Functional Connectivity in the Pontomedullary Respiratory Network

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ABSTRACT

Current models propose that a neuronal network in the ventrolateral medulla generates the basic respiratory rhythm and that this ventrolateral respiratory column (VRC) is profoundly influenced by the neurons of the pontine respiratory group (PRG). However, functional connectivity among PRG and VRC neurons is poorly understood. This study addressed four model-based hypotheses: (i) the respiratory modulation of PRG neuron populations reflects paucisynaptic actions of multiple VRC populations, (ii) functional connections among PRG neurons shape and coordinate their respiratory-modulated activities, (iii) the PRG acts upon multiple VRC populations, contributing to phase-switching, and (iv) neurons with no respiratory modulation located in close proximity to the VRC and PRG have widely distributed actions on respiratory-modulated cells. Two arrays of microelectrodes with individual depth adjustment were used to record sets of spike trains from a total of 145 PRG and 282 VRC neurons in 10 decerebrate, vagotomized, neuromuscularly blocked, ventilated cats. Data were evaluated for respiratory modulation with respect to efferent phrenic motoneuron activity and short-time scale correlations indicative of paucisynaptic functional connectivity using cross-correlation analysis and the “gravity” method. Correlogram features were found for 109 (3%) of the 3,218 pairs composed of a PRG and a VRC neuron, 126 (12%) of the 1,043 PRG-PRG pairs, and 319 (7%) of the 4,340 VRC-VRC neuron pairs evaluated. Correlation linkage maps generated for the data support our four motivating hypotheses and suggest network mechanisms for proposed modulatory functions of the PRG.
INTRODUCTION

Since the work of Marckwald (1887), many studies have demonstrated that the pons plays an important role in shaping the respiratory motor pattern (Lumsden 1923), and that removal of the rostral pons in vagotomized animals converts eupnea to apneusis (Stella 1938), which is characterized by a dramatically increased inspiratory duration. Connections between the pons and the medulla must be intact for the generation of a eupneic-like breathing pattern in the anesthetized or decerebrate, vagotomized cat (Berger et al. 1978; Bertrand and Hugelin 1971; Cohen 1958).

Pontine neurons with respiratory-modulated discharge patterns were first identified in the 1950s (Cohen and Wang 1959; Takagi and Nakayama 1958). Current models propose that a neuronal network in the ventrolateral medulla generates the basic respiratory rhythm and motor patterns for breathing, and that this ventrolateral respiratory column (VRC) (Rybak et al. 2004; Smith et al. 2007) is profoundly influenced by neurons of the “pontine respiratory group” (PRG) in the parabrachial-Kölliker-Fuse-pons/mesencephalic region (Alheid et al. 2004; Bianchi et al. 1995; Cohen 1979; Dick et al. 1994; St. John 1985, 1986).

The PRG has been shown to modulate both respiratory rate and drive. Differential effects on inspiratory and expiratory phase durations are correlated with the anatomic site of experimental interventions within the PRG (Chamberlin and Saper 1998; Cohen 1979; Jodkowski et al. 1997; Okazaki et al. 2002; St. John and Zhou 1991). Lesions in the PRG affect respiratory drive by attenuating the response to hypoxia and hypercapnia (Fung and St. John 1994; Mizusawa et al. 1995; St. John 1979), blocking post-stimulus plasticity evoked by chemoreceptor and other afferent stimulation (Coles and Dick 1996; Dick and Coles 2000; Siniaia et al. 2000), and decreasing breathing pattern stability (Oku and Dick 1992). Moreover, sectioning studies have suggested that the rostral pons is capable of generating a (possibly respiratory-related) rhythm
when separated from the medulla, and, together with other data, suggest that the PRG may also contribute to respiratory system redundancy as a back-up rhythm generator (St. John 1983, 1985; St. John and Bledsoe 1985).

Recent models have proposed that the respiratory modulation of the firing rates of PRG neurons is a consequence of direct efferent projections from VRC neurons with similar respiratory discharge profiles (Rybak et al. 2004). In addition, recent data and reviews suggest that the dorsolateral pons may be a critical component of eupneic pattern generation (Dutschmann and Herbert 2006; Smith et al. 2007; St. John and Paton 2004). However, the functional connectivity among PRG and VRC neurons is not well understood. While antidromic microstimulation (Bianchi and St. John 1981, 1982; Ezure and Tanaka 2006; Gang et al. 1998) and anatomical tract tracing studies (Herbert et al. 1990; Kalia 1977; King 1980; Smith et al. 1989) have suggested reciprocal projections, a 1985 study from this laboratory remains the sole published effort to address the functional connectivity between individual neurons in PRG and the VRC (Segers et al. 1985). At that time, our data suggested that monosynaptic interactions between medullary and rostral pontine respiratory neurons play a limited role in the control of the respiratory cycle in the decerebrate vagotomized cat.

Although the locations, cytoarchitecture, and respiratory-modulated firing rates of PRG neurons have been described (Bertrand et al. 1974; Dick et al. 1994; Ezure and Tanaka 2006; Song et al. 2006), circuits within the PRG that may shape pontine respiratory neuron discharge patterns remain largely unexplored. Alterations in membrane potentials have been interpreted to suggest that connections among Kölliker-Fuse neurons are similar to those in the core medullary network (Dick et al. 1994), but the sources of such synaptic activity remain unknown. To our knowledge, there have been only two published studies of simultaneously recorded neurons in the region of the PRG in the cat. One of those earlier works was based on a total of 40 neuron
pairs (Harper and Sieck 1980) and the other on a subset of the same data (Frostig et al. 1984); the respiratory modulation of the correlated neurons was not described in either study.

Neurons with no respiratory modulation (NRM) of their firing rates are found in the regions of the PRG and the VRC (Bianchi and St. John 1981, 1982; Dick et al. 1994). The extent to which these cells influence neighboring neurons with respiratory-modulated firing rates is not well understood, nor is it known whether NRM neurons in each of these two regions functionally influence or are influenced by respiratory-modulated cells in the other area. Although anatomic findings indicate projections between the ventrolateral medulla and the rostral pons (Herbert et al. 1990; Kalia 1977; King 1980; Smith et al. 1989), electrophysiological studies have found a relative paucity of respiratory-modulated activity in the pons (Cohen and Wang 1959; Dick et al. 1994; Segers et al. 1985). It is possible that NRM neurons have a key role in the respiratory control function attributed to the dorsolateral pons.

The present work was motivated by the gaps in knowledge enumerated above and addressed the following four model-based hypotheses: (i) the respiratory modulation of PRG neurons reflects, at least in part, paucisynaptic actions of multiple VRC populations, (ii) functional connectivity among PRG neurons shapes and coordinates their respiratory-modulated activities, (iii) the PRG acts upon multiple VRC populations, contributing to respiratory phase-switching, and (iv) NRM neurons located in close proximity to the VRC and PRG have widely distributed actions on respiratory-modulated cells. Our approach used multiple arrays of microelectrodes with fine individual depth adjustment and computational methods to screen large datasets of simultaneously recorded spike trains for short-time scale correlations indicative of paucisynaptic functional connectivity.

The results document correlation linkages among PRG and VRC neurons and suggest network mechanisms underlying the proposed modulatory functions of the PRG. A companion
paper (Rybak et al. submitted with this manuscript) describes a computational model of the pontomedullary respiratory network developed in conjunction with the present work. Preliminary accounts of the results have been reported (Morris et al. 2006; Nuding et al. 2006, 2007).

METHODS

General methods and surgical procedures

Experiments were performed under protocols approved by the University of South Florida’s Institutional Animal Care and Use Committee and were performed 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 10 adult cats (2.8–5.6 kg) of either sex. Surgical procedures were similar to those described previously (Baekey et al. 2001; Shannon et al. 1998, 2000). Briefly, animals were initially anesthetized with isoflurane (2–5%; n = 4) or with an intramuscular ketamine hydrochloride injection (5.5 mg kg\(^{-1}\); n = 6) followed by isoflurane and later decerebrated using a technique adapted from Kirsten and St. John (1978). The level of anesthesia was assessed periodically by noxious stimuli (toe pinch); if the withdrawal reflex occurred or there was an increase in blood pressure or respiration, the percentage of isoflurane in the inspired gas was increased until the response was absent. Animals were artificially ventilated through a tracheal cannula with a respirator. End-tidal CO\(_2\) was monitored continuously and maintained at 4-4.5%. Femoral arteries and veins were catheterized to monitor arterial blood pressure, administer fluids and drugs intravenously, and acquire arterial blood samples for the periodic measurement of PO\(_2\), PCO\(_2\), and pH. These parameters were maintained within normal limits with solutions of 6% Dextran 70 in 0.9% sodium chloride, 0.04 to 0.1% dopamine or 0.075-0.3 mg mL\(^{-1}\) phenylephrine in lactated Ringer’s solution; sodium bicarbonate solution (8%) was used
to correct metabolic acidosis. Atropine (0.5 mg kg\(^{-1}\), im) and, in 5 experiments, diphenhydramine hydrochloride (1.8 mg kg\(^{-1}\), iv) were administered to reduce mucus secretion in the airways, and dexamethasone (initial bolus of 2.0 mg kg\(^{-1}\) followed by 4.5 mg kg\(^{-1}\) hr\(^{-1}\), iv) was administered to help prevent hypotension and minimize brainstem swelling. The trachea was periodically suctioned and the lungs hyper-inflated to counteract atelectasis. A urinary catheter was inserted to monitor urine flow as an indication of renal function. Rectal temperature was maintained at 38±0.5\(^{\circ}\)C.

In preparation for decerebration, the external carotid arteries were bilaterally ligated rostral to the lingual arteries. The animals were placed prone in a stereotaxic frame. A parietal craniotomy was performed and the cerebellum was partially removed by suction to expose the dorsal surface of the brainstem. At this point, an anesthetic assessment was performed, animals were neuromuscularly blocked by pancuronium bromide (initial bolus of 0.1 mg kg\(^{-1}\) followed by 0.2 mg kg\(^{-1}\) hr\(^{-1}\), iv), and the brainstem was immediately transected at the midcollicular level. Brain tissue rostral to the transection was aspirated. Isoflurane was removed from the inhaled gas circuit after the decerebration was complete and the flow rate was increased to aid elimination of residual isoflurane (Sasano et al. 2001; Vesely et al. 2003). During this period, sufficient CO\(_2\) was added to the gas mixture to maintain PCO\(_2\) at normocapnic levels of at least 30 mmHg (Herbert and Mitchell 1971; Lovering et al. 2003) to prevent decreased brainstem microcirculation resulting from hypocapnia.

The T\(_4\) vertebra was exposed and clamped to suspend the abdomen and thorax and a bilateral thoracotomy was performed to minimize brainstem movement. Vagosympathetic nerve trunks were isolated and sectioned bilaterally to eliminate vagal afferent feedback from pulmonary stretch receptors and aortic baroreceptors. When necessary, the fraction of inspired O\(_2\) was increased to prevent the hypoxemia resulting from ventilation-perfusion mismatching caused by
the open chest. At the end of the experiments, cats were euthanized with an injection of sodium pentobarbital (28 mg kg\(^{-1}\)) followed by 5 mL of a saturated solution of KCl in water.

**Recording nerve activity.** The left C\(_5\) phrenic nerve rootlet was exposed, desheathed, cut and its efferent activity recorded with bipolar silver electrodes covered with mineral oil. Nerve signals were amplified, filtered (band-pass 10 Hz–10 kHz), and integrated (full-wave rectified signal to a resistor-capacitor integrator; 0.2 second time constant) to obtain a moving time average of activity in the respective nerves. Nerve discharges were observed using oscilloscopes and audio monitors and the integrated signal was recorded continually on a polygraph to monitor the respiratory motor pattern and to define the respiratory phases.

**Neural recordings and data acquisition.** The brainstem surface was covered by warm mineral oil. Extracellular recordings of pontine and medullary neuronal activity were made using two arrays, each with 8 to 32 tungsten microelectrodes (10-12 M\(\Omega\)). Electrode placement was guided by appropriate stereotaxic coordinates (see Results) derived from Berman (1968) and numerous previous studies reviewed herein. The electrodes in the arrays were arranged either linearly or in a rectangular grid with adjacent tips 150-400 microns apart and individually advanced in submicron steps allowing isolation of signals from single neurons. These signals were band-pass filtered (0.1–5 kHz) and, together with multi-fiber efferent nerve activities, systemic arterial blood pressure, tracheal pressure, and end-tidal CO\(_2\), stored on digital recorders (16-bit accuracy, 24 or 25 kHz sampling frequency per channel).

**Data analysis**

Most of the analytic methods used have been described in detail previously (Li et al. 1999a,b; Morris et al. 1996). Briefly, action potentials from single neurons were converted to arrays of occurrence times with spike-sorting software (Datawave Tech. Corp; O'Connor et al. 2005).
Time stamps indicating the onset of each inspiratory and expiratory phase were derived from the phrenic nerve signal. Coordinates of recording sites were mapped into the three-dimensional space of a computer-based brainstem atlas (Segers et al. 1987) using a common origin (obex), rotating the Berman coordinate axes to align with our coordinate frame of reference, and calculating a scaling factor to match both coordinate systems. The version of the program used in this study incorporated open source code of the IBM Open Visualization Data Explorer and included the coordinates of 19 frontal section traces from the cat brainstem together with outlined substructures derived from *The Brainstem of the Cat: A Cytoarchitectonic Atlas with Stereotaxic Coordinates* (Berman 1968) with permission of the University of Wisconsin Press.

*Respiratory modulation of firing rates.* Both standard and normalized respiratory cycle-triggered histograms (CTHs) were computed for all recorded neurons. The normalized CTH was calculated using a spike train in which the durations of the inspiratory and expiratory phases were normalized to the average phase lengths; individual spike times within each phase were proportionally shifted to fit within the normalized phases. The normalization procedure reduced temporal dispersion of the average activity pattern caused by varying cycle lengths during the recordings. The CTHs were used to identify the phase (inspiration: I, expiration: E) or phase transition (IE and EI) in which the neuron was most active (Cohen 1968). Each neuronal spike train was additionally evaluated for respiratory modulation using two statistical tests (Morris et al. 1996). Neurons with no preferred phase of maximum activity as assessed by both statistical tests were designated “non-respiratory modulated” neurons (NRM). A measure of respiratory modulation, \( \eta^2 \), was calculated for each neuron (Orem and Dick 1983).

*Cross-correlation analysis.* Cross-correlation histograms (CCHs) were calculated for each pair of simultaneously recorded spike trains to detect and evaluate effective neuronal connectivity (Moore et al. 1970; Perkel et al. 1967b). The CCH gives an estimate of the
probability that an action potential in one (reference) spike train will be preceded or followed by action potentials in a second (target) spike train. The significance of correlogram features (peaks and troughs) was evaluated by calculating a ‘detectability index’ (DI, equal to the ratio of the maximum amplitude of departure from the background to the background, divided by the standard deviation of the correlogram noise (Aertsen and Gerstein 1985; Melssen and Epping 1987)). Features with DI values greater than 3 were considered significant. Cumulative sum histograms with statistical confidence limits set at ± 3 SD (Davey et al. 1986) were calculated for all CCHs with significant DI values to confirm the significance of the correlogram feature (Ellaway 1978). Autocorrelograms were calculated for each spike train to verify that the activity of only a single neuron was represented and to aid interpretation of CCHs (Moore et al. 1970; Perkel et al. 1967a,b).

Correlation linkage maps. All significant CCH features, together with descriptions of each neuron’s respiratory modulation, were placed in a standard relational database. Correlation linkage maps for groups of simultaneously monitored neurons were generated automatically by database queries using software based on the open source graph visualization tool Graphviz. The plotted positions of the represented neurons corresponded to their relative anterior-posterior recording site coordinates. The thickness of lines connecting the neuronal “nodes” of the graph provided an indication of correlation strength. Correlation features were represented by line color and type (e.g., Fig. 7A).

Gravity analysis. The gravity method for the analysis and visualization of groups of simultaneously recorded neurons (Gerstein and Aertsen 1985; Gerstein et al. 1985) was also used to confirm assembly identification in selected data sets. This method represents each neuron as a particle in N-space with a time-varying charge that is a filtered version of the corresponding spike train. Movement of a particular particle in the N-space is calculated by evaluating a vector
sum of pair forces with all other particles. Resulting trajectories of particles and their aggregation reflect neuronal timing relationships (e.g., Fig. 7C). A recently described “tuning” enhancement to the gravity method that improves detection of both short- and long-time lag correlations was incorporated into the analysis (Lindsey and Gerstein 2006). Charge kernels used for each pair of particles were offset by an amount determined by time lags to peaks or troughs in corresponding CCHs. The significance of particle aggregation was evaluated using confidence limits defined with an empirical Monte-Carlo method (Lindsey et al. 1992a).

RESULTS

Recording sites and respiratory modulation of neurons

Four hundred and twenty seven single neuron spike trains were recorded with electrode arrays in the region of the pontine respiratory group (PRG; 145 neurons) and ventral respiratory column (VRC; 282 neurons) in 10 decerebrate, vagotomized, and ventilated cats. Fig. 1A shows the coordinates of the recording sites of neurons included in this study mapped in a standard brainstem atlas (Berman 1968). Cells recorded at the same coordinates are shown as vertically displaced spheres, color-coded to indicate the presence (red) or absence (blue) of respiratory-modulated activity. Recording sites in the PRG ranged from 2.0 mm anterior to 2.0 mm posterior to the caudal border of the inferior colliculus, 2.5 to 5.8 mm lateral to the midline, and 1.2 to 4.5 mm below the dorsal surface of the pons. Cells recorded in the VRC were located 3.0 mm caudal to 8.6 mm rostral to the obex, 3.0 to 4.5 mm lateral to the midline, and 2.1 to 6.5 mm below the dorsal surface of the medulla. The spike trains of up to 82 neurons were simultaneously monitored in a single animal. Figure 1B shows representative signals from 8 electrodes (left) and corresponding sections of the spike trains of 10 neurons isolated from them (right). Firing rate
histograms of those spike trains along with those of 36 other concurrently monitored neurons are shown in Fig. 1C.

Seventy-two percent (203) of the 282 neurons recorded at sites within the VRC were respiratory modulated; the remaining cells were classified as non-respiratory modulated (NRM) neurons. For some respiratory-modulated cell types, a further distinction was made: 106 neurons had a phasic discharge pattern (zero firing probability during part of the respiratory cycle as assessed in the CTH average). The remaining 97 respiratory-modulated VRC neurons were tonically active, i.e., exhibiting some activity throughout the respiratory cycle in CTHs.

Ventrolateral respiratory column respiratory-modulated neurons were initially classified into one of four major categories according to the timing of their peak firing rate: inspiratory (I), expiratory (E), or IE or EI if the peak activity occurred at a phase transition. Inspiratory and expiratory neurons with peak firing rates in the first or second half of their phases were further characterized as decrementing (Dec) or augmenting (Aug), respectively. The number and proportion of VRC neurons in each category of respiratory modulation are reported in Fig. 2A (left). We note that our standard classification of VRC E-Dec-P neurons includes “post-inspiratory” neurons. In this study, these neurons were distinguished from the sample of VRC IE neurons, which included phasic cells transiently active late in the inspiratory phase and as phrenic inspiratory activity declined into the early-expiratory or post-inspiratory interval. Neurons with such spiking profiles at the phase transition have been considered variously as sub-types of heterogeneous “late-I” or E-Dec neuron populations by different authors (Haji et al. 2002; Shannon et al. 2000). In addition, four VRC neurons were designated I-EI neurons. The onset of their increase in activity began slightly before phrenic nerve discharge; their firing rates peaked in the early I phase and then slowly decremented before abruptly decreasing at the I-to-E
phase transition. The firing characteristics of these neurons were consistent with possible roles as “I-driver” neurons (Morris et al. 1996; Segers et al. 1987).

Sixty-six (46%) of the 145 cells recorded within the region of the PRG were respiratory modulated (Fig. 2A, right); only 3 were classified as phasic. For the 63 pontine respiratory-modulated neurons that discharged tonically, $\eta^2$ values ranged from 0.01 to 0.45. Neurons with peak firing rates during the second half of the inspiratory or expiratory phase following a generally augmenting “ramp” of activity were designated I and E cells, respectively. Pontine neurons with peak firing rates either during the transition from one phase to the other or at the beginning of a phase followed by a decrementing firing rate were designated either IE or EI, depending on the phase transition temporally juxtaposed to the peak rate. Examples of respiratory-modulated discharge pattern in each category are shown in Fig. 2B.

Cross-correlation analysis

Spike trains of 8,601 pairs of neurons were screened for short-time scale correlations. Overall, CCHs for 554 (6.4%) of the neuron pairs displayed a significant feature indicative of mono- or pauci-synaptic connectivity (Table 1). Correlations were detected between the spike trains of 3.4% of the pairs composed of a PRG and VRC neuron, 12.1% of PRG-PRG pairs, and 7.4% of VRC-VRC neuron pairs. Primary CCH features included an offset peak or trough ($n = 282, 3.3\%$; 204 peaks, 78 troughs) or a central feature ($n = 272, 3.2\%$; 232 peaks, 40 troughs).

The mean half-widths of the offset peaks and troughs were less than those of the corresponding central features (Kolmogorov-Smirnov test; peaks: $p = 3.7 \times 10^{-11}$; troughs: $p = 1.4 \times 10^{-5}$; see Table 1 legend for mean values), suggesting that the offset and central correlogram features in our sample reflected the consequences of distinct circuit properties. Offset and central features are considered separately in the Results. We note that several simple
classes of connectivity are commonly inferred from features in the CCHs (Aertsen and Gerstein 1985; Kirkwood 1979; Moore et al. 1970). A central peak is indicative of shared inputs with similar actions, while a central trough can be attributed to functional inputs with opposite actions on each of the neurons. A peak offset in time relative to the trigger event origin suggests excitation of the target neuron or an unobserved shared input that influences both cells with different conduction delays. Conversely, an offset trough suggests an inhibitory process, operationally defined as a mono- or pauci-synaptic relationship that reduces target cell firing probability following trigger neuron spikes. Cross-correlation and related approaches thus define an abbreviated set of possible connections (i.e., the simplest neuronal model) that replicate the experimentally observed features (Aertsen et al. 1989).

Cross-correlation feature summary diagrams (e.g., Fig. 3A) provide an overview of offset features for pairs of neurons with the indicated respiratory-modulated discharge profiles and recording sites. Reference neurons are represented on the left side of each diagram; target neuron categories are shown across the top. The lines indicate the correlation linkage for that particular reference and target neuron pair type. Each small circle on the target cell at the end of each line indicates an offset feature present in at least one CCH. Numbers enclosed in circles reference the corresponding CCH shown in the figure or elsewhere in Results. For example, correlogram features for different VRC IE → PRG IE neuron pairs (Fig. 3A) included an offset peak (e.g., Fig. 3B, CCH 8) and a trough (not shown).

**Evidence for VRC → PRG functional connectivity**

Short-time scale correlations simply interpreted as evidence of excitatory and inhibitory functional connections from VRC to PRG neurons were detected in the spike trains from 48 of the 3,218 VRC-PRG neuron pairs analyzed (Fig. 3A). Primary offset correlogram features
included 33 peaks and 15 troughs. Table 2 provides details on these features. In this and subsequent tables, VRC neurons are grouped into their major categories of respiratory modulation to facilitate discussion of correlations of similar and dissimilar respiratory types. Offset peaks were found in CCHs for 8 of 466 respiratory-modulated neuron pairs in which both neurons shared the same category of respiratory modulation; one additional pair of such neurons had an offset trough. Of the 1,075 neuron pairs composed of respiratory-modulated neurons with different firing profiles, 17 had offset features (8 peaks and 9 troughs).

PRG I neurons had increased short-time scale firing probabilities following spikes in several categories of VRC respiratory-modulated neurons (e.g., Fig. 3B, CCH 1); troughs or transient reductions in PRG I neuron firing probability primarily followed spikes in VRC E and EI neurons (Fig. 3B, CCH 3). Pontine IE neurons were also correlated with multiple classes of VRC neurons; offset peaks and troughs were detected in CCHs triggered by neurons with similar and different respiratory discharge profiles (Fig. 3B, CCHs 5, 6, and 8). An offset peak was identified in one of 268 correlograms with a PRG E target neuron; the VRC reference neuron also had an E-Aug discharge pattern (Fig. 3B, CCH 4). One of 263 CCHs calculated for VRC-PRG respiratory-modulated neuron pairs that included a PRG EI target neuron also had an offset peak; the VRC reference neuron had a similar respiratory modulated profile.

Correlations were detected for 15 of 1,381 pairs of neurons (1.1%) composed of a VRC NRM or PRG neuron and a respiratory-modulated neuron in the PRG or VRC, respectively. VRC NRM neurons were correlated with PRG I and IE cells (Fig. 3B, CCH 2); PRG NRM cells had primary offset positive-lag peaks following spikes in VRC I-EI (Fig. 3B, CCH 7), I-Dec, I-Aug, and E-Aug neurons. Of 296 cell pairs composed of VRC and PRG NRM neurons, 7 pairs (2.4%) were significantly correlated.
Evidence for PRG → PRG functional connectivity

Spike trains from 1,043 pontine neuron pairs were analyzed for short-time scale correlations. A summary of the 54 (5.2%) CCHs with significant offset features is shown in Fig. 4A. Primary offset features included 48 peaks and 6 troughs (Table 3). Offset peaks were detected for 4 of 100 pairs composed of neurons with similar respiratory profiles (Fig. 4B, CCHs 9 and 15). A total of 220 pairs of respiratory-modulated neurons with different activity patterns were evaluated; 10 pairs had offset peaks and 2 had offset troughs in their correlograms.

PRG I neurons had increased firing probabilities following spikes in each category of pontine respiratory-modulated neuron (Fig. 4B, CCHs 9, 11, and 13). PRG I, IE, and EI neurons had increased firing probabilities following spikes in a PRG I neuron (Fig. 4B, CCHs 9 and 10). PRG IE neurons were also correlated with multiple types of PRG respiratory-modulated neurons, as shown by the presence of offset peaks (Fig. 4B, CCHs 11 and 14) and troughs (Fig. 4B, CCHs 10 and 12) in CCHs for pairs containing at least one PRG IE neuron.

The majority of PRG-PRG neuron pairs with offset correlation features contained at least one NRM neuron (38 of 54, 70.4%). PRG NRM neurons were involved in 22 correlated pairings with PRG respiratory-modulated cells (21 offset peaks and 1 offset trough; see Fig. 4B, CCHs 16 and 17). Cross-correlograms for pairs of PRG NRM neurons contained offset features in 16 cases (13 peaks and 3 troughs; 29.6% of offset features detected for PRG neuron pairs).

Evidence for PRG → VRC functional connectivity

Offset features consistent with paucisynaptic actions of PRG neurons upon the VRC were detected in 37 of 3,218 PRG-VRC neuron pairs (Fig. 5A). These primary features included 22 peaks and 15 troughs (Table 4).
Correlograms from 10 of the 1,075 pairs of neurons with different categories of respiratory discharge had offset peaks (e.g., Fig. 5B, CCH 24); offset troughs were found in another 10 pairs (Fig. 5B, CCHs 20 and 21). Among the 466 pairs of neurons that shared the same category of respiratory activity, two had correlations suggestive of a PRG-to-VRC connection, including an offset peak for a pair of IE neurons (Fig. 5B, CCH 19) and an offset trough in an PRG I to VRC I-Dec correlogram (not shown).

Five peaks (Fig. 5, CCHs 19 and 24) and 6 troughs were detected in pairs composed of a VRC respiratory-modulated neuron and a PRG IE cell. VRC I-Dec neurons had decreased short-time scale firing probabilities following spikes in each type of PRG respiratory-modulated neuron (Fig. 5A; e.g., Fig. 5B, CCH 20). Neurons in other VRC respiratory categories exhibited increased and decreased firing rates following spikes in several types of PRG respiratory cells.

Twelve offset feature correlations were detected for PRG-VRC pairs composed of a NRM neuron and a respiratory-modulated cell. Peaks were detected in VRC NRM neuron firing probabilities in CCHs triggered by several types of PRG respiratory neurons (e.g., Fig. 5B, CCH 18). Correlograms for several categories of VRC respiratory neurons triggered by PRG NRM neuron spikes also exhibited offset peaks and troughs (e.g., Fig. 5B, CCHs 23 and 25). Three of 296 CCHs from pairs of VRC and PRG NRM neurons had offset features consistent with PRG-to-VRC neuron interactions.

Two PRG neurons—an IE and an NRM neuron—were correlated with the same VRC I-EI target neuron. Correlogram features for these two pairs suggested opposite actions on the VRC neuron. The CTHs for the PRG IE neuron (529) and the VRC target (103) are shown in Fig. 5C (top). The CCH for this pair (Fig. 5C, CCH 24) identified an increased firing probability in the VRC target cell following spikes in the PRG IE neuron. The second correlogram, calculated
using the PRG NRM neuron as the reference cell (532; CTH not shown), had an offset trough (Fig. 5C, CCH 25).

**Evidence for VRC → VRC functional connectivity**

The diagram in Fig. 6A summarizes correlation features obtained from the analysis of 4,340 pairs of VRC neuron spike trains. Offset features were detected in 143 of these pairs (3.3%; 101 peaks and 42 troughs; Table 5).

Primary offset peaks (n = 34) or troughs (n = 8) were detected in CCHs of the 932 pairs in which the VRC cells shared the same category of respiratory activity (e.g., Fig. 6B, CCHs 29 and 30). Pairs of VRC I neurons accounted for 26 of the offset peaks (e.g., Fig. 6B, CCH 35) and 6 offset troughs. Of the 1,558 pairs of respiratory-modulated neurons with different firing profiles, 52 had offset correlogram peaks (Fig. 6B, CCH 34) and 17 had offset troughs (Fig. 6B, CCHs 31 and 33). Most sub-categories of VRC respiratory-modulated neurons exhibited short-time scale changes in firing rate following spikes in VRC I-Dec, I-Aug, IE, and E-Dec neurons. VRC I-Aug, IE, E-Dec, and EI neurons had changes in firing probability following spikes in multiple categories of respiratory-modulated VRC neurons.

Primary offset features (13 peaks, 16 troughs) were found in CCHs from a total of 1,482 pairs of VRC neurons in which one cell was respiratory-modulated and the other was not (Fig. 6B, CCHs 26, 27, 28, and 32). VRC NRM neurons were correlated with cells of each broad respiratory category (Table 5). Cross-correlograms of 3 of the 368 pairs of VRC NRM neurons included 2 offset peaks and 1 trough.
Correlations between contralateral neurons

Most recordings were made from ipsilateral respiratory groups. In 2 cases, however, electrode arrays were placed in contralateral areas. Cross-correlograms for 3 of 48 contralateral VRC-VRC neuron pairs contained offset features. Offset peaks were detected in two pairs of VRC E-Dec neurons. The third VRC pair contained an offset trough, indicating that the E-Dec neuron had decreased firing probability following spikes in the contralateral I-Dec cell. One of 200 contralateral VRC-PRG pairs contained an offset peak; the VRC E-Dec cell had increased firing probability following spikes in the PRG IE neuron. Contralateral pairs of PRG neurons were not recorded.

Extended correlation linkages

Our use of arrays of microelectrodes with individual depth adjustment permitted detection of correlations among neurons recorded in parallel at multiple sites and provided evidence for distributed functional circuits. The correlation linkage map of a group of 16 simultaneously recorded PRG neurons shown in Fig. 7A includes information on short-time scale correlations among the neurons. Line color, type, and thickness reflect the type (peak or trough), location (offset or central), and strength (DI) of the feature in the CCH constructed for the corresponding pair of neurons. The rectangles in the extended correlation linkage map contain each neuron’s ID code and discharge pattern, which is also reflected by the color of the rectangle. For example, the central peak in the CCH for neurons 501 and 529 (Fig. 7B) can be simply interpreted as evidence of a shared input. The linkage map reported central peaks for several other cell pairs that include one of these two neurons: 501-521, 501-539, 521-529, 501-507, 507-529, and 529-539.

This group of 16 PRG neurons was also screened with the gravity method for evidence of nonrandom temporal relationships. Figure 7C shows the final frame of the animated sequence of
projections of the particle trajectories during a 351-second sample of spike train data. The 
aggregation of the particles representing neurons 501, 507, 521, 529, and 539 documents their 
coordinated activity. Because of information loss in projections from the gravity N-space to a 
plane, a particle distance as a function of time plot (PDFT, Fig. 7D) was generated to show 
distances between particles corresponding to neurons in each of the 120 pairs in the group as a 
function of time in the gravity run. The PDFT plot for a particular neuron pair, 501-529, was 
isolated (Fig. 7E, dark line) to document significant aggregation of these two particles. 
Interparticle distances were less than the lower bounds of the Monte-Carlo empirical confidence 
band (lower thin line). The black time intervals represented in each row of the plot in Fig. 7F 
indicate time intervals within the 351-second sample period when the distance between particles 
for each neuron pair was less than expected; the row corresponding to the PDFT plot for pair 501-529 (Fig. 7E) is shown in red.

The linkage map in Fig. 8A gives a summary of 44 different correlogram features detected in 
the analysis of a group of 46 simultaneously recorded PRG and VRC neurons. In addition to 
many offset features, a central trough and several central peaks indicative of shared influences 
were also identified. The central trough in CCH 38 (Fig. 8B) is from a pair of PRG neurons most 
active at opposing phase transitions. This feature is indicative of an unobserved shared influence 
that altered the activities of the neurons oppositely. Shift control correlograms (not shown) 
suggest that the central trough was not solely attributable to the difference in the neuron’s 
discharge patterns. The same PRG IE neuron tended to discharge in near synchrony with a VRC 
E-Dec neuron (Fig. 8B, CCH 39); both neurons were most active during or immediately 
following the I-to-E phase transition. Pontine IE neurons were correlated with multiple classes of 
VRC neurons in this dataset.
Evidence for shared inputs

Another example of a central peak from the group represented in Fig. 8A was found in the CCH for a VRC I-Aug cell and a VRC NRM neuron (Fig. 8B, CCH 40). Overall, 49% (272 of 554) of the detected primary features were central peaks or troughs. Table 6 provides details on primary central features with regard to the respiratory modulation profile and location of each neuron in a pair. In each group – PRG-VRC, PRG-PRG, and VRC-VRC – the ratio of central peaks to troughs was similar.

DISCUSSION

This study identified correlation linkages among pontine and medullary neurons, detecting evidence of functional associations between approximately 3% of the PRG-VRC, 12% of the PRG, and 7% of the VRC neuron pairs. Simple interpretations of the primary correlogram features support and suggest a large variety of functional interactions within the pontomedullary respiratory network. The summary of functional connectivity represented in Fig. 9 as a ball-and-stick model diagram has a spatial or compartmental organization incorporating the PRG and three component regions of the VRC: the Bötzinger complex (BötC), the pre-Bötzinger complex (pre-BötC), and the ventral respiratory group (VRG). Each neuron population is represented by a colored ball labeled with its respiratory activity pattern. Heterogeneity within these classes of respiratory neurons was considered; some types of neurons described in the literature were relabeled or grouped together, either for simplicity or because, as has been noted elsewhere (e.g., Ezure 1990), various conventions have been used to label neurons according to their discharge patterns during the respiratory cycle (see the figure legend for more details about grouping of neuron types in this diagram).
The diagram incorporates functional connectivity based upon both the results of earlier work (lines labeled with green squares; see the legend of Fig. 9 for references) and the present study (stars). Interactions proposed in other studies and models were also incorporated and are labeled with triangles (Rybak et al. submitted with this manuscript). Previously suggested functional connections supported by the present results are labeled with both a yellow star and a green square or blue triangle. Similarly, axonal projections between the PRG and VRC previously identified by antidromic activation studies (Bianchi and St. John 1981, 1982; Ezure and Tanaka 2006) are noted with green arrows; those supported by inferences from the correlogram features in this study are additionally marked with stars.

The starred connections in Fig. 9 represent simple interpretations of offset correlogram peaks and troughs. Inferred shared inputs consonant with central correlogram features detected in this study can also be seen in the diagram. For example, PRG IE neurons exciting other IE cells and inhibiting PRG EI neurons would be a source of shared inputs of opposite sign that generate a central trough (Fig. 8B, CCH 38).

The results support the motivating model-based hypotheses

The catalog of inferred functional connectivity generated in the present work informed the coordinated development of a new computational model of the pontomedullary respiratory network. A companion paper describes the connections incorporated into the new model, together with related hypotheses on network organization, and other coordinated experiments that tested model predictions (Rybak et al. submitted with this manuscript). Figure 10 highlights some pontine and medullary neuron interactions suggested by the present work that support the four motivating model-based hypotheses. We note here that detected offset features between different categories of neurons were, in general, relatively sparse, as indicated by the respective
percentages of correlated pairs to total pairs for the inferred actions summarized in Tables 2 through 5 and further detailed in Fig 10. We consider possible explanations for the overall paucity of identified interactions later in the Discussion.

(i) The respiratory modulation of PRG neuron populations reflects paucisynaptic actions of multiple VRC populations. Figure 10A shows inferred VRC-to-PRG excitatory and inhibitory functional connections (blue and red arrows, respectively) appropriate for roles in the generation and shaping of the respiratory modulation of PRG neuron activity. The core VRC circuit in this and subsequent diagrams is abstracted from the computational model. Each category of PRG neuron – I, IE, E, and EI – receives excitatory input from VRC neurons with similar respiratory profiles. The PRG I and IE patterns are further shaped by excitation or inhibition from VRC neurons with dissimilar respiratory-modulated activities. The VRC EI to PRG IE inhibition is consistent with a previous intracellular recording study in which PRG post-inspiratory neurons (classified here as IE neurons) were shown to be strongly inhibited during early I (Dick et al. 1994). VRC EI neurons in the present study were typically most active near the onset of the inspiratory phase. We note that evidence of inferred VRC-to-PRG projections was detected for 27 of the 203 (13.3%) VRC neurons with respiratory modulated discharge profiles, an incidence similar to the percentage (13%) of axonal projections to or through the pons from the VRC detected in the antidromic microstimulation studies of Bianchi and St. John (1981).

(ii) Functional connections among PRG neurons shape and coordinate their respiratory-modulated activities. Inferred interactions among PRG respiratory neurons are predominately excitatory (Fig. 10B), with most respiratory types receiving inputs from other PRG neurons with similar as well as dissimilar discharge patterns. The inferred inhibitory actions of PRG IE on PRG EI neurons involve two categories of neurons most active at opposing phase-transitions.
(iii) *Neurons of the PRG exert modulatory actions upon multiple VRC populations.* The PRG is considered to be involved in the determination of respiratory phase durations. The diagrams in Fig. 10C summarize inferred excitatory and inhibitory actions of each category of respiratory neuron in the PRG region upon various VRC respiratory cell types. The proposed connectivity suggests a basis for PRG facilitation of I-to-E phase-switching. Inhibition of I cells and excitation of E neurons within the VRC network by PRG IE neurons increases E-Dec and E-Aug neuron activity directly and via disinhibition (due to inhibition of VRC I-Dec neurons), leading to further inhibition of the VRC I populations and the transition from I to E.

In turn, PRG EI neurons may promote the E-to-I transition by exciting VRC I-Aug neurons and reducing VRC E-Dec neuron inhibition of the I-Driver population, leading to the inspiratory phase. Evidence for inferred PRG-to-VRC projections was detected in 27.3% (18 of 66) of recorded PRG neurons with respiratory-modulated firing patterns.

(iv) *Non-respiratory-modulated neurons located within and near the PRG and VRC exert widely-distributed actions upon neurons with respiratory-related discharge properties.* Neurons with no respiratory modulation of their firing rates are intermingled with respiratory-modulated cells, both within and near the VRC and the PRG (Fig. 1). Our results suggest that these medullary peri-columnar and pontine NRM neurons provide a tonic bias and modulatory influence to multiple classes of respiratory neurons located within their respective regions or in the opposite region (Fig. 10D). On the basis of the present results and preliminary data (Nuding et al. 2007), we suggest that these neurons have modulatory functions, including involvement in the transmission and transformation of central and peripheral chemosensory information.

It is noteworthy that some NRM neurons have presumptive excitatory and inhibitory inputs from other cells with diverse patterns of respiratory-modulated firing rates (Fig. 9). There are potential network mechanisms that could account for this apparently paradoxical observation,
including the balanced or “blended” effects of multiple convergent respiratory-modulated inputs of like or opposite sign (Rybak et al. submitted with this manuscript). These absorbed respiratory influences may subsequently affect other cells in the network. An alternate hypothesis is that NRM cells are members of a single population composed of neurons with a variety of respiratory discharge patterns. Elsewhere (Morris et al. 2007), we have described a network model with recurrent inhibition suggested by previous correlational data (Lindsey et al. 1992a,b,c). The model includes one target population with two inputs: a direct inspiratory-modulated input and a recurrent inhibitory input driven by the same inspiratory population. The two sets of inputs are “tuned” to generate tonic firing in the target population as a whole. Because of the randomness of distributed synapses, some individual neurons in the target population are not respiratory modulated, while others are classified as I or E using conventional criteria. Such a model also offers a parsimonious circuit mechanism for a previous observation of respiratory phase-dependent firing synchrony among neurons with no respiratory modulation of their individual firing rates (Lindsey et al. 1992b).

The $\eta^2$ value calculated for each neuron in this study is an indication of the strength and consistency of its respiratory discharge pattern (Orem and Dick 1983). Cells with high $\eta^2$ values are robustly respiratory modulated; neurons with low $\eta^2$ values are, to varying degrees, more weakly modulated, presumably reflecting, at least in some cases, a mixture of inputs with both respiratory and non-respiratory forms. Orem and co-workers have proposed that high $\eta^2$-valued cells represent the “automatic” system for breathing and low $\eta^2$-valued cells are the interface between non-respiratory inputs, such as those for behavioral control, and the automatic system (Orem and Trotter 1994). The present results support a respiratory network model with both high and low $\eta^2$-valued cells. We found evidence for functional connections involving high and low $\eta^2$-valued cells within each broad class of respiratory neuron – inspiratory, expiratory, and
phase-spanning.

_Sparseness of detected connectivity and relationship to other studies_

Previous studies have established that cross-correlation analysis is sufficiently sensitive to detect evidence for functional interactions among brainstem respiratory neurons (Bianchi et al. 1995; Feldman and Speck 1983; Lindsey et al. 2000; Segers et al. 1985, 1987). Advantages and limitations of cross-correlation and gravity methods have been discussed elsewhere (Lindsey et al. 1992c, 1994). As considered in previous studies on functional connectivity between brainstem domains (Lindsey et al. 1994), the results may reflect a paucity of functional links between neurons in the sampled domains, or the regions may be tightly coupled through many distributed connections, each with limited divergence and convergence. Both arrangements would result in a low probability that sampled neurons would be directly linked. Connection properties may also preclude generation of transient changes in neuronal firing probability time-locked to spikes in a trigger neuron that are detectable with cross-correlation. The size and time course of underlying conductance and potential changes and whether the connections are mono- or polysynaptic would influence detectability. Moreover, excitation may be more difficult to detect when target neurons have high firing rates or when the rate of impulses is limited by refractoriness. Conversely, inhibition is more difficult to detect than excitation when target neurons have low rates (Aertsen and Gerstein 1985).

We used both cross-correlation and gravity analysis methods to screen our data for indications of functional connectivity. Signs of interactions between neurons with transient periods of correlated activity may be averaged out in cross-correlation analysis. This limitation, and the requirement of the cross-correlation method for stationary data, does not apply to the gravity method for neuronal assembly analysis. We have previously unmasked transient
relationships not identified by cross-correlation techniques when the gravity method was applied to the same data (Lindsey et al. 1992b). Interactions identified with the gravity method in this study were confirmed with cross-correlation analysis.

A 1985 study from this lab is the lone previously published effort to address functional connectivity between individual PRG and VRC neurons (Segers et al. 1985). In that study, 11 of 255 (4.3%) respiratory-modulated PRG-VRC neuron pairs were correlated with a short-latency offset or central feature. Three offset correlations were suggestive of a paucisynaptic projection from the PRG to the VRC (1.2%; all peaks). Offset features consistent with VRC-to-PRG functional connections were detected in 5 pairs (2.0%; 4 peaks, 1 trough). Correlograms for the remaining 3 correlated pairs contained a central peak. Neurons with no respiratory modulation were not considered in that study. When PRG-VRC pairs that include an NRM neuron(s) are excluded from the present study, the results are similar to our earlier work. Sixty-one of 1,541 respiratory-modulated PRG-VRC neuron pairs (4.0%) were significantly correlated with offset or central peaks or troughs. Evidence was detected for 22 pontomedullary and 26 medullopontine functional projections (1.4% and 1.7%, respectively).

The small percentages of correlations between PRG and VRG neurons in both studies are consistent with a sparse distribution of functional connections, as were earlier studies using antidromic stimulation methods (Bianchi and St. John 1981, 1982). Given the strong influence of the pons upon the medullary respiratory network, it is reasonable to suggest, as noted above, that widely distributed or “weaker” effective connections not detectable with our cross-correlation methods are also present. Another possibility is that interactions between neurons of the PRG and VRC also include less direct pathways. Supporting the latter hypothesis, we have recently identified multiple short-time scale correlations among simultaneously recorded PRG, brainstem midline, and VRC neurons consistent with serial and recurrent paucisynaptic PRG -
VRC pathways that include intervening midline neurons (Nuding et al. 2006).

A preliminary report of a cross-correlation study of PRG neurons in the rat indicated a correlation in approximately 6% of the neuron pairs analyzed (Yu et al. 2006). All of the correlogram features reported were offset peaks. The respiratory modulation of the cells was not described. Offset peaks were detected in 48 of 1,043 (4.6%) pontine pairs in the present study and the incidence of intra-PRG correlation was greater (12%). Furthermore, correlogram features included central peaks and central and offset troughs in addition to offset peaks.

The current study largely focused on ipsilateral connectivity because labeling (Bystrzycka 1980; Denavit-Saubie and Riche 1977) and stimulation studies (Bianchi and St. John 1981, 1982; Ezure and Tanaka 2006) in the cat and the rat indicate that projections between the PRG and VRC are predominantly ipsilateral. Moreover, although the pontine and medullary respiratory groups are distributed bilaterally, each side of the cat brainstem is capable of producing a respiratory rhythm (Eldridge and Paydarfar 1989).
GRANTS

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Insights from computational models of acute vagotomy. Program No. 230.11. 2007


**Figure 1.** A. Spheres mark coordinates at which signals from single neurons were recorded. Locations in which signals from different neurons were monitored on the same electrode are indicated by adjacent vertically “stacked” spheres. Spheres are color-coded to indicate the presence (red) or absence (blue) of respiratory-modulated impulse activity. B. Illustrative traces of simultaneously recorded spike trains from two multi-electrode arrays. Corresponding spike times derived from sorted waveforms are shown to the right of each trace. In some cases, waveforms from two or more neurons were separated, e.g. neurons designated 809 and 839. C. Firing rate histograms for 46 of 82 simultaneously monitored PRG and VRC neuron spike trains. For each trace, the pattern of respiratory modulation, cell identification number, and peak firing rate are shown. Highlighted histograms (red numbers) were derived from neurons represented in panel B.
**Figure 2.** A. Activity discharge classifications of all recorded cells in the VRC (left) and the PRG (right). See text. B. Normalized PRG neuron respiratory cycle-triggered histograms grouped by the respiratory phase (transition) with peak average activity. Respiratory phases were defined by efferent phrenic nerve activity (gray plots). The $\eta^2$ value, numbers of respiratory cycles averaged, bin widths, and maximum firing rates, respectively, for each CTH were as follows (top to bottom). I neurons: 0.05, 471 cycles, 85.0 ms, 1.2 spikes s$^{-1}$; 0.45, 471 cycles, 85.0 ms, 7.4 spikes s$^{-1}$; 0.12, 497 cycles, 45.0 ms, 5.5 spikes s$^{-1}$; 0.27, 471 cycles, 85.0 ms, 6.7 spikes s$^{-1}$; 0.06, 471 cycles, 85.0 ms, 5.2 spikes s$^{-1}$. IE neurons: 0.02, 490 cycles, 60.5 ms, 4.2 spikes s$^{-1}$; 0.02, 491 cycles, 25.0 ms, 5.7 spikes s$^{-1}$; 0.05, 496 cycles, 50.3 ms, 8.6 spikes s$^{-1}$; 0.02, 490 cycles, 60.5 ms, 4.1 spikes s$^{-1}$; 0.02, 496 cycles, 50.3 ms, 25.8 spikes s$^{-1}$. E neurons: 0.91, 490 cycles, 80.3 ms, 23.1 spikes s$^{-1}$; 0.07, 491 cycles, 50.0 ms, 6.7 spikes s$^{-1}$; 0.21, 471 cycles, 85.0 ms, 8.4 spikes s$^{-1}$; 0.04, 471 cycles, 85.0 ms, 3.9 spikes s$^{-1}$; 0.02, 496 cycles, 50.3 ms, 47.3 spikes s$^{-1}$. EI neurons: 0.13, 471 cycles, 85.0 ms, 5.5 spikes s$^{-1}$; 0.01, 449 cycles, 70.0 ms, 11.4 spikes s$^{-1}$; 0.03, 449 cycles, 70.0 ms, 4.6 spikes s$^{-1}$; 0.02, 471 cycles, 85.0 ms, 0.8 spikes s$^{-1}$; 0.02, 497 cycles, 45.0 ms, 3.6 spikes s$^{-1}$.
**Figure 3.** A. Cross-correlation feature summary diagram (CFSD) represents the offset peaks (+) or troughs (−) at positive time-lags in CCHs for VRC reference neuron-to-PRG target neuron pairs with the indicated respiratory-modulated firing patterns. B. Cross-correlograms with circled numbers have features corresponding to similarly labeled correlation linkages represented in the CFSD. 1: peak, detectability index (DI) = 3.55, 23,617 reference and 23,410 target spikes; 2: trough, DI = 12.52, 18,554 reference and 40,145 target spikes; 3: trough, DI = 3.99, 64,025 reference and 7,051 target spikes; 4: peak, DI = 3.58, 91,929 reference and 46,659 target spikes; 5: peak, DI = 5.81, 80,313 reference and 79,330 target spikes; 6: peak, DI = 7.51, 17,957 reference and 79,330 target spikes; 7: peak, DI = 3.22, 65,722 reference and 35,057 target spikes; 8: peak, DI = 3.29, 41,968 reference and 19,775 target spikes.

**Figure 5.** A. PRG reference neuron-to-VRC target neuron correlation feature summary diagram. B. Examples of cross-correlograms with positive time-lag offset primary features. Same labeling conventions as Fig. 3. 18: peak, DI = 7.32, 8,437 reference and 13,127 target spikes; 19: peak, DI = 3.87, 11,022 reference and 78,153 target spikes; 20: trough, DI = 3.90, 3,526 reference and 133,522 target spikes; 21: trough, DI = 3.73, 54,835 reference and 5,269 target spikes; 22: peak, DI = 3.15, 61,962 reference and 13,127 target spikes; 23: peak, DI = 3.95, 123,339 reference and 47,206 target spikes. C. The top 2 histograms are normalized CTHs of the neurons in CCH 24. The CTH of each neuron is drawn on the same axes with, but at a different scale than, the CTH of concurrent phrenic nerve activity (shown in gray). 24: peak, DI = 3.87, 7,513 reference and 65,722 target spikes; 25: trough, DI = 3.08, 12,392 reference and 72,332 target spikes.

**Figure 6.** A. VRC reference neuron-to-VRC target neuron correlation feature summary diagram. B. Examples of cross-correlograms with positive time-lag offset primary features. Same labeling conventions as Fig. 3. 26: trough, DI = 6.05, 55,503 reference and 37,847 target spikes; 27: trough, DI = 19.84, 96,204 reference and 329,666 target spikes; 28: peak, DI = 34.03, 55,503 reference and 47,203 target spikes; 29: peak, DI = 7.32, 35,737 reference and 40,809 target spikes; 30: peak, DI = 3.83, 24,080 reference and 5,778 target spikes; 31: trough, DI = 3.69, 60,634 reference and 87,600 target spikes; 32: peak, DI = 11.09, 12,686 reference and 26,164 target spikes; 33: trough, DI = 8.26, 438,039 reference and 85,471 target spikes; 34: peak, DI = 6.46, 79,463 reference and 6,111 target spikes; 35: peak, DI = 6.35, 47,203 reference and 23,617 target spikes.
Figure 7. A. Correlation linkage map shows feature and strength of extended correlation linkages among simultaneously monitored PRG neurons (see Key). Note that some represented linkages include a “secondary” feature. These features were juxtaposed on either side of the correlogram origin or more offset from the origin than primary features (e.g., Fig 6B, CCH 27 with a primary trough and secondary peak). B. Cross-correlogram with central peak and bilateral troughs from PRG neuron pair 501-529 represented in panel A; 36: DI = 15.91; 61,962 reference and 7,513 target spikes. C. Tuned gravity particle trajectories projected from N-space to a plane. Data were from a 351 second sample of the 16 neuron spike trains represented in panel A. The acceptor charge decay was set backward and the effector charge decay forward; both charge decay time constants were 5.0 ms. Numbers of spikes - neuron 501: 4,929; 502: 2,682; 504: 1,588; 505: 6,633; 506: 4,057; 507: 1,725; 509: 675; 510: 5,468; 514: 1,172; 516: 1,511; 519: 577; 521: 858; 523: 496; 529: 760; 537: 494; 539: 1,266. D. Particle distance as a function of time (PDFT) plot for all cell pairs represented in the projected particle trajectories. E. PDFT plot (labeled heavy line) documents significant aggregation of particles for the same neuron pair (501-529) represented in CCH 36 (panel B). The top and bottom thin lines define maximum and minimum distances, respectively, between particles at each time step in 100 shuffled surrogate data sets; this Monte Carlo test indicated significant particle pair aggregation. The middle thin line indicates the mean inter-particle distance at each time step for the 100 runs. F. Black regions in each row of the graph indicate when the distance between the represented pair of particles was less than expected under the Monte-Carlo test. The red highlighted row corresponds to the pair represented in the 501-529 PDFT plot (red arrow). See text for details.
**Figure 8.** A. Correlation linkage map from a multi-array recording revealed extended functional linkages, including PRG IE neurons with multiple relationships. Encircled numbers less than 38 correspond to CCHs depicted in previous figures. B. Illustrative cross-correlograms for 3 neuron pairs from the recording depicted in panel A. Same labeling conventions as Fig. 3. 38: CCH of 2 PRG neurons, central trough, DI = 6.12, 11,022 reference and 37,619 target spikes; 39: CCH of the same PRG neuron and a VRC cell, central peak, DI = 3.55, 339,189 reference and 11,022 target spikes; 40: CCH of 2 VRC neurons, DI = 153.29, 102,970 reference and 18,554 target spikes.

**Figure 9.** Detailed functional circuit diagram of the pontomedullary respiratory network. To simplify the diagram, each neuron type has only one “output” line, which then divides as necessary to provide input(s) to other cell populations. The first division of each output line is marked with a white circle; all other network junctions are labeled with a black circle. These connection lines are not meant to imply, for example, that a single VRC EI neuron is functionally connected to 4 separate target neurons within the VRC, but, rather, that there is evidence for connections of neurons of the VRC EI respiratory type to four other types of VRC neurons (NRM, I-Aug, IE, and other EI neurons). The synapse type for a particular connection is represented by a small colored circle next to the target neuron population; excitatory and inhibitory synapses are colored blue and red, respectively. Green squares denote functional connectivity inferred from previously published studies that used spike train cross-correlation and spike-triggered averaging of synaptic potentials (Anders et al. 1991; Bianchi et al. 1995; Ezure 1990; Feldman and Speck 1983; Jiang and Lipski 1990; Li et al. 1999a; Lindsey et al. 1987, 1989a,b, 1998; Morris et al. 1996; Rekling et al. 2000; Segers et al. 1985, 1987; Shannon et al. 1998, 2000). Connections between VRC propriobulbar neurons or bulbospinal neurons and
their target motoneurons were based on spike-triggered averages of synaptic potentials or multi-unit nerve recordings (Baekey et al. 2001, 2004; Ezure 1990; Ezure et al. 1989; Feldman and Speck 1983; Jiang and Lipski 1990; Lindsey et al. 1998). Pump cells of the nucleus tractus solitarius (NTS) project to the ipsilateral pons (Ezure et al. 1998, 2002) and have been shown to influence VRC populations (Ezure and Tanaka 2004; Kubin et al. 2006); it has been suggested that Pump cells inhibit ipsilateral PRG neurons presynaptically (Dick et al. 2002; Feldman et al. 1976; Kubin et al. 2006; Morris et al. 2007). Connections proposed in other studies and models are labeled with blue triangles (Rybak et al. submitted with this manuscript). Yellow stars indicate inferred functional connections that are documented for the first time (star alone) or confirmed in this study (star enclosed in green box or blue triangle).

Some neuron types were grouped. Neurons with the I-EI discharge pattern characteristic of some neurons proposed to have an ‘I-_DRIVER’ function include neurons with the ‘inspiratory constant’ (I-CON) pattern (Ezure 1990). E-Aug-Early and E-CON were considered equivalent, and the E-Dec-Tonic category was assumed to have inputs similar to those of the E-Dec-P population. The IE population included the overlapping designations of late I, early E-Dec, and post-inspiratory used by various authors. I-Aug and bulbospinal I-Aug neurons were collapsed into a single population for simplicity. NTS, nucleus tractus solitarius; BötC, Bötzing complex. Pre-BötC, pre-Bötzing complex; VRG, Ventral Respiratory Group.
**Figure 10.** Ball-and-stick models of some proposed functions of pontomedullary neuron interactions suggested or supported by the results. PRG and VRC populations are shown as large balls labeled with a corresponding respiratory discharge pattern. The small circles at the ends of the connecting lines indicate the particular synaptic relationship: excitatory and inhibitory synapses are shown as light and dark circles, respectively. A and B. Inferred VRC-to-PRG (panel A) and intra-PRG interactions (panel B) proposed to contribute to the respiratory modulated firing patterns of PRG neurons. C. Some proposed sites of action of PRG neurons in the VRC. D. NRM neuron actions within the PRG and VRC inferred from the results. See text for further discussion. Each arrow is labeled with the number and percentage of neuronal pairs of each “type” that exhibited evidence for the indicated suggested action.
Table 1. Summary of results of cross-correlation analysis of 8,601 neuron pairs.

<table>
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<tr>
<th>Significant correlations / Total pairs</th>
<th>VRC - PRG</th>
<th>PRG - PRG</th>
<th>VRC - VRC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>109 / 3,218 (3.4%)</td>
<td>126 / 1,043 (12.1%)</td>
<td>319 / 4,340 (7.4%)</td>
</tr>
<tr>
<td>Central Peaks</td>
<td>21</td>
<td>60</td>
<td>151</td>
</tr>
<tr>
<td>Offset Peaks</td>
<td>33</td>
<td>48</td>
<td>101</td>
</tr>
<tr>
<td>Troughs</td>
<td>3</td>
<td>12</td>
<td>25</td>
</tr>
<tr>
<td>Offset Troughs</td>
<td>15</td>
<td>6</td>
<td>42</td>
</tr>
<tr>
<td>TOTAL</td>
<td>24</td>
<td>72</td>
<td>176</td>
</tr>
<tr>
<td>Offset</td>
<td>48</td>
<td>54</td>
<td>143</td>
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Offset features involving VRC-PRG pairs are separated according to the simplest interpretation of their CCHs. Mean DI, half-width, and time-lag from origin of the feature (mean ± SD) for all offset features: 5.3 ± 4.0, 19.6 ± 29.0 ms, 26.2 ± 30.4 ms; all central features: 10.8 ± 25.3, 42.1 ± 47.4 ms, 0.0 ms; all offset peaks: 5.5 ± 4.4, 20.9 ± 31.1 ms, 28.2 ± 32.7 ms; all offset troughs: 4.8 ± 2.8, 21.0 ± 22.6 ms, 21.0 ± 22.7 ms; all central peaks: 11.7 ± 27.3, 37.4 ± 38.8 ms, 0.0 ms; all central troughs: 5.7 ± 2.6, 69.5 ± 76.1 ms, 0.0 ms.
Table 2. VRC–to–PRG significant offset features with positive time lags detected in the analysis of 3,218 VRC–PRG neuron pairs.

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Detected peaks and troughs simply interpreted as evidence for a functional connection from the VRC to a PRG neuron. Mean DI, half-width, and time-lag from origin (mean ± SD) for offset peaks: 4.1 ± 1.0, 23.8 ± 38.0 ms, 30.1 ± 26.9 ms; offset troughs: 5.6 ± 2.9, 17.5 ± 17.0 ms, 21.1 ± 23.4 ms. In this and subsequent tables, Pk = Peak, Tr = Trough, and Tot = total number of such pairs evaluated for evidence of correlation.
Table 3. *PRG–to–PRG significant offset features with positive time-lags detected in the analysis of 1,043 PRG-PRG neuron pairs.*

Detected peaks and troughs simply interpreted as evidence for a functional connection from one PRG neuron to another. Correlated neuron pairs are organized so that offset correlogram features have positive time-lags. Shaded numbers indicate the total number of pairs composed of neurons with the discharge patterns indicated by the row and column labels. These numbers were used to calculate the percentages of neurons correlated (e.g., of the 122 pairs composed of an IE and an NRM neuron, an IE $\rightarrow$ NRM connection may be inferred for 9 pairs (7.4%) and an NRM $\rightarrow$ IE connection for 3 pairs (2.5%)). The shaded numbers were summed to calculate the total number of PRG-PRG pairs analyzed. Mean DI, half-width, and time-lag from origin (mean ± SD) for *offset peaks*: 4.9 ± 3.4, 30.2 ± 33.4 ms, 24.3 ± 27.0 ms; *offset troughs*: 4.0 ± 0.6, 13.3 ± 18.4 ms, 9.7 ± 8.9 ms.

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Table 4. PRG-to-VRC significant offset features with positive time-lags detected in the analysis of 3,218 VRC-PRG neuron pairs.

Detected peaks and troughs simply interpreted as evidence for a functional connection from the PRG to a VRC neuron. Mean DI, half-width, and time-lag from origin (mean ± SD) for offset peaks: 4.0 ± 1.3, 9.6 ± 15.2 ms, 21.5 ± 17.4 ms; offset troughs: 3.4 ± 0.4, 8.9 ± 7.7 ms, 35.5 ± 30.1 ms.
Table 5. *VRC–to–VRC significant offset features with positive time-lags detected in the analysis of 4,340 VRC-VRC neuron pairs.*

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Detected peaks and troughs simply interpreted as evidence for a functional connection from one VRC neuron to another. Correlated neuron pairs are organized so that offset correlogram features have positive time-lags. Shaded numbers indicate the total number of pairs composed of neurons with the discharge patterns indicated by the row and column labels. These numbers were used to calculate the percentages of neurons correlated (e.g., of the 133 pairs composed of an IE and an NRM neuron, an IE → NRM connection may be inferred for 0 pairs (0.0%) and an NRM → IE connection for 5 pairs (3.8%)). The shaded numbers were summed to calculate the total number of VRC-VRC pairs analyzed. Mean DI, half-width, and time-lag from origin (mean ± SD) for *offset peaks*: 6.5 ± 5.5, 20.8 ± 32.7 ms, 28.1 ± 36.5 ms; *offset troughs*: 5.0 ± 3.3, 18.3 ± 27.3 ms, 16.9 ± 18.0 ms.
Table 6. Significant central features detected in the analysis of 3,218 VRC-PRG neuron pairs, 1,043 PRG-PRG neuron pairs, and 4,340 VRC-VRC neuron pairs (top to bottom).

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Detected peaks and troughs simply interpreted as evidence for the influence of a shared input upon the activity of both neurons of a pair. VRC-PRG neuron pairs (top): Mean DI and half-width of the feature (mean ± SD) for central peaks: 7.3 ± 4.9, 61.4 ± 39.5 ms; central troughs: 5.3 ± 3.5, 48.3 ± 53.6 ms. PRG-PRG neuron pairs (middle): Mean DI and half-width of the feature (mean ± SD) for central peaks: 6.6 ± 6.4, 54.0 ± 36.7 ms; central troughs: 7.1 ± 3.7, 69.8 ± 53.3 ms. VRC-VRC neuron pairs (bottom): Mean DI and half-width of the feature (mean ± SD) for central peaks: 14.3 ± 33.3, 27.6 ± 36.0 ms; central troughs: 5.1 ± 1.6, 71.8 ± 88.4 ms.
Electrode Neuron PRG Sorted spike times Signals from electrodes

Phrenic

Phrenic

PRG Phrenic

VRC

Firing rate histograms from a multi-array recording

Spikes s
A  Respiratory modulation of recorded cells

Ventral Respiratory Column

Pontine Respiratory Group

B  PRG neuron cycle-triggered histograms
A

PRG-to-PRG offset correlation feature summary diagram

B

- 775  775 ms
- 375  375 ms
- 275  275 ms
- 525  525 ms
- 25   25 ms
- 125  125 ms
- 75   75 ms
- 125  125 ms

KEY

- offset peak
- offset trough

0.8 spikes s^{-1}
2.4 spikes s^{-1}
5.2 spikes s^{-1}
12.4 spikes s^{-1}
3.6 spikes s^{-1}
10.0 spikes s^{-1}
0.7 spikes s^{-1}

Scaled up 1.53x
A

PRG-to-VRC offset correlation feature summary diagram

VRC

PRG

I

IE

E

EI

NRM

KEY

offset peak

offset trough

B

IE → NRM

E → I-Dec

NRM → NRM

IE → IE

EI → E-Dec-T

NRM → IE

C

Normalized CTHs

Neuron 529

Neuron 103

Phrenic
A Correlation linkage map

B Cross-correlation histograms

- IE → EI (510 → 515)
- E-DEC-T → IE (829 → 510)
- I-AUG → NRM (803 → 805)

Correlogram features
- Primary
- Peak
- Secondary
- Trough
- Location
  - Offset
  - Central

KEY

Neuron ID
Respiratory modulation
Network actions inferred from correlation linkage maps

A

PRG

VRC

B

PRG

C

PRG

VRC

D

PRG

VRC

KEY

- excitation
- inhibition

Graphical representations showing network actions between different nodes (PRG, VRC, E-DEC, T, E-AUG, I-DEC, I-AUG, I-Driver, IE, EI) with percentage values indicating the strength of the connections.