Central chemoreceptor modulation of breathing via multipath
tuning in medullary ventrolateral respiratory column circuits

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ABSTRACT

Ventral respiratory column (VRC) circuits that modulate breathing in response to changes in central chemoreceptor drive are incompletely understood. We employed multi-electrode arrays and spike train correlation methods to test predictions of the hypothesis that pre-Bötzinger complex (pre-BötC) and retrotrapezoid nucleus/parafacial (RTN-pF) circuits cooperate in chemoreceptor-evoked tuning of ventral respiratory group (VRG) inspiratory neurons. Central chemoreceptors were selectively stimulated by injections of CO₂-saturated saline into the vertebral artery in 7 decerebrate, vagotomized, neuromuscularly blocked, and artificially ventilated cats. Among sampled neurons in the Bötzinger complex (BötC)-to-VRG region, 70% (161 of 231) had a significant change in firing rate following chemoreceptor stimulation, as did 70% (101 of 144) of the RTN-pF neurons. Other responsive neurons (24 BötC-VRG; 11 RTN-pF) had a change in the depth of respiratory modulation without a significant change in average firing rate. Seventy BötC-VRG chemoresponsive neurons triggered 189 offset-feature correlograms (96 peaks; 93 troughs) with at least one responsive BötC-VRG cell. Functional input from at least one RTN-pF cell could be inferred for 45 BötC-VRG neurons (19%). Eleven RTN-pF cells were correlated with more than one BötC-VRG target neuron, providing evidence for divergent connectivity. Thirty-seven RTN-pF neurons, 24 of which were chemoresponsive, were correlated with at least one chemoresponsive BötC-VRG neuron. Correlation linkage maps and spike-triggered averages of phrenic nerve signals suggest transmission of chemoreceptor drive via a multipath network architecture: RTN-pF modulation of pre-BötC-VRG rostral-to-caudal excitatory inspiratory neuron chains is tuned by feed-forward and recurrent inhibition from other inspiratory neurons and from “tonic” expiratory neurons.
INTRODUCTION

Central chemoreceptors monitor brain CO₂/pH and, with their chemoresponsive follower neurons, provide an essential component of the drive to breathe. The retrotrapezoid nucleus/parafacial region of the brainstem (RTN-pF) contains central chemoreceptors (Abbott et al. 2009; Gourine et al. 2010; Guyenet et al. 2010) and is a rostral extension of the medullary ventrolateral respiratory column (VRC), a network containing circuits essential for generating and modulating the motor pattern for breathing (Onimaru et al. 2008; Smith et al. 2009). The VRC extends caudally through the Bötzing complex (BötC), largely composed of inhibitory expiratory neurons (Fedorko et al. 1989; Jiang and Lipski 1990; Lindsey et al. 1989; Merrill and Fedorko 1984), and the pre-Bötzing complex (pre-BötC), a core “compartment” for inspiratory rhythm generation (Smith et al. 1991) which may also have a CO₂/pH chemosensory function (Koizumi et al. 2010; Nattie 2001; Peever et al. 2001; Solomon et al. 2000). The most caudal region of the VRC is the ventral respiratory group (VRG) with its premotor and motor neuron populations. The VRG includes bulbospinal neurons that drive phrenic motor neurons innervating the diaphragm (Bianchi et al. 1995; Lois et al. 2009) and may also contain chemoreceptors (Nattie and Li 1996).

Although axonal projections of RTN-pF neurons with properties characteristic of chemoreceptors have been traced to every segment of the VRC (Abbott et al. 2009), the organization of chemoreceptor reflex circuits within the VRC remains incompletely understood (Guyenet et al. 2010). In a recent study, we employed multi-electrode arrays and spike train analysis to test hypotheses on network mechanisms for the respiratory modulation and central chemoreceptor-evoked responses of RTN-pF neurons (Ott et al. 2011). Here, we report complementary results from that work. Correlational linkages support a new network model for chemoreceptor-mediated tuning of pre-BötC-VRG circuits and respiratory drive. The model incorporates tonic columnar expiratory neurons in a multipath architecture.
Preliminary accounts of this work have been presented (Lindsey et al. 2011; Ott et al. 2008).

METHODS

General methods and surgical preparation

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.

As detailed descriptions of the methods have recently been published (Nuding et al. 2009b; Ott et al. 2011; Segers et al. 2008), only a brief review is included here. Data were obtained from 7 adult cats (2.9 – 4.3 kg) of either sex. Animals were initially anesthetized with 5% isoflurane mixed with air and maintained with 0.5 – 3.0% isoflurane until decerebration.

Arterial blood pressure, end-tidal CO₂, and tracheal pressure were monitored continuously; arterial PO₂, PCO₂, and pH were measured periodically. The left and right vago-sympathetic nerve trunks were isolated in the neck and sectioned to remove vagal sensory feedback from pulmonary stretch receptors. A 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). At the end of each experiment, animals were overdosed with Beuthanasia (0.97 mg kg⁻¹; Schering-Plough Animal Health) and perfused using a 10% neutral-buffered formalin solution.

Efferent phrenic nerve activity - used as an indication of respiratory drive, to assess stimulus effectiveness, and to identify the phases of breathing - was monitored together with signals from two multi-electrode arrays with individually adjustable high impedance tungsten microelectrodes (1 μm tip diameter; 10-12 MΩ). A 24-electrode array (4 x 6 arrangement) was placed in the rostral region of the medulla to monitor RTN-pF neurons and a 32-electrode array
(2 x 16) was placed in the region of the BötC-VRG. Electrode placement was guided by anatomical landmarks (obex, brainstem midline), appropriate stereotaxic coordinates derived from Berman (1968), and the results of previous studies (Baekey et al. 2004; Connelly et al. 1992; Schwarzacher et al. 1995). Stereotaxic coordinates of recording sites were mapped into the three-dimensional space of a computer-based brainstem 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).

**Stimulation of central chemoreceptors**

Control neuronal activity was recorded for a 30-minute period before any stimuli were presented. Central chemoreceptors were then selectively stimulated by 30-s injections of 1.0 mL of a CO₂-saturated 0.9% saline solution into the vertebral artery (Arita et al. 1988a, 1988b; Nuding et al. 2009b; Ott et al. 2011). The stimulus protocol included at least 5 trials separated by 4.5-min intervals to allow phrenic nerve activity to return to prestimulus levels. Stimulus effectiveness was confirmed by measures of the peak amplitude of the integrated phrenic nerve signal; effective reflexes were identified by a change greater than 2 standard deviations in the peak integrated phrenic nerve amplitude from the mean of prestimulus values (Nuding et al. 2009b). Control saline injections did not evoke significant changes in phrenic nerve frequency or amplitude.

**Post-experimental processing and data analysis**

**Classification of neuronal responses**

Signals from single neurons were isolated using interactive spike sorting software (O'Connor et al. 2005). Methods used to evaluate and classify neuronal responses have been described previously (for details see Fig.3C in Ott et al. 2011). Briefly, we compared neuronal firing rates during a 90-s “response” window (30-s stimulus injection plus 60-s post-injection).
with those during the immediately preceding 90-s of “control” in order to measure significant firing rate changes. Note that window duration extended beyond the injection period to ensure that responses with varying time lags would be detected. Results reported are averages of at least 5 trials. Cumulative sum histograms (Ellaway 1978) were calculated from peri-stimulus time histograms. Changes in activity that exceeded 3 SD (Davey et al. 1986) were confirmed 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 were classified into one of 5 response categories: increase (↑), decrease (↓), biphasic response [increase-decrease (↑↓) or decrease-increase (↓↑)], or no change (→).

Neurons were also assessed for significant changes in the depth of respiratory modulation [i.e., “rate ratio” (↕)]; the rate ratio is a measure of cross-phase modulation. This parameter was evaluated by dividing each respiratory cycle within the stimulus response period and its corresponding 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. This test measured the ratio of the maximum to the minimum mean firing rate. The bootstrap method was used to evaluate whether or not there was a significant change in the degree of modulation.

Respiratory modulation of firing rates

Normalized respiratory cycle-triggered histograms (CTH) were constructed from the 30-minute control period recordings for each neuron (Cohen 1968). Neurons were classified as respiratory modulated if either of two complementary statistical tests (Morris et al. 1996a; Netick and Orem 1981; Orem and Netick 1982) rejected the null hypothesis ($p < 0.05$); neurons with no preferred phase of maximum activity were considered non-respiratory modulated (NRM). 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 (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 their firing probability was essentially zero during any part of the respiratory cycle, or tonic (T) otherwise (Morris et al. 1996b).

Cross-correlation analysis

Cross-correlation histograms (CCHs) were calculated using the entire recording for all pairs of simultaneously monitored neurons. The goal of this approach is to define simple circuit models that reproduce experimentally observed features (Aertsen and Gerstein 1985; Kirkwood 1979; Moore et al. 1970; Ostojic et al. 2009). For example, central peaks or troughs can be simply interpreted as evidence of a shared input that influences both cells’ firing rates with similar or opposite effects, respectively. An offset peak with a positive time-lag suggests excitation of the target neuron by the trigger (or reference) cell, whereas an offset trough is evidence for an inhibitory process. All offset-feature data are presented with a positive time lag.

Peak or trough features were identified as departures $\geq 3$ SD from the mean of shift-predictor control correlograms calculated using 20 respiratory cycles at a time with all possible shifts of these cycles (Nuding et al. 2009a). Detectability indices (equal to the ratio of the maximum amplitude of feature departure from background activity, divided by the standard deviation of the correlogram noise) were calculated (Aertsen and Gerstein 1985; Melssen and Epping 1987). Autocorrelation histograms aided interpretation of the cross-correlogram features (Moore et al. 1970; Perkel et al. 1967a, 1967b). Correlation linkage maps for groups of simultaneously monitored neurons were generated (Segers et al. 2008).
The spike times of 186 neurons in 5 recordings were used to generate triggered averages of full-wave rectified contralateral phrenic nerve signals (e.g., Shannon et al. 2000). Averages were screened for short-time scale characteristics in the efferent signal time-locked to the trigger events. Peaks and troughs in the averages were evaluated for significance ($p < 0.05$) using an adaptation of the “multiple fragment” statistical analysis method (Poliakov and Schieber 1998) applied to all bins of the average. A spike-triggered average (STA) was calculated for each pair of adjacent even-numbered respiratory cycles, bin-by-bin. Control averages were calculated from the same two cycles by taking trigger events from one cycle and the analog signal from the other. Using signals from every other cycle minimized the interference of local serial correlations on the statistics. A two-sided Wilcoxon signed-rank test with a Bonferroni correction for multiple testing was used to determine whether the mean of each STA bin significantly differed from the mean of the corresponding control STA bin. Signals from odd numbered cycles were similarly evaluated. Features were classified as significant if reported as such for either data subset.

RESULTS

This work was part of a larger study on VRC network organization and central chemoreceptor reflex circuits. Complementary results on functional connections for shaping the respiratory modulation and chemoreceptor-evoked responses of RTN-pF neurons have been reported (Ott et al. 2011). The present data were acquired during 8 recording sessions in 7 animals. A 32-electrode array monitored neurons ($n = 231$) within the BötC-to-VRG domain, extending from 1.5 caudal to 5.1 mm rostral to the obex, 3.3 to 4.6 mm lateral to the midline, and 2.6 to 5.8 mm below the dorsal surface of the medulla. A 24-electrode array recorded RTN-pF neurons ($n = 144$) in the region extending from 5.0 to 7.0 mm rostral to the obex, 1.4 to 4.2 mm lateral to the midline, and 3.3 to 7.8 mm below the dorsal surface of the medulla.
Table 1 shows the numbers of neurons found in each region grouped by category of respiratory modulation. The majority of neurons in the BötC-VRG and RTN-pF domains were respiratory modulated (79% and 66%, respectively). During the initial control recording period, a greater proportion of respiratory-modulated BötC-VRG neurons had a phasic discharge pattern (62%), whereas most respiratory-modulated RTN-pF neurons (94%) were tonic with some activity throughout the respiratory cycle. The respiratory cycle-triggered histograms (CTHs) in Fig. 1A illustrate the variety of discharge patterns relative to phrenic nerve activity found in one simultaneously-recorded group of neurons in the pre-BötC-VRG region; firing patterns are shown for several I-Aug-P and I-Dec-P neurons, two tonic expiratory neurons [cells 813 (E-Dec-T) and 815 (E-Aug-T)], an E-Aug-P neuron (820), and an E-Dec-P cell (848).

Responses to central chemoreceptor stimulation

Firing rate histograms generated from the activity of neurons shown in Fig. 1A (marked by colored boxes around each cell’s identification code) and three additional I-Dec-P cells during one of five central chemoreceptor challenges are shown in Fig. 1B together with integrated efferent phrenic activity and arterial blood pressure. The 30-s stimulus injection period is delineated by the horizontal line at the bottom; the respiratory modulation and response of each neuron (arrow) based on analysis of all 5 stimulus trials are indicated to the left of each trace. Cells with similar respiratory modulated discharge patterns had diverse responses. The maximum firing rate represented in each histogram is shown to the right.

Recording sites mapped to stereotaxic coordinates (Fig. 1C) include color-coded responses of the corresponding neurons (see Key). The plots in Fig. 1D show peak firing rates per respiratory cycle for paired control (black traces) and 90-s response evaluation periods (red traces) for 3 of 5 central chemoreceptor stimulus trials. The representative traces for neurons 808 (Fig. 1D₁) and 820 (Fig. 1D₂) show significant (p < 0.05) peak rate increases and decreases, respectively.
Seventy percent of neurons in both the BötC-VRG and RTN-pF responded to central chemoreceptor stimulation with a significant change in firing rate (Table 2). The more common direction of change differed for neurons in the two regions, however: 69% of rate-changers in the BötC-VRG responded with an increased firing rate, whereas the rates of 61% of those in the RTN-pF initially decreased. There was no significant change in firing rate in response to chemoreceptor stimulation for cell 822; however, this cell’s depth of respiratory modulation increased (denoted by double-headed arrow, ↕; see METHODS). We observed examples of neurons from both regions (24 BötC-VRG; 11 RTN-pF) that had this significant change in the rate ratio metric unaccompanied by a significant change in firing rate relative to the control. These neurons were also considered responsive. Some cells (57 RTN-pF; 125 BötC-VRG) responded with an alteration in depth of respiratory modulation as well as a change in firing rate; these cases were classified according to the rate change, not the rate ratio.

Functional connectivity among BötC-VRG neurons

Cross-correlation analysis was applied to 3,831 pairs of BötC-VRG neurons. Table 3 summarizes the total number of central and offset peaks and troughs. BötC-VRG neuron pairs are grouped according to the respiratory discharge pattern of the trigger (left side) and target neurons (across the top). Overall, 161 different BötC-VRG neurons (70%) had short-time scale spike train correlations with at least one other BötC-VRG neuron. A total of 146 BötC-VRG neurons (63%) were elements of BötC-VRG neuron pairs with correlogram central peaks (n = 269) or troughs (n = 103) indicative of a shared input of like or opposite sign, respectively. Seventy-six neurons triggered 202 cross-correlograms with other BötC-VRG target neurons that featured offset peaks or troughs, correlational signatures of excitation or inhibition (104 peaks, 98 troughs; Table 3). Forty-five of these reference neurons were correlated with more than one target neuron, a result consistent with local functional divergence. The majority of correlations were among chemoresponsive BötC-VRG neurons: When this same analysis is limited to the
2,619 pairs comprised of chemoresponsive BötC-VRG cells, 70 BötC-VRG neurons triggered 189 offset-feature correlograms (96 peaks and 93 troughs) with at least one other chemoresponsive BötC-VRG cell (Table 4); 42 cells were correlated with more than one target neuron.

Correlational linkages of chemoresponsive pre-BötC-VRG neurons

Cross-correlograms (Fig. 2A) from the group of neurons represented in Fig. 1 had offset peaks (Fig. 2A, 1-3), offset troughs (Fig. 2A, 5-7 and 9-18), and central peaks (Fig. 2A, 4, 8-11, and 19); note that some correlograms had both central peak and offset trough features. The correlation linkage map (Fig. 2C) shows a compilation of pair-wise correlations for the group of neurons and provides a framework to aid visualization of the detected relationships and correlogram feature sets. In this and subsequent maps, each large “sphere” represents a neuron with the corresponding identification number, color-coded respiratory modulation pattern, and chemoreceptor-evoked response (arrow). Inspiratory neurons are represented in the left region of the map; inspiratory neurons that triggered correlograms with positive-lag offset troughs are distinguished by an alternate color (pink). Tonic and phasic expiratory neurons are represented in the right column. Small white and black circles at the ends of the numerically labeled lines between spheres represent offset peaks or troughs, respectively, in the correspondingly numbered correlogram. The curved gray lines with filled circles at both ends indicate central correlogram peaks.

Correlations among inspiratory neurons represented by the green spheres included a “chain” of successive offset peaks extending from pre-BötC region inspiratory neurons 831, 802 and 808 to more caudal neurons 851, 822, and 817; cells 822 and 817 then converge upon 821. The central peak feature (Fig. 2A, 4) for the correlogram calculated for neurons 802 and 808 indicates a shared coordinating influence. Offset-peak correlograms triggered by neuron 802 indicate additional links with inspiratory target neurons 853 and 842 (pink spheres). Each of
these neurons, in turn, triggered correlograms with offset troughs, as did two other putative inhibitory inspiratory neurons, 826 and 812 (Fig. 2A, 5 and 6). We note that cell 853 was (recurrently) linked with an offset trough to inspiratory neuron 802 (Fig. 2A, 7) and also correlated with E-Aug-P neuron 820 (blue sphere). The central peak feature for 853 and 812 (Fig. 2A, 8) indicates short-time scale coordination of the spike activity by a shared influence. Neuron 842 triggered offset-trough correlograms with E-Dec-T (yellow) target neuron 813 (Fig. 2A, 14), as did inspiratory neuron 826 (Fig. 2A, 12), which was also linked to E-Dec-P neuron 848 (Fig. 2A, 13).

Correlograms triggered by inspiratory neuron 816 (Fig. 2A, 9-11) had offset troughs adjacent to asymmetrical central peaks (target neurons 826, 812, and 851). These features, coupled with the neurons’ common phase of peak firing and responses to chemoreceptor stimulation, suggest the operation of shared influences upon the neurons and a sequential inhibitory chain (e.g., 816-to-812-to-802) for modulating the pre-BötC node of the excitatory inspiratory neuron chain.

Tonic expiratory neuron 815 (yellow) was tightly coordinated with E-Aug-P neuron 820 (Fig. 2A, 19). Both neurons had augmenting activity patterns as the expiratory phase developed (Fig. 1A) and both responded to central chemoreceptor stimulation with transient reductions in firing rates. The two neurons also had divergent offset-trough functional connections with inspiratory neurons distributed throughout the sampled region (e.g., Fig. 2A, 15-18). The gray “substrate” under sections of the linkage map highlights the distributed associations of cell 815. The chemoreceptor-evoked reduction in the firing rate of this tonic neuron, most notably during the inspiratory phase (Fig. 1B), is consistent with disinhibitory influence upon the linked inspiratory neurons.

Results from spike-triggered averaging of the full wave rectified contralateral phrenic nerve signal were consistent with hypotheses suggested by other relationships represented in the linkage map. The average triggered by inspiratory neuron 802 revealed an offset peak
(arrow, Fig. 2Ba) superimposed upon a broader central trough. The peak supports the excitatory chain hypothesis; the broader trough is consistent with the influence of a recurrent inhibitory circuit also driven in part by neuron 802. The offset trough (arrow, Fig. 2Bb) in the average triggered by tonic expiratory neuron 815 is consistent with functional inhibition of the phrenic motor neurons or antecedent elements of the excitatory inspiratory neuron chain. Overall, 42 of 186 STAs contained characteristics indicative of a cell’s influence upon phrenic motor nerve activity. The majority of these involved BötC-VRG neurons: significant features in STAs calculated for 39 of 113 BötC-VRG cells had central (n = 23) or offset (n = 4) peaks or troughs, or combinations of these features (n = 12). An effect upon phrenic activity could be inferred for only 3 of 73 evaluated RTN-pF neurons; in each case, the STA contained a central trough.

Further examples of spike triggered averages are shown in subsequent figures.

Distributed linkages of tonic expiratory neurons

Results from a second animal provide additional evidence for wide-spread actions of tonic expiratory neurons upon pre-BötC-VRG inspiratory neurons. Figure 3A shows the recording sites and chemoreceptor-evoked responses of a group of 14 simultaneously monitored neurons. Cycle-triggered histograms from 4 of 12 inspiratory neurons and two tonic expiratory neurons are shown in Fig. 3B. We note that, as for the data shown in Figure 1, the tonic expiratory neurons (879, 847 in Fig. 3A) were recorded at sites well caudal to the coordinates of the pre-BötC. The average peak firing rates of both expiratory neurons increased in response to central chemoreceptor stimulation as did their depth of respiratory modulation, reflecting in part a reduced firing rate during the inspiratory phase. Cross-correlogram 20 (Fig. 3C) for pre-BötC inspiratory neuron 810 and caudal target inspiratory neuron 862 featured an offset peak. Correlograms triggered by neuron 862 with targets cells 857 and 814 revealed additional offset peaks represented in the correlation linkage map for this group (Fig. 3E).
In addition to the inspiratory neurons linked by offset-peak correlations, we identified overlapping sets of mutually correlated inspiratory neuron pairs with central peak features. These associations are represented by colored circles within the green spheres in the linkage map (Fig. 3E). For example, all of the correlograms calculated for every pair comprised of the 7 neurons marked with a blue circle contain a central peak; one example is shown in correlogram 21 from the 829-862 pair (Fig. 3C). A central peak (Fig. 3C, 22) was also identified in the cross-correlogram for tonic expiratory neurons 847 and 879.

All of the inspiratory neurons shown in this sample (within the gray background) had transient reductions in their discharge probability following trigger spikes of tonic expiratory neuron 847; the offset troughs in correlograms 23-27 (Fig. 3C) document 5 of these 12 inferred inhibitory relationships. Correlograms triggered by tonic expiratory neuron 879 for three of these inspiratory target cells also had offset troughs, providing evidence for a functional convergence of coordinated tonic expiratory neurons upon the same pre-BötC target neurons (cells 810, 862, and 890; Fig. 3E).

The average of the phrenic nerve signal triggered by inspiratory neuron 821 included a narrow offset peak superimposed upon a broader peak that spanned the trigger origin (Fig. 3Dc). Together, these features suggest the influence of other correlated premotor inspiratory neurons. The offset trough (Fig. 3Dd) in the average triggered by tonic expiratory neuron 847 provides further support for functional inhibition of the inspiratory neuron chain.

Evidence for functional connectivity of RTN-pF neurons with the BötC-to-VRG domain

The spike trains of 4,028 neuron pairs, each composed of an RTN-pF and a BötC-VRG neuron, were evaluated for short-time scale correlations. Table 5 shows the numbers of offset and central peaks and troughs detected in correlograms calculated using an RTN-pF trigger neuron and a BötC-VRG target. As in Table 3, pairs are arranged according to the respective respiratory discharge patterns of the RTN-pF reference (left) and BötC-VRG target neuron (top).
A total of 37 RTN-pF neurons (26%) were correlated with at least one BötC-VRG neuron. Of these, 24 were triggers of 68 correlograms with positive time lag offset peaks \( (n = 47) \) or troughs \( (n = 21) \), results consistent with paucisynaptic functional neuronal connectivity from the RTN-pF to BötC-VRG neurons. Eleven RTN-pF reference neurons were correlated with more than one BötC-VRG target neuron, evidence for divergent connectivity. Functional input from at least one RTN-pF cell could be inferred for 45 BötC-VRG neurons (19%); in 12 cases, there was evidence for the convergent influences of two or more RTN-pF cells upon a single BötC-VRG neuron.

Twenty-eight (19%) RTN-pF neurons were associated with one or more BötC-VRG neurons via shared influences as indicated by 85 correlograms with a central peak or trough. In 13 instances, correlograms triggered by the same RTN-pF neuron but with RTN-pF and BötC-VRG target neurons had offset features, while the correlogram for those two target neurons had a central feature. Each such result from a trio of neurons is consistent with a particular RTN-pF neuron serving as a putative input source shared by the target cells.

Twenty-four chemoresponsive RTN-pF neurons were correlated with at least one chemoresponsive BötC-VRG neuron as indicated by an offset or central feature in the correlogram. Table 6 shows the numbers of correlograms with offset peaks and troughs for chemoresponsive RTN-pF – BötC-VRG cell pairs arranged by respiratory modulation category and response to chemoreceptor stimulation. The recording site coordinates and responses of three neurons from this data subset are represented in Fig. 4A. The rostral tonic decrementing expiratory RTN-pF neuron 415 had a transient decline in average activity late in the inspiratory phase (CTH, Fig. 4B, top). This neuron responded to chemoreceptor stimulation with a reduced firing rate, whereas the rates of phasic VRG inspiratory neuron 818 and augmenting expiratory cell 826 (Fig. 4B, middle and bottom CTHs) increased. The correlograms triggered by the RTN-pF neuron and computed for both VRG target spike trains had offset troughs (Fig. 4C, 28 and 29). These features and the neurons' responses represented in the linkage map (Fig. 4D) are
consistent with inhibition of both VRG target neurons by cell 415 and suggest the hypothesis
that evoked disinhibition contributes to the increased VRG neuron activity with chemoreceptor
stimulation; see DISCUSSION.

Multiple correlations were identified in a group of 14 RTN-pF and VRG neurons from a
fourth animal; recording site coordinates and responses are represented in Fig 5A. Firing rate
histograms and CTHs from a subset of the neurons (Fig. 5B and C, respectively) show that tonic
decrementing RTN-pF neurons 418 and 402 both had transient rate reductions late in the
inspiratory phase similar to that of neuron 415 in Fig. 4B. The VRG sample included phasic
augmenting (817) and decrementing (829) inspiratory neurons and tonic decrementing (820)
and phasic augmenting (831) expiratory neurons (Fig 5C, right).

Cross-correlograms from RTN-pF neuron pairs in this data set have been previously
reported (Ott et al. 2011). Here we document specific correlogram features (Fig. 5D) and a
corresponding correlation linkage map that incorporates identified functional associations with
VRG neurons (Fig 5F). Central peaks were the primary features in correlograms for pairs
composed of RTN-pF neurons 402, 408, and 418. This feature set is represented by the small
blue circles (Fig 5F; e.g., Fig. 5D, 30) and is indicative of shared coordinating influences.

This trio had other associations with RTN-pF neurons (Fig. 5F). Notable features in
correlograms triggered by neuron 402 included an offset “peak-trough” with target neuron 427
(Fig. 5D, 31), an offset peak with target 416 (Fig. 5D, 32), and an indirect link with neuron 427
via the offset trough in the correlogram triggered by cell 416 (Fig. 5D, 33). Neuron 418 had
similar correlational relationships with cells 416 and 427 (Fig. 5D, 35, 37). These results are
consistent with the operation of parallel excitatory and feed-forward inhibitory RTN-pF circuits
and are considered further in the DISCUSSION. In addition to triggering offset trough-feature
correlograms with RTN-pF targets (Fig 5F; e.g., Fig. 5D, 36), cells 402, 408, and 418 also
triggered correlograms with offset peaks for VRG augmenting expiratory target neuron 831
(e.g., 5D, 34, 39).
Evidence for multiple sources of pre-BötC-VRG tonic expiratory neuron modulation

Other correlation features identified in this set of neurons included offset peaks in correlograms triggered by neurons 408 and 418 with pre-BötC-VRG tonic expiratory target neuron 820. The 418-820 correlogram (Fig. 5D, 38) had an offset peak "superimposed" upon a broader central peak, the latter feature indicative of a shared influence. The correlogram triggered by decrementing inspiratory neuron 829 with target cell 820 had an offset trough (Fig. 5D, 45). These and other results shown in Tables 5 and 6 suggest that multiple influences shape the discharge pattern of pre-BötC-VRG tonic expiratory neurons; see DISCUSSION.

Correlograms triggered by tonic expiratory neuron 820 with each of four VRG inspiratory target neurons (809, 812, 815, and 817, grouped within the gray background) all had positive time lag offset troughs (Fig. 5D, 40-43). Each pair of neurons in this group of 4 tended to discharge synchronously as illustrated by the central correlogram peak for pair 815-817 (Fig. 5D, 44). The spike triggered averages of phrenic nerve signals triggered by three of the VRC inspiratory neurons (Fig. 5Ee-g) had short-latency narrow offset peaks (3.9 ± 0.4 ms lag to peak; 1.7 ± 0.3 ms half-width) superimposed upon broader peaks. These results are consistent with the influence of tightly synchronized premotor neurons correlated by functionally antecedent shared inputs. The offset trough in the average triggered by neuron 820 (Fig. 5Eh) shows a functional inhibition of inspiratory drive correlated with the spikes of this expiratory neuron.

DISCUSSION

The combinations of responses and correlogram features identified in this study suggest that eight distinct operations among VRC neurons contribute to central chemoreceptor modulation of breathing. As shown in Figure 6A, the increases (↑) and reductions (↓) in the reference and target neuron firing rates in response to chemoreceptor challenge together with the associated offset correlogram feature (peak or trough) are simply interpreted as distinct
functional actions (excitation, disfacilitation, inhibition, and disinhibition) that either promote or limit changes in target neuron activity following chemoreceptor stimulation.

The mosaic of identified correlational sub-assemblies incorporating these operations (Fig. 6B) offers a new perspective on respiratory network architecture and includes multiple sites for regulating the motor pattern for breathing. Selective stimulation of central CO₂/pH chemoreceptors evokes increased firing rates in the rostral-to-caudal VRC excitatory inspiratory neuron chain (Rekling and Feldman 1998; Segers et al. 1987) that extends from the pre-BötC region to more caudal VRC populations (Fig. 6B, square 1). The chain includes putative pre-motor bulbospinal neurons identified by offset peaks in spike-triggered averages of phrenic motor neuron signals. Neurons in the chain excite inhibitory inspiratory neurons (Fig. 6B, square 2) with recurrent and feed-forward connections to both the excitatory and other inhibitory inspiratory circuit elements, shaping their discharge pattern. Inhibitory inspiratory neurons modulate the inspiratory-phase firing rates of tonic expiratory neurons (Fig. 6B, square 3) that inhibit pre-BötC and more caudal inspiratory neurons, thereby also influencing phrenic motor output. Divergent actions of inhibitory inspiratory neurons also suppress spiking in phasic augmenting expiratory (E-Aug-P) populations.

The RTN-pF modulation of the pre-BötC-VRG in this scheme includes 6 distinct categories of “indirect” actions through tonic expiratory neurons (Fig. 6B, square 4). Several chemoreceptor-evoked operations converge upon phasic decrementing expiratory (E-Dec) neurons to reduce and limit their activity (Fig. 6B, operations 3, 4, and 5). Neurons with this discharge pattern are widely considered to play a role in controlling expiratory phase duration (e.g., Hayashi et al. 1996; Rybak et al. 2004).

Recent studies have proposed that RTN-pF E-Aug-P neurons, distinct from inhibitory BötC neurons (Fedorko et al. 1989; Jiang and Lipski 1990; Lindsey et al. 1989; Merrill and Fedorko 1984), are involved in generating active expiration (Molkov et al. 2010; Pagliardini et al. 2011), complementing prior related observations on expiratory neuron discharge patterns during
the expiration reflex and their connectivity (Baekey et al. 2004). Although this study did not
specifically address hypotheses on the control of expiratory drive, we did identify correlogram
features consistent with excitatory and inhibitory actions of tonic RTN-pF neurons (Fig. 6B,
square 5) upon phasic augmenting expiratory neurons in the caudal VRG, a region containing
bulbospinal neurons that drive active expiration (Baekey et al. 2004; De Almeida et al. 2010; De Troyer et al. 2005; Iscoe 1998). We note that while there is strong evidence for excitatory
glutamatergic projections of chemoresponsive and putative chemoreceptor RTN-pF cells to the
BötC-VRG (Abbott et al. 2009; Mulkey et al. 2004), the inhibitory interactions in the present
results suggest other parallel projections that are modulated by chemoresponsive circuitry within
the RTN-pF (Ott et al. 2011) or sign-changing multisynaptic connectivity.

Consideration of methods
The decerebrate cat model used in this study avoids the confounding effects of
anesthetics and has ventilatory responses to hypercapnia similar to those in the awake intact
cat although supra-pontine influences are absent (Tenney and Ou 1977). We identified
chemoresponsive neurons using selective stimulation of central chemoreceptors (Arita et al.
1988a, 1988b). We evaluated responses and correlations only in animals with an enhanced
peak amplitude of integrated phrenic nerve activity following stimulus onset, an established
metric for identifying a change in inspiratory drive with chemoreceptor stimulation in animals
vagotomized to eliminate the effects of pulmonary afferent feedback upon the medullary
respiratory network (e.g., Clark and von Euler 1972; Hwang et al. 1983; Scott 1908).
Our multi-array approach with sub-micron depth adjustment for each electrode is an
effective method for monitoring neurons in widely-distributed sites deep within the brainstem
with high temporal resolution. By recording many neurons simultaneously, we identified
extended correlational linkages of neurons assessed during the same pre- and post-stimulus
conditions with the goal of defining simple circuit models that reproduce experimentally
observed features (Aertsen et al. 1989). Advantages and limitations of the approach have been considered elsewhere (Nuding et al. 2009a; Ott et al. 2011; Shannon et al. 2000).

Diversity of correlogram features

The present data set included offset correlogram peaks and troughs with half-widths ranging from 0.7 ms (Fig. 3C, 20) to over 100 ms and is consistent with actions of diverse neurotransmitters and receptors associated with different categories of neurons in the VRC (Alheid and McCrimmon 2008; Haji et al. 2000; Stornetta 2008). Glutamate is a predominant excitatory transmitter found in inspiratory interneuron and premotor neuron populations. GABA and glycine both act as inhibitory neurotransmitters in other types of inspiratory and expiratory populations (Bongianni et al. 2010; Shao and Feldman 1997). Phasic BötC E-Aug and E-Dec or “post-inspiratory” neuron populations use glycine as an inhibitory transmitter (Büsselberg et al. 2003; Ezure et al. 2003; Schreihofer et al. 1999). GABA acts as a gain modulator limiting both control and reflex-evoked activity of inspiratory and expiratory neurons and, via distinct receptors, contributes to the silent phase of phasic neurons (Tonkovic-Capin et al. 2003; Zuperku and McCrimmon 2002). The present observation of functional inhibition of inhibitory inspiratory neurons by other inspiratory neurons (e.g., neuron 816 in Fig. 2) is consistent with the concept that glycinergic inspiratory neurons act on both excitatory and other glycinergic inspiratory neurons (Winter et al. 2009).

Differential modulation of nodes within the inspiratory neuron chain

When considered together with prior work (reviewed in Segers et al. 2008), the results support a circuit architecture with an excitatory inspiratory chain (Fig. 7A, green populations) embedded within a partly hierarchical structure that incorporates both feed-forward and recurrent inhibition by other inspiratory neurons (Fig. 7B and C, pink populations). In this model, the inhibitory inspiratory neurons participate in several temporally overlapping tuning functions,
including adjustment of burst onset time and the slope of augmenting burst ramps in other
inspiratory neurons, as well as directly moderating chemoreceptor-evoked increases in firing
rates and inspiratory drive. Notably, chemoreceptor-evoked changes in firing rate included
reduced activity in some pre-BötC-VRG inspiratory neurons and increased activity in others
(arrows). In this regard, the present results are reminiscent of the differential modulation of
rostral I-Driver and caudal inspiratory neurons in response to selective peripheral

Central peaks detected in correlations of some inspiratory neuron pairs are consistent
with the effects of shared synaptic drive from antecedent sources. These sets of mutually
correlated inspiratory neuron pairs suggest the operation of several distinct excitatory (or
inhibitory) influences acting upon the cluster. Functional heterogeneity of our sample of VRG
inspiratory neurons is likely. For example, neurons without short-latency offset peaks in the
phrenic spike triggered averages may include somatic motor neurons innervating laryngeal
muscles or their premotor drivers (Baekey et al. 2001), as well as pre-ganglionic vagal motor
neurons (McAllen and Spyer 1978).

Tonic expiratory neurons provide a “reservoir” for inspiratory drive modulation. The
enhanced respiratory modulation of tonic E neurons during central chemoreceptor stimulation -
presumably attributable in part to increased inhibition by inspiratory neurons relative to control
conditions - diminishes the inspiratory-phase inhibition of inspiratory neurons, thereby
contributing to the observed augmented inspiratory drive (Fig. 7C and D). We identified several
instances of short latency positive-lag offset troughs in both phrenic signal averages and in
multiple cross-correlograms with inspiratory target neurons, all triggered by spikes of the same
tonic expiratory neuron. These results suggest divergent actions of individual tonic expiratory
neurons upon clusters of inspiratory neurons, perhaps amplified by spike synchrony among
expiratory neurons with common targets (e.g., neurons 847 and 879 in Fig. 3).
When considered together with prior evidence of a role for tonic expiratory neurons in baroreceptor reflex inhibition of phrenic motor neurons and inspiratory drive (Lindsey et al. 1998), the present results support the concept that tonic VRC expiratory neurons constitute a node for converging afferent systems that regulate inspiratory drive intensity and thus tidal volume (Fig. 7E). These tonic expiratory neurons may also contribute to the well-known tonic inhibitory bias on inspiratory activity under conditions of hypocapnia (Nuding et al. 2009b; Sears et al. 1982).

The correlation linkage map in Fig. 5F complements previously reported pair-wise relationships between chemoresponsive RTN-pF neurons (Ott et al. 2011). Two of the correlograms represented in that map include offset “peak-trough” sequences (31 and 37 in Fig. 5D). Simple interpretations of this feature include i) dual actions of a particular transmitter or co-transmitter (Stornetta 2008), and ii) connections composed of excitatory and multi-synaptic inhibitory actions evoked by the same trigger neuron. The multiple correlations among a trio of neurons in the circuit (correlograms 31 through 33, Fig. 5D) are simply interpreted as evidence for the second conjecture: neuron 402 excites neurons 427 and 416 with neuron 416 also inhibiting cell 427. The offset peak superimposed upon a broader central peak in correlogram 38 (Fig. 5D) is consistent with shared synaptic influences amplifying an excitatory RTN-pF-to-BötC-VRG interaction.

Collectively, the correlogram features in Fig. 5 suggest distributed circuit mechanisms that include interactions between RTN-pF cells and more caudal tonic VRC expiratory neurons (Fig. 7F). As considered previously, parallel paths in the respiratory network (e.g., Lindsey et al. 1987; Nuding et al. 2009a; Segers et al. 2008), including circuits mediating chemoreceptor modulation of breathing (Abbott et al. 2009; Nuding et al. 2009b; Song and Poon 2009; Spyer and Gourine 2009), provide a substrate for multiple regulatory mechanisms and enhanced system robustness through redundancy - the duplication of critical components - and degeneracy, where different operations yield similar changes in output (Tononi et al. 1999).
ACKNOWLEDGEMENTS

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Figure Legends

Figure 1. Discharge patterns, responses, and recording sites of a group of pre-BötC-VRG neurons. A: Subset of normalized respiratory cycle-triggered histograms (CTHs) grouped by the respiratory phase in which the neuron fires with peak average activity; respiratory phases were defined by efferent phrenic nerve activity (gray trace). Colors represent respiratory modulated categories and correspond to the colored boxes surrounding the cell ID numbers and respiratory pattern labels in B. A total of 255 respiratory cycles were averaged and displayed using 90.0 ms bin widths. B: Firing rate histograms for 17 of 39 simultaneously monitored neuron spike trains during one of five sequential central chemoreceptor stimulation trials. For each trace, the respiratory modulated pattern, cell identification number, response to central chemoreceptor stimulus (average of 5 trials), and maximum firing rate are shown. The 30-s stimulus injection is denoted by the black horizontal line at the bottom; the 90-s response evaluation period is highlighted in yellow. C: Sagittal view of stereotaxic coordinates of recording sites of the neuronal activity shown in part B mapped onto a 3D atlas of the cat brainstem. In this and subsequent recording site schematics, colored “spheres” denote each neuron’s response to central chemoreceptor stimulation (see Key). D: Graphs 1 and 2 (corresponding to starred neurons 808 and 820 in B) display the peak firing rate per respiratory cycle for 3 of the 5 paired control (black traces) and stimulus periods (red traces) used to determine statistical significance of neuronal response. Abbreviations: pre-BötC-VRG, the region including and extending caudally from the pre-Bötzinger complex (pre-BötC) through the ventral respiratory group (VRG); I, inspiratory; E, expiratory; I-EI, neuron with a discharge pattern that begins before phrenic nerve discharge with a firing probability that peaks early in the inspiratory phase and slowly decrements before abruptly decreasing at the I-to-E phase transition; Dec, decrementing; Aug, augmenting; P, phasic; Phr, phrenic; BP, arterial blood pressure.
Figure 2. Correlations of pre-BötC-VRG neurons represented in Fig. 1. A: Cross-correlation histograms (CCHs) for neuron pairs labeled with circled yellow numbers have features corresponding to similarly labeled connections diagrammed in the correlation linkage map (C).

The minimum and maximum bin values, normalized to spikes/sec/reference event, are shown for each correlogram. Feature description, detectability index, half-width, bin width, and numbers of trigger neuron and target neuron spikes for each CCH are as follows:

1: offset peak, 7.71, 1.0 ms, 0.5 ms, 69,128 and 254,296; 2: offset peak, 31.30, 1.0 ms, 0.5 ms, 254,296 and 102,992; 3: offset peak, 8.91, 2.5 ms, 0.5 ms, 248,746 and 102,992; 4: central peak, 7.60, 3.0 ms, 0.5 ms, 254,296 and 248,746; 5: offset trough, 7.94, 10.0 ms, 2.5 ms, 77,740 and 254,296; 6: offset trough, 6.80, 10.0 ms, 2.5 ms, 101,667 and 254,296; 7: offset trough, 7.28, 12.5 ms, 2.5 ms, 54,214 and 254,296; 8: central peak, 9.27, 12.5 ms, 2.5 ms, 101,667 and 54,214; 9: offset trough, 3.81, 7.5 ms, 2.5 ms, 32,253 and 77,740; 10: offset trough, 4.74, 12.5 ms, 2.5 ms, 32,253 and 101,667; 11: offset trough, 4.76, 10.0 ms, 2.5 ms, 32,253 and 102,992; 12: offset trough, 6.67, 19.5 ms, 1.5 ms, 77,740 and 157,239; 13: offset trough, 4.94, 12.0 ms, 1.5 ms, 77,740 and 157,239; 14: offset trough, 4.94, 7.5 ms, 2.5 ms, 307,189 and 101,667; 15: offset trough, 6.00, 35.0 ms, 2.5 ms, 307,189 and 69,128; 16: offset trough, 12.30, 12.5 ms, 2.5 ms, 307,189 and 248,746; 17: offset trough, 3.58, 16.5 ms, 5.5 ms, 307,189 and 32,253; 18: offset trough, 19.66, 2.0 ms, 0.5 ms, 307,189 and 231,212.

B: Spike-triggered averages of contralateral phrenic nerve activity. Binwidth for both STAs = 0.1 ms. a: Neuron 802, peak (lag to peak = 3.6 ms, half-width = 3.8 ms); b: Neuron 815, trough (part of a broader trough with lag = 19 ms and half-width = 20 ms).

C: In this and subsequent correlation linkage maps, each large “sphere” represents a neuron with its identification number, respiratory modulation pattern (see Key to left), and chemoreceptor-evoked response (arrow; see Key to right). Small white and black circles at the ends of the lines between spheres represent offset peaks or troughs, respectively; circled yellow numbers indicate the corresponding cross-correlogram shown in A. The gray curved lines with
The gray background illustrates the distributed associations of tonic expiratory neuron 815.

**Figure 3.** Recording sites, discharge patterns, and correlational linkages of pre-BötC-VRG neurons from a second animal. **A**: Sagittal view of stereotaxic coordinates of recording sites for the neuronal subset illustrated in **E**. **B**: A subset of pre-BötC-VRG CTHs with colors corresponding to the colored spheres in **E**. A total of 418 respiratory cycles were averaged and displayed using 70.0 ms bin widths. **C**: CCHs calculated among pre-BötC-VRG neuron pairs. Feature description, detectability index, half-width, bin width, and numbers of trigger neuron and target neuron spikes for each CCH are as follows: 20: offset peak, 6.26, 0.7 ms, 0.1 ms, 22,599 and 61,703; 21: central peak, 10.36, 2.0 ms, 0.5 ms, 54,453 and 61,703; 22: central peak, 13.14, 1.5 ms, 0.5 ms, 107,761 and 22,599; 24: offset trough, 13.06, 7.5 ms, 1.5 ms, 107,761 and 61,703; 25: offset trough, 6.40, 10.5 ms, 1.5 ms, 107,761 and 22,988. **D**: spike-triggered averages of contralateral phrenic nerve activity. **C**: neuron 821, peak (lag = 4.9 ms, half-width = 0.9 ms, binwidth = 0.1 ms); **D**: neuron 847, trough (lag = 4.3 ms, half-width = 23.7 ms, binwidth = 0.5 ms). **E**: correlation linkage map. Cells grouped within the gray background are target neurons with decreased activity following spikes in trigger cell 847; colored circles mark overlapping subsets of neuron pairs with central peaks in their correlograms. See text for details.

**Figure 4.** Recording sites, discharge patterns, and correlational linkages of RTN-pF and VRG neurons in a third animal. **A**: Sagittal view of stereotaxic coordinates of recording sites for this neuronal subset are marked by spheres colored to indicate each neuron’s response. **B**: Normalized CTHs from one RTN-pF neuron (tonic cell 415) and its two pre-BötC-VRG targets.
(phasic inspiratory cell 818 and phasic expiratory cell 826). A total of 942 respiratory cycles were averaged and displayed using 35.0 ms bin widths. C: Cross-correlation histograms.

Feature description, detectability index, half-width, bin width, and numbers of trigger neuron and target neuron spikes for each correlogram are as follows: 28: offset trough, 4.99, 7.5 ms, 7.5 ms, 134,779 and 41,750; 29: offset trough, 4.22, 16.5 ms, 5.5 ms, 134,779 and 103,327.

Figure 5. Recording sites (sagittal view), discharge patterns, and correlational linkages of 14 RTN-pF and VRG neurons monitored in a fourth animal. See Figure 4 legend/text for details. C: CTHs: A total of 403 respiratory cycles were averaged and displayed using 80.0 ms bin widths. D: Cross-correlation histograms. Feature description, detectability index, half-width, bin width, and numbers of trigger neuron and target neuron spikes for each correlogram are as follows:

30: central peak, 144.25, 22.5 ms, 2.5 ms, 162,941 and 91,386; 31: offset trough, 11.84, 126.0 ms, 10.5 ms, 162,941 and 36,042; 32: offset peak, 12.84, 73.5 ms, 10.5 ms, 162,941 and 36,042; 33: offset trough, 4.08, 42.0 ms, 10.5 ms, 162,941 and 28,460; 34: offset peak, 5.05, 62.0 ms, 15.5 ms, 71,132 and 25,541; 35: offset peak, 9.42, 52.5 ms, 7.5 ms, 91,386 and 25,541; 36: offset trough, 7.41, 4.5 ms, 0.5 ms, 353,652 and 202,921; 37: offset trough, 8.46, 4.0 ms, 0.5 ms, 353,652 and 242,479; 38: offset trough, 11.4, 5.0 ms, 0.5 ms, 353,652 and 243,589; 39: offset trough, 10.04, 4.5 ms, 0.5 ms, 353,652 and 104,166; 40: central peak, 28.83, 7.5 ms, 2.5 ms, 243,589 and 104,166; 41: offset trough, 19.61, 15.0 ms, 2.5 ms, 306,493 and 353,652. E: spike-triggered averages of contralateral phrenic nerve activity. Binwidth for all STAs = 0.1 ms. g: neuron 809, peak (lag = 3.9 ms, half-width = 1.4 ms); h: neuron 812, peak, (lag = 3.6 ms, half-width = 1.9 ms); i: neuron 815, peak (lag = 3.4 ms, half-width = 1.7 ms); j: neuron 820, trough, (lag = 5.2 ms, half-width = 5.9 ms). F: correlation linkage map; represented inspiratory neurons grouped...
within the gray background were target neurons with increased activity following spikes in tonic
expiratory cell 820.

Figure 6. Proposed multipath tuning operations among VRC neurons for central chemoreceptor
modulation of breathing. A: combinations of trigger (reference) and target neuron responses and
correlogram features simply interpreted as distinct functional actions that promote or limit
changes in target cell activity following central chemoreceptor stimulation. B: Mosaic of
identified correlational sub-assemblies incorporating operations outlined in A. See text for
discussion.

Figure 7. Schematic summary of RTN-pF-to-preBötC-to-VRG functional connectivity inferred
from correlation linkages. Neurons are represented by color-coded respiratory cycle triggered
histograms as detailed in the RESULTS. Changes in firing rate or depth of respiratory modulation
in response to central chemoreceptor stimulation are represented by the arrow symbols on the
histograms. Interactions inferred from the correlational linkages are indicated by the small
circles at the ends of the connecting lines (see Key). The actions of central chemoreceptors
(CO₂/pH) target both the rostral region of the RTN-pF and more caudal regions of the column,
including the pre-BötC, reflecting the current state of knowledge regarding the distribution and
mechanisms of action of central chemoreceptors as summarized in the INTRODUCTION. See text
for details.
Table Legends

Table 1. Control period respiratory modulated discharge patterns. RTN-pF, retrotrapezoid nucleus/parafacial area. BötC-VRG, Bötzheimer and pre-Bötzheimer complexes along with the rostral and caudal respiratory groups. I, inspiratory neuron; E, expiratory neuron; IE, phase-spanning neuron; Aug, augmenting firing pattern; Dec, decrementing firing pattern; NRM, non-respiratory modulated neuron. No EI phase-spanning neurons were recorded.

Table 2. Responses of single neurons to central chemoreceptor stimulation. ↑, Increased neuronal firing rate; ↓, decreased firing rate; →, no significant change in firing rate to central chemoreceptor stimulus; Rate Ratio Δ (↑), significant change in the depth of respiratory modulation not accompanied by a significant change in neuronal firing rate.

Table 3. Offset and central feature correlations detected among 3,831 BötC-VRG neuron pairs grouped according to respiratory discharge patterns. In this and subsequent tables, trigger (left) and target neurons (top) are organized so that offset correlogram features have positive time-lags. Offset peaks (OP; upper left quadrant of each data “block”) and troughs (OT; upper right) as well as central peaks and troughs (CP, CT; lower left and right, respectively) are shown; shaded cells show the total number of pairs composed of neurons with the discharge patterns indicated by the row and column labels. Central features are reported only in the upper right half of the table because the designation of trigger and target neuron is irrelevant for intra-group central feature correlations: the central feature will still be present after switching the two spike trains. For example, a total of 416 BötC-VRG cell pairs composed of an I-Aug and an I-Dec neuron were analyzed. Correlograms calculated for 41 of these pairs (10%) contained an offset feature: 17 were suggestive of an I-Aug → I-Dec influence (11 peaks, 6 troughs) and 24 of an I-
Dec → I-Aug effect (16 peaks, 8 troughs). In addition, 62 central features were detected (55 peaks, 7 troughs) for pairs of this type.

**Table 4.** Offset correlogram features detected among 2,619 chemoresponsive BötC-VRG neuron pairs grouped according to the phase of the respiratory cycle during which each cell was most active and the cells’ responses to central chemoreceptor stimulation. In addition to the data shown in this table, 5 chemoresponsive BötC-VRG cells were triggers in 7 offset correlations with non-chemoresponsive BötC-VRG neurons, 4 non-responsive cells were triggers in 5 correlations with chemoresponsive neurons, and another non-responsive cell had an inferred influence upon a non-responsive cell. OP, offset peak; OT, offset trough; changes in neuronal firing rate in response to central chemoreceptor stimulation indicated by ↑ (increase), ↓ (decrease), and Rate ratio Δ (significant change in the depth of respiratory modulation not accompanied by a significant change in neuronal firing rate).

**Table 5.** Offset and central features detected among 4,028 RTN-pF – BötC-VRG neuron pairs grouped according to respiratory discharge patterns. RTN-pF respiratory modulation categories have been further “collapsed” in this table. Results within each data “block” are presented as in Table 3. For example, a total of 154 cell pairs composed of an RTN-pF inspiratory neuron and a BötC-VRG E-Tonic cell were analyzed. Correlograms calculated for 14 (9%) of these pairs contained a significant feature: 8 were suggestive of an RTN-pF I → BötC-VRG E-Tonic influence (3 peaks, 5 troughs), whereas 6 central features were detected (2 peaks, 4 troughs) for pairs of this type.

**Table 6.** Offset features detected among 2,539 chemoresponsive RTN-pF → BötC-VRG neuron pairs grouped according to the cells’ respiratory discharge patterns and responses to central
chemoreceptor stimulation. Abbreviations and symbols are as in Table 4. In addition to the data shown in this table, 9 chemoresponsive RTN-pF neurons were triggers in 11 offset correlations with non-responsive BötC-VRG neurons; 4 non-responsive cells had inferred influences upon 4 chemoresponsive BötC-VRG neurons. One non-responsive RTN-pF cell triggered offset correlations with 2 non-responsive BötC-VRG neurons.
A. Depth mm

B. Phasic inspiratory neurons

C. Spikes/s (min.; max.)

D. Respiratory modulation

E. Correlogram feature

- Increase
- Decrease
- Rate ratio
- No change

- Offset peak
- Offset trough
- Central peak
- Branch point
- Groups of mutually correlated cell pairs (central peaks)
A

Depth mm

Caudal

level of obex

Rostral

2 mm

818

826

818

415

= Increase

= Decrease

B

17.8 spikes/sec

415

16.7 spikes/sec

818

25.5 spikes/sec

826

3.5 s

C

Spikes/s (min.; max.)

415 - 818

(4.4; 5.0)

(12.1; 13.0)

415 - 826

-375 0 375 ms

-275 0 275 ms

D

Respiratory modulation

RTN-
pF

I

Aug-P

Correlogram feature

Offset peak

Offset trough

Branch point

Response

Increased firing rate

Decreased firing rate
**Figure A**

A 3D graph showing the depth (mm) in the A/P plane with landmarks such as obex, rostral, and caudal. The graph includes markers for different responses and cell types, such as E-Dec-T, NRM, and E-Aug-P.

**Figure B**

A table showing the response type (E-Dec-T, NRM, E-Aug-P, I-Dec-P, E-Aug-T, I-Aug-P, etc.), cell ID, level of obex, and maximum spikes/sec. The table also indicates whether the response is an increase, decrease, or rate ratio (INC/DEC).

**Figure C**

Graphs showing correlograms for different cell pairs, such as 402 - 418, 427 - 416, and 820 - 815, with offset peaks, troughs, central peaks, and branch points.

**Figure D**

Graphs showing the spikes/s response for different time intervals, such as 402 - 418, 418 - 427, and 416 - 427.

**Figure E**

Graphs showing stimulus injection and response over time, with markers for 809 > phrenic, 812 > phrenic, and 815 > phrenic.

**Figure F**

Diagram showing the respiratory modulation response with markers for RTN-pF, BötC-VRG, and Phrenic, with response types and rate ratios indicated.
A

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B

Connections:
- RTN-pF to preBöt-VRG
- Within preBöt-VRG
- Branch point
Distributed control of inspiratory drive

- pre-BötC-VRG
- RTN-pF
- CO₂/pH

Response:
- Increased firing rate
- Decreased firing rate
- Rate ratio Δ

Excitation
Inhibition
Branch point

Increased firing rate
Decreased firing rate
Rate ratio Δ
Table 1. Control Respiratory Modulated Discharge Patterns

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Table 2. Responses of single neurons to central chemoreceptor stimulation

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<th>BötC-VRG</th>
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<td>OP OT</td>
<td>BötC–VRG Trigger Neuron</td>
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Table 4: Offset features detected among 2,619 BötC-VRG neuron pairs responsive to central chemoreceptor stimulation

| BötC-VRG Trigger Neuron | BötC-VRG Target Neuron | Rate ratio Δ | Rate ratio Δ | Rate ratio Δ |
|-------------------------|-------------------------|--------------|--------------|
|                         |                         | I            | E            | NRM          |
|                         | ↑                       | ↓            | ↑            | ↓            |
| OP                      | 44                      | 5            | 1            | 2            |
| OT                      | 16                      | 3            | 11           | 1            |
|                         |                         | I            | E            | NRM          |
| OP                      | 5                       | 1            | 1            | 2            |
| OT                      | 1                       | 1            | 1            | 1            |
| Rate ratio Δ            | OP                      | 3            | 1            | 1            |
|                         | OT                      | 2            | –            | –            |
| Rate ratio Δ            | OP                      | –            | –            | –            |
|                         | OT                      | –            | –            | –            |
| Rate ratio Δ            | OP                      | –            | –            | –            |
|                         | OT                      | –            | –            | –            |
| NRM                     |                         | –            | –            | –            |
|                         |                         | –            | –            | –            |
| Rate ratio Δ            | OP                      | –            | –            | –            |
|                         | OT                      | –            | –            | –            |
Table 5: Offset and central features (peaks and troughs) detected in 4,028 RTN-pF → BötC-VRG neuron pairs

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Table 6: Offset features detected among 2,539 RTN-pF → BötC-VRG neuron pairs responsive to central chemoreceptor stimulation

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