
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<tr>
<td>DC injected current (mV)</td>
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<td>10.0</td>
</tr>
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</table>

Parameters for the three populations of neurons in the simulated circuit. The variables used for each population were as described in Rybak (2008); abbreviated names from MacGregor (1987). Briefly, the resting threshold was normally distributed around the value of THO with a standard deviation equal to the variability parameter value. Noise amplitude is the conductance added to synaptic conductances produced by each cell’s internal noise generator, which acts like two synapses, one with an equilibrium potential of 70 mV above and the other 70 mV below resting threshold. These inputs have a time constant of 1.5 ms and firing probabilities of .05 per time step. The DC injected current, specified in mV, raises the membrane potential by an amount that is inversely proportional to the membrane conductance and is interpreted as the current that is required to raise the membrane potential by the specified number of mV when the membrane conductance is at its resting value. At other membrane conductances, the effect on the membrane potential is inversely proportional to the conductance.
### Table 3. Connectivity parameters for the simulated circuit shown in Fig. 2.

<table>
<thead>
<tr>
<th>Connectivity parameter</th>
<th>Simulation parameter value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fiber connections to population 1</strong></td>
<td></td>
</tr>
<tr>
<td>Number of fibers</td>
<td>30</td>
</tr>
<tr>
<td>Number of terminals</td>
<td>10</td>
</tr>
<tr>
<td>Firing probability at each simulation time step (0.5 ms)</td>
<td>0.03</td>
</tr>
<tr>
<td>Synaptic weight</td>
<td>0.01</td>
</tr>
<tr>
<td>Synaptic type and time constant (ms)</td>
<td>Excitatory: 1.5</td>
</tr>
<tr>
<td>Number of terminals</td>
<td>Inhibitory: 1.5</td>
</tr>
<tr>
<td><strong>Connections between populations</strong></td>
<td>1 → 2</td>
</tr>
<tr>
<td>Synaptic type and time constant (ms)</td>
<td>Excitatory: 7.5</td>
</tr>
<tr>
<td>Min. conduction time (ms)</td>
<td>Inhibitory: 1.5</td>
</tr>
<tr>
<td>Max. conduction time (ms)</td>
<td>3.0</td>
</tr>
<tr>
<td>Number of terminals</td>
<td>20</td>
</tr>
<tr>
<td>Synaptic weight</td>
<td>0.18</td>
</tr>
<tr>
<td>Synaptic type and time constant (ms)</td>
<td>Inhibitory: 0.03</td>
</tr>
<tr>
<td>Mean (SD) divergence: No. of target neurons innervated by each source neuron</td>
<td>8.8 (0.837) 9.4 (0.516)</td>
</tr>
<tr>
<td>Mean no. of terminals from each source neuron to each target</td>
<td>2.27 3.40</td>
</tr>
<tr>
<td>Mean (SD) convergence: No. of source neurons that innervated each target neuron</td>
<td>4.4 (0.699) 9.4 (0.699)</td>
</tr>
</tbody>
</table>

Parameter values for connections between the three cell population layers and “fiber” populations that provided some modulation of the layer 1 population. Excitatory synapses had an equilibrium potential of 115.0 mV; inhibitory synapses had an equilibrium potential of -25.0 mV.
Table 4. *Overall responses of neurons to peripheral chemoreceptor stimulation*

<table>
<thead>
<tr>
<th>Response to Peripheral Chemoreceptor Stimulation</th>
<th>↓</th>
<th>↑</th>
<th>↓↑</th>
<th>↑↓</th>
<th>→</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>62</td>
<td>10</td>
<td>2</td>
<td>12</td>
<td>18</td>
<td>113</td>
</tr>
<tr>
<td>E</td>
<td>30</td>
<td>3</td>
<td>10</td>
<td>16</td>
<td>24</td>
<td>89</td>
</tr>
<tr>
<td>NRM</td>
<td>9</td>
<td>0</td>
<td>1</td>
<td>6</td>
<td>3</td>
<td>38</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td>101</td>
<td>13</td>
<td>13</td>
<td>34</td>
<td>18</td>
<td>240</td>
</tr>
</tbody>
</table>

Arrows indicate direction of significant change in neuronal firing rate over the entire respiratory cycle. ↑, increase; ↓, decrease; ↑↓, increase-decrease; ↓↑, decrease-increase; →, no significant change; ‧, significant change in the depth of respiratory modulation not accompanied by a significant overall change in neuronal firing rate. For neurons with biphasic rate responses, only the initial response is indicated in subsequent tables and figures.
Table 5. Correlogram features detected among 5,728 neuron pairs in which both cells were evaluated for responses to peripheral chemoreceptor stimulation.

<table>
<thead>
<tr>
<th>Type and overall response correlogram of trigger neuron</th>
<th>Location of correlogram</th>
<th>Respiratory type and overall response of target neuron</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>↑ Offset 13 5 2 1</td>
<td>1* 1*</td>
<td>1* 1* 4*</td>
</tr>
<tr>
<td>Central 83 4 7 25</td>
<td>3 (2) 7 (4)</td>
<td>3* 6*†</td>
</tr>
<tr>
<td>Total 449 169 98 207</td>
<td>400 (233) 375 (301)</td>
<td>32 (39) 285 (235)</td>
</tr>
<tr>
<td>↓ Offset 5 2 1</td>
<td>1*</td>
<td>1* 2*</td>
</tr>
<tr>
<td>Central 2 1</td>
<td>1*</td>
<td>2*</td>
</tr>
<tr>
<td>Total 32 22 25</td>
<td>94 (27) 79 (54)</td>
<td>20 (10) 23 (18)</td>
</tr>
<tr>
<td>↑ Offset 3 - - -</td>
<td>1*</td>
<td>1*</td>
</tr>
<tr>
<td>Central 1 - 1</td>
<td>1*</td>
<td>4 (3)</td>
</tr>
<tr>
<td>Total 12 9</td>
<td>25 (9) 30 (19)</td>
<td>9 (2) 37 (22)</td>
</tr>
<tr>
<td>↓ Offset 3 - - -</td>
<td>1*</td>
<td>1*</td>
</tr>
<tr>
<td>Central -</td>
<td>1*</td>
<td>1*</td>
</tr>
<tr>
<td>Total 38 93 (44)</td>
<td>53 (49) 21 (12)</td>
<td>83 (57)</td>
</tr>
<tr>
<td>↓ Offset 2* 1</td>
<td>1*</td>
<td>1*</td>
</tr>
<tr>
<td>Central -</td>
<td>2*</td>
<td>1*</td>
</tr>
<tr>
<td>Total 156 (116)</td>
<td>224 (199) 27 (18)</td>
<td>191 (178)</td>
</tr>
<tr>
<td>↓ Offset 3* 26*†</td>
<td>2*</td>
<td>2*</td>
</tr>
<tr>
<td>Central 4*</td>
<td>3 (2) 3 (2)</td>
<td>4*</td>
</tr>
<tr>
<td>Total 76 (73)</td>
<td>23 (19) 111 (107)</td>
<td>68 (52)</td>
</tr>
<tr>
<td>↑ Offset -</td>
<td>1*</td>
<td>1*</td>
</tr>
<tr>
<td>Central -</td>
<td>4 (3)</td>
<td>1*</td>
</tr>
<tr>
<td>Total 2 (2)</td>
<td>10 (5)</td>
<td>6 (3)</td>
</tr>
</tbody>
</table>

| E                                                      |                         |                                                     |
| ↑ Offset 1*                                          | 1*                     | 1*                                                   |
| Central -                                           | 4 (3)                  | 1*                                                   |
| Total 2 (2)                                         | 10 (5)                 | 6 (3)                                                |

| NRM                                                   |                         |                                                     |
| ↑ Offset 1                                           | 1*                     | 1*                                                   |
| Central -                                           | 1*                     | 1*                                                   |
| Total 2 (2)                                         | 10 (5)                 | 6 (3)                                                |
Offset and central correlogram features detected among 5,728 VRC neuron pairs grouped according to respiratory discharge pattern and response over the entire respiratory cycle to carotid chemoreceptor stimulation (arrows). 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 italics are the total number of pairs composed of neurons with the respiratory discharge patterns and stimulation responses indicated by the row and column labels. Central features are reported only in the top right portion of the table because the designation of trigger and target neuron is irrelevant for intragroup central feature correlations. The central feature will still be present after switching the two spike trains. Similarly, total numbers of specific pairings are found only in the top right portion of the table. Numbers marked with * indicate that all of the E neurons within those pairs were t-E cells; otherwise, numbers in parentheses indicate the number of pairs containing a t-E neuron. For example, see the shaded numbers within the table and note that a total of 375 cell pairs composed of an I↑ and an E↓ neuron were analyzed; in 301 of such pairs, the E cell discharged tonically. Correlograms calculated for 38 of these pairs contained an offset feature: 9 were suggestive of an I↑ → E↓ influence (2 peaks, 7 troughs) and 29 of an E↓ → I↑ effect (3 peaks, 26 troughs). In addition, 9 central features were detected (3 peaks, 6 troughs) for pairs of this type. All of the E cells involved in
these detected correlations were t-E cells. The values marked with † specifically indicate the 32 correlated pairs consistent with t-E neuron inhibition of I neurons and disinhibitory enhancement of inspiratory drive during response to peripheral chemoreceptor stimulation.
Table 6. *I*-phase responses of 26 t-E neurons tested with peripheral and central chemoreceptor stimulation

<table>
<thead>
<tr>
<th></th>
<th>Peripheral</th>
<th>Central</th>
</tr>
</thead>
<tbody>
<tr>
<td>↑</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>↓</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>→</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>