Pontine – Ventral Respiratory Column Interactions through Raphé Circuits

Detected Using Multi-Array Spike Train Recordings

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ABSTRACT

Recently, Segers et al. (2008) identified functional connectivity between the ventrolateral respiratory column (VRC) and the pontine respiratory group (PRG). The apparent sparseness of detected paucisynaptic interactions motivated consideration of other potential functional pathways between these two regions. We report here evidence for “indirect” serial functional linkages between the PRG and VRC via intermediary brain stem midline raphé neurons. Arrays of microelectrodes were used to record sets of spike trains from a total of 145 PRG, 282 VRC, and 340 midline neurons in 11 decerebrate, vagotomized, neuromuscularly blocked, ventilated cats. Spike trains of 13,843 pairs of neurons that included at least one raphé cell were screened for respiratory modulation and short-time scale correlations. Significant correlogram features were detected in 7.2% of raphé–raphé (291/4,021), 4.3% of VRC–raphé (292/6,755), and 4.0% of the PRG–raphé (124/3,067) neuron pairs. Central peaks indicative of shared influences were the most common feature in correlations between pairs of raphé neurons, while correlated raphé-PRG and raphé-VRC neuron pairs displayed predominantly offset peaks and troughs, features suggesting a paucisynaptic influence of one neuron upon the other. Overall, offset correlogram features provided evidence for 33 VRC-to-raphé-to-PRG and 45 PRG-to-raphé-to-VRC correlational linkage chains with 1 or 2 intermediate raphé neurons. The results support a respiratory network architecture with parallel VRC-to-PRG and PRG-to-VRC links operating through intervening midline circuits, and suggest that raphé neurons contribute to the respiratory modulation of PRG neurons and shape the respiratory motor pattern through coordinated divergent actions upon both the PRG and VRC.
INTRODUCTION

Neurons in the medullary ventrolateral respiratory column (VRC) and the dorsolateral pontine respiratory group (PRG) cooperate in the generation of the motor pattern for breathing (Alheid et al. 2004; Bianchi et al. 1995; Feldman et al. 2003; Segers et al. 1985; Smith et al. 1991, 2007; Spyer 1994; St. John 1998). Recently, Segers et al. (2008) identified functional linkages within this pontomedullary respiratory network. The results suggested and supported model-based hypotheses on circuit mechanisms for the generation of respiratory-modulated pontine neuron activity and phase-switching (Rybak et al. 2008). The data were also consistent with evidence for distributed but apparently sparse projections between the two domains based on antidromic stimulation studies (Bianchi and St. John 1981, 1982).

Given the profound influence of the pons upon the medullary respiratory network and the evidence of anatomical projections between the PRG and VRC (Herbert et al. 1990; Kalia 1977; King 1980; Song and Poon 2006; Smith et al. 1989), the scarcity of detected interactions between the VRC and PRG motivated consideration of other potential functional pathways. Bianchi et al. (1995) proposed that medullary raphé circuits serve as “intermediate relays” in VRC–PRG interactions. There is anatomical evidence for axonal projections between the region of the medullary raphé nuclei and both the VRC (Smith et al. 1989) and dorsolateral pons (Gang et al. 1990, 1991; Hermann et al. 1997; Holstege 1988). In addition, midline neurons exhibit respiratory-modulated firing rates or phase-dependent synchrony, properties consistent with VRC and PRG influences (Bennett and St. John 1985; Dick et al. 2008; Hosogai et al. 1993; Lindsey et al. 1992a,c, 1994, 1998; Mason et al. 2007). The primary aim of the present study was to test the intermediate relay hypothesis using
correlational analysis of spike trains monitored simultaneously in these three brain stem regions. The results support a model of respiratory network architecture with reciprocal VRC-to-PRG and PRG-to-VRC paucisynaptic linkages operating through intermediary midline circuits. The data also suggest that raphé neurons modulate the PRG and VRC, confirming and extending earlier studies on midline neuron functional connectivity with the pontomedullary network (Li et al. 1999a; Lindsey et al. 1994, 1998; Morris et al. 1996b). Preliminary accounts have been reported (Nuding et al. 2004, 2006, 2007b).

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 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 11 adult cats (2.8–5.6 kg) of either sex as part of a larger study on the brain stem respiratory network; a detailed description of the methods has been published (Segers et al. 2008). Briefly, animals were initially anesthetized with isoflurane (2–5%; n = 5) 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
CO₂, rectal temperature, and arterial blood pressure were monitored continuously; arterial PO₂, PCO₂, and pH were measured periodically. These parameters were maintained within normal limits. Prior to decerebration, 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 brain stem 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. Animals were bilaterally vagotomized to eliminate vagal afferent feedback from pulmonary stretch receptors. 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.

**Neural recordings and data analysis**

Efferent phrenic nerve activity was monitored together with signals from arrays of extracellular electrodes with individual depth adjustment positioned in the dorsolateral pons, VRC, and medullary midline. Electrode placement was guided by appropriate stereotaxic coordinates (see Fig. 1, legend) derived from Berman (1968) and numerous previous studies as described in Segers et al. (2008) and herein. These signals, together with systemic arterial blood pressure, tracheal pressure, and end-tidal CO₂, were recorded and spike trains from single neurons were converted to occurrence times with spike-sorting software. Stereotaxic coordinates of recording sites were mapped into the three-dimensional space of a computer-based brain stem atlas derived from *The Brain Stem of the Cat: A Cytoarchitectonic Atlas with Stereotaxic Coordinates* (Berman 1968) with permission of the University of Wisconsin Press.
Two statistical tests were used to evaluate each spike train for the presence of respiratory-modulated activity. The first test, a subjects-by-treatments analysis of variance, partitioned each respiratory cycle ("subject" variable) into twenty equal time segments ("treatment" variable) (Netick and Orem 1981; Orem and Netick 1982). A complementary nonparametric sign test was also used to determine if the probability of occurrence of an increased firing rate in one half of the respiratory cycle, over the length of the recording, was greater than chance (Morris et al. 1996a). A neuron was classified as respiratory modulated if either test rejected the null hypothesis ($p < 0.05$). Both standard and normalized respiratory cycle-triggered histograms (CTHs) were computed for each neuron and used to identify the phase (inspiration [I]; expiration: [E]) or phase transition (IE and EI) in which a respiratory-modulated cell was most active (Cohen 1968). Neurons with no preferred phase of maximum activity as determined by both statistical tests were monitored in close proximity to the respiratory-modulated cells within all three areas; these neurons were classified as "nonrespiratory-modulated" (NRM).

Cross-correlation histograms (CCHs) were calculated for each pair of simultaneously recorded spike trains to detect and evaluate effective neuronal connectivity. The significance of cross-correlogram features (peaks and troughs) was evaluated by calculating a "detectability index" (DI): the ratio of the maximum amplitude of departure from the background to the standard deviation of the correlogram noise; features with DI values $\geq 3$ were considered significant (Aertsen and Gerstein 1985; Melssen and Epping 1987). Correlation linkage maps for groups of simultaneously monitored neurons were generated automatically by database queries using software employing the open source graph visualization tool Graphviz.
RESULTS

Recording sites and respiratory modulation of neurons

Spike trains were monitored simultaneously in the VRC, PRG, and medullary raphé nuclei during 13 recording sessions in 9 animals. In 2 additional animals, only raphé and PRG activity was sampled and for one of these, only raphé neurons (n=32) could be isolated. Recording durations ranged from 47 to 186 minutes; from 32 to 116 neurons were monitored simultaneously in the different recordings. Stereotaxic coordinates of the midline recording sites were mapped in a brain stem atlas (Fig. 1A, B). More than one neuron was recorded at some represented locations, due either to recording the activities of two neurons with the same electrode or to replicating stereotaxic coordinates in different recording sessions; these cells are represented as vertically displaced spheres in the sagittal perspective (Fig. 1A). The recording sites of the midline neurons were also projected together with sites of the sampled VRC and PRG neurons in the dorsal view (Fig. 1B).

Of the 340 midline neurons sampled from all animals, 138 (41%) were respiratory modulated. These cells were classified according to the timing of their peak firing rates during the respiratory cycle (inspiratory [I], expiratory [E], or IE or EI if the peak activity occurred at a phase transition) and further characterized as “decrementing” (Dec) or “augmenting” (Aug) if peak rates were in the first or second half of the phase, respectively. Of 282 VRC neurons, 203 (72%) were respiratory modulated, as were 65 (45%) of the 145 PRG neurons. Classification of respiratory-modulated neurons in these two regions followed previously specified conventions (Segers et al. 2008). PRG neurons with peak firing rates at a phase transition 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; neurons with peak firing rates during the second half of the inspiratory or 
expiratory phase and with a generally augmenting “ramp” of activity were characterized as I 
and E, respectively. Two “stacks” of cycle-triggered histograms from one recording are 
shown in Fig. 1C and illustrate the variety of observed respiratory-modulated discharge 
profiles. Nine VRC neurons had properties consistent with possible roles as “I-driver” 
neurons (Morris et al. 1996a; Segers et al. 1987): each cell was located more than 2.0 mm 
rostral to obex and there was no evidence of a functional inhibitory influence upon any other 
cell. In addition, at least one of the following criteria was met: 1) the cell’s firing rate began 
increasing slightly prior to phrenic nerve discharge, peaked in early I, and slowly 
decremented before abruptly decreasing at the I-to-E transition, or 2) the cell was most active 
during the I phase with a functional excitatory influence upon a more caudally-located VRC I neuron.

Cross-correlation analysis

The use of arrays of microelectrodes with individual depth adjustment allowed us to 
acquire spike train data from relatively many neurons monitored in parallel at multiple sites. 
Spike trains of 13,843 pairs of neurons that included at least one raphé cell (i.e., PRG–raphé, 
raphé–raphé, and raphé–VRC neuron pairs) were screened for short-time scale correlations. 
Of these, 707 (5.1%) correlograms displayed features indicative of mono- or paucisynaptic 
connectivity (Table 1). Significant correlogram features were detected in 7.2% of raphé– 
raphé (291/4,021), 4.3% of VRC–raphé (292/6,755), and 4.0% of the PRG–raphé (124/3,067) 
neuron pairs. Primary features detected included 266 offset peaks, 95 offset troughs, 291 
central peaks, and 55 central troughs. Complementary results from a coordinated study of
functional interactions between sets of VRC–PRG, PRG–PRG, and VRC–VRC neuron pairs have been reported (Segers et al. 2008).

A goal of spike train correlation analysis is to “define simple circuit models that replicate experimentally observed features” (Aertsen et al. 1989). As elaborated elsewhere (Duffin 2000; Moore et al. 1970; Perkel et al. 1967), primary peaks and troughs offset from the correlogram origin reflect transient changes in the short-time scale firing probability of the target cell following spikes in the reference neuron and may be simply interpreted as signs of functional excitation and inhibition, respectively. Central peaks suggest similar influences shared by the two neurons, while central troughs are indicative of opposite actions resulting from a common input. Central features were detected in a greater percentage of within-raphé correlated pairs (58%, 168/291) whereas the majority of between-region correlation features were offset [VRC–raphé: 53% (156/292); PRG–raphé: 66% (82/124)]. The mean half-widths of the offset peaks and troughs were less than those of the corresponding central features (Kolmogorov-Smirnov test; peaks: $p = 2.2 \times 10^{-16}$; troughs: $p = 1.5 \times 10^{-5}$; see Table 1 legend for mean values), suggesting that the offset and central correlogram features in our sample reflected distinct circuit properties. These two types of features are considered separately.

Subsequent sections of the results are divided into 2 parts. The first part documents and highlights offset features in correlograms for raphé–PRG neuron pairs, and provides evidence for local interactions within the midline raphé and for raphé–VRC connectivity. The second part details extended correlational linkages among neurons simultaneously recorded in all three domains. This organization is intended to provide a concise summary of a large data set and to facilitate further consideration of functional implications in the discussion and the development of future distributed respiratory network models that include raphé circuits.
Part 1: Details of pairwise correlations

Evidence for Midline → PRG functional connectivity

Thirty-five of 308 raphé neurons evaluated (11.4%) were elements of correlated raphé–PRG pairs with positive-lag features consistent with excitatory and inhibitory functional connections from the midline to the PRG; 20 of these had a respiratory-modulated discharge. Overall, correlograms for 44 of the 3,067 raphé–PRG pairs had an offset peak (n=35) or trough (n=9); two examples are shown in Fig. 2A (CCHs 1 and 2). Table 2 (right) details the numbers of correlated and total pairs evaluated. Offset correlogram features, including 2 peaks and 3 troughs, were identified in 5 of 208 pairs composed of neurons with similar respiratory-modulated discharge patterns (see categories enumerated in METHODS). Of the 653 neuron pairs composed of neurons with different firing profiles, 13 had offset features (10 peaks, 3 troughs).

The cross-correlation feature summary diagram in Fig. 2C provides a graphical overview of positive-lag peaks and troughs found in raphé–PRG correlograms when midline raphé neurons served as reference cells and PRG neurons were targets. In this and subsequent feature summary diagrams, respiratory modulation categories for reference neurons are represented on the left side; target neuron profiles are shown across the top. Connecting lines indicate that at least one correlational linkage was identified for that particular combination of reference–target neuron categories. Each small circle at the end of the line indicates a peak or trough; the circled number on some lines indicates a corresponding correlogram shown elsewhere in the RESULTS.
Evidence for PRG → Midline functional connectivity

Thirty-one of 145 PRG neurons (21.4%) were members of correlated PRG-raphé pairs with offset features suggestive of functional connections from PRG cells to the midline; the activities of 19 of these were respiratory modulated. Correlograms for 38 of the 3,067 raphé–PRG pairs had an offset peak (n=27) or trough (n=11) (Table 2, left); examples are shown in Fig. 2B (CCHs 3 and 4). These data are graphically summarized in Fig. 2D. One positive-lag offset peak was detected in 208 pairs of neurons with similar respiratory profiles, while 17 of the 653 pairs of neurons with dissimilar respiratory-modulated discharge profiles had offset features (12 peaks, 5 troughs).

Midline neuron interactions

Eighty-five of 340 raphé neurons (25%) were elements of correlated raphé–raphé pairs with positive time-lag features consistent with a functional connection from one cell to the other; 51 of the raphé reference cells were respiratory modulated. Overall, correlograms for 123 of 4,021 raphé neuron pairs had significant offset features (92 peaks and 31 troughs; Table 3). Offset features were detected for 22 of 374 pairs composed of neurons with similar respiratory discharge profiles (19 peaks, 3 troughs; Fig. 3A, CCH 6). A total of 539 pairs composed of neurons different patterns were evaluated; 23 pairs had offset peaks and 9 had offset troughs in their correlograms. Over half of the correlated raphé neuron pairs included at least one NRM neuron (69 of 123, 56%). Features in cross-correlograms for pairs of NRM neurons included 16 offset peaks and 10 offset troughs, or 21% of the offset features detected for all raphé neuron pairs.
Evidence for VRC → Midline functional connectivity

Fifty-nine of 282 VRC neurons (20.9%) were members of correlated VRC–raphé pairs with offset features suggestive of excitatory and inhibitory functional connections from the VRC to the midline; of these, 44 had a respiratory-modulated discharge. Offset features were detected in 89 of 6,755 VRC–midline neuron pairs; these features included 58 peaks and 31 troughs (Table 4, left). Among the 682 pairs of neurons that shared the same category of respiratory activity, 10 had offset features (7 peaks, 3 troughs). Correlograms from 34 of the 1,539 pairs of neurons with different respiratory patterns had offset features; 24 of these were peaks. Offset features (23 peaks, 14 troughs) were found in CCHs from 3,430 VRC-to-raphé neuron pairs in which one cell was respiratory modulated and the other was not (Fig. 3B; CCHs 5, 8, and 19); 21 of these 37 pairs involved a VRC NRM reference neuron. Eight of 1,104 pairs composed of two NRM neurons had positive time-lag features (4 peaks, 4 troughs).

Evidence for Midline → VRC functional connectivity

Forty-five of 285 raphé cells evaluated (15.8%) were elements of correlated raphé–VRC pairs with positive-lag features consistent with functional connections from the midline to the VRC; 24 of these had a respiratory-modulated activity pattern. Overall, correlograms for 67 of 6,755 midline–VRC pairs had an offset peak (n=54) or trough (n=13). These results are summarized in Fig. 3C and Table 4 (right). Among the 682 pairs composed of neurons with the same respiratory profile, 10 had correlations suggestive of a raphé-to-VRC connection (8 peaks, 2 troughs). Correlograms from 15 of the 1,539 pairs of neurons with dissimilar respiratory discharge patterns had offset features (10 peaks, 5 troughs; Fig. 3D, CCH 15).
Thirty-three offset features (30 peaks, 3 troughs) were detected in raphé–VRC pairs composed of an NRM neuron and a respiratory-modulated cell; for 23 pairs, the midline reference cell was not respiratory modulated. Nine NRM neuron pairs had correlogram features consistent with raphé-to-VRC interactions (6 peaks, 3 troughs).

**Overview of central peaks and troughs: Evidence for shared inputs**

For completeness, we note that nearly half (346 of 707, or 49%) of the detected primary features were central peaks or troughs. Table 5 details the respiratory discharge profiles and locations of each neuron in pairs with central correlogram features. For two groups of neuron pairs – raphé–raphé and VRC–raphé – the ratios of central peaks to troughs were similar (4.4 and 4.9, respectively). For PRG–raphé pairs, the ratio was 41: one central trough was detected among 3,067 pairs (0.03%). Although less than 30% of all neuron pairs were composed of midline neurons, these pairings accounted for 49% of the detected central features.

**Part 2: Extended correlational linkages**

Correlation linkage maps summarized the pairwise connectivities detected among a group of simultaneously recorded neurons and identified evidence of extended relationships, including multi-synaptic “serial” functional connections and convergent and divergent associations. In these maps (e.g., Fig. 4A), each rectangle corresponds to a neuron with the indicated respiratory modulation and identification number. Line color indicates the primary feature of the correlation between the represented neurons; line width reflects the detectability index of the feature.
Evidence for VRC→PRG functional connectivity via intermediary raphé neurons

Overall, offset correlogram features provided evidence for 33 VRC-to-raphé-to-PRG serial connections, 7 of which included an interaction between 2 midline neurons. A correlation linkage map generated from one recording (Fig. 4A) illustrates some of the 35 correlogram features detected in the analysis of 27 PRG, raphé, and VRC neurons. This group was a subset of a larger sample of 116 simultaneously recorded neurons, among which 265 of 6,670 (4.0%) distinct neuron pairs were correlated. The mapped features are consistent with serial chains of paucisynaptic connections directed from the VRC to PRG via intermediary raphé neurons.

The offset correlogram features in Fig. 4B (CCHs 5–7) provide evidence of one such chain linking VRC neuron 805 to PRG cell 515 via the correlation between raphé cells 902 and 988 (805→902→988→515). The last link in this connectivity chain involves the reduction in activity of PRG EI cell 515 following spikes in midline cell 988 (CCH 7). The correlogram for the VRC–PRG neuron pair in the represented chain had no feature, a result consistent with an "asynchronous" or weakly connected chain, as considered further in the DISCUSSION. The linkage map also shows that the first VRC reference neuron in the chain (805) was involved in several other correlations, including those with another midline target cell (906), which had a decreased firing probability, and two PRG cells (507 and 521), with offset peak and trough features, respectively.

The correlation linkage map in Fig. 4A includes two other sets of divergent functional associations involving different VRC cells and raphé and PRG target neurons. Both VRC neurons 839 and 825 are correlated with raphé and PRG targets in opposite ways (see CCHs
8 and 9 in Figure 4C). Several features suggestive of functional convergence are also apparent in the correlation linkage map: offset peaks in cross-correlograms triggered by spikes from numerous cells (e.g., VRC cells 891, 829, 825, 879, and 847; raphé neurons 902 and 984) document time-locked transient increases in the firing probability of midline neuron 988.

Evidence for PRG→VRC functional connectivity through raphé circuits

A correlation linkage map from another animal (Fig. 5A) represents some of the 36 offset correlogram features detected in the analysis of a subset of 21 simultaneously recorded PRG, midline, and VRC neurons. The analysis of the spike trains from all 77 cells in the recording identified 302 correlated pairs out of 2,926 (10.3%). The cross-correlograms in adjacent panels document evidence of functional connectivity linking the PRG to the midline (Fig. 5B), within the raphé (Fig. 5C), and from the midline to the VRC (Fig. 5D). These pairwise correlations delineate at least two overlapping chains of functional connections between PRG cell 513 and VRC neuron 117, one including raphé neuron 909 and the other including raphé cells 909 and 902 (diverging black arrows). Overall, in all recordings, 45 PRG-to-raphé-to-VRC functional connectivity chains were detected, 19 of which included 2 midline neurons.

In addition to the connections noted in the preceding paragraph, the map shown in Fig. 5A includes correlations indicative of convergent functional connections from neighboring raphé cells and/or PRG neurons onto a midline cell (i.e., 914, 912, 909, and 904). Other suggested interactions include VRC neurons with two or more putative raphé inputs (VRC cells 112, 114, 117, and 118). The offset peaks in correlograms for neuron pairs 902–912 (Fig. 5C, CCH 12) and 902–911 (Fig. 5C, CCH 14) are consistent with divergent connections
from neuron 902, as is the central peak in the cross-correlogram for neurons 911 and 912 (not shown).

*Evidence for parallel and overlapping functional pathways between the VRC and PRG*

The correlation linkage map in Fig. 6A includes bidirectional functional connectivity chains with midline neurons interposed between VRC and PRG neurons, as well as linkages for more direct routes that bypass raphé circuits. The represented features were detected in the analysis of a subset of 13 neurons derived from a larger 54-cell recording in which 89 of the 1,431 pairs (6.2%) exhibited short-time scale correlations. Evidence for three parallel VRC-to-PRG links included: (i) the offset trough in the correlogram for neuron pair 810–520 (Fig. 6B) and a pair of serial correlations, both characterized by offset peaks in correlograms between the same VRC trigger cell (810) and (ii) raphé follower neuron 921, and its target PRG neuron 520 (Fig. 6C), and (iii) raphé follower cell 902 and its target PRG neuron 520 (Fig. 6D, left). In addition, a serial link from raphé cell 902 to PRG cell 520 via VRC cell 815 is consistent with detected offset peaks in correlograms 23 and 24 (Fig. 6D, right). The yellow-filled rectangle indicates that neuron 921 (Fig. 6A) also had correlations consistent with participation in a PRG-to-VRC paucisynaptic linkage (correlograms not shown). In all recordings, a total of 12 neurons were associated with functional links suggestive of such an involvement in bidirectional interactions.

*Divergent actions of raphé neurons upon the PRG and VRC*

Offset features consistent with actions of individual raphé neurons on both PRG and VRC target cells were detected for 12 raphé neurons (4.2% of the 285 raphé cells recorded
concurrently with PRG and VRC neurons, 14.3% of the 84 raphé cells with evidence of projection to either region), half of which affected the firing probability of more than one cell in either target area (as many as 3 PRG or 4 VRC target cells). Correlograms for 9 of these raphé reference neurons had offset peaks with both PRG and VRC target cells, features consistent with divergent excitatory influences on the pontomedullary neurons. Relationships in one such trio are documented in the correlograms for neuron pairs 902–520 and 902–815 in Fig. 6D (CCHs 22 and 23). Two raphé reference cells were associated with features of opposite sign in correlograms with PRG and VRC target neurons (e.g., see correlation linkage map for midline neuron 921, Fig. 6A). Correlograms for the remaining raphé neuron suggested an inhibitory influence on a PRG cell with excitatory and inhibitory actions on separate VRC neurons. Seven of these 12 raphé cells were also involved in correlations with features suggesting influences on at least one other raphé neuron. In each case but one, the raphé–raphé cross-correlograms contained an offset peak.

DISCUSSION

The results of this study support a network organization that includes parallel and reciprocal VRC-to-PRG and PRG-to-VRC paucisynaptic linkages operating through intermediary midline circuits. The present work and a recent complementary study (Segers et al. 2008) have the common observation that more of the detected correlations for within-region neuron pairs had central features presumably due to shared inputs, while the majority of features in correlations for between-region neuron pairs were more simply interpreted as evidence for the paucisynaptic influence of one neuron upon the other, with offset peaks
(inferred excitation) more common than offset troughs (inferred inhibition) by a margin of two or three to one.

Simple interpretations of these features suggest a variety of functional relationships between raphé neurons and widely-distributed elements of the pontomedullary respiratory network. Figure 7 provides a graphical summary of suggested connectivity among raphé neurons and various categories of PRG and VRC cells. This “ball-and-stick” diagram enumerates a large set of inferred interactions based on the results obtained from the present study (interpretations of offset correlogram features noted with stars) and previously published work on correlations of respiratory-related raphé neurons (lines marked with green squares; see Fig. 7 legend for references). Correlogram features confirmed in the present study are labeled with a yellow star inside a green square. We note that a complementary diagram with connectivity within the PRG and VRC and between those two regions can be found in Segers et al. (2008).

Detailed inspection of the diagram (Fig. 7) revealed, among others, 42 distinct “indirect” routes (via single midline intermediates) between particular categories of PRG and VRC neuron types, each of which corresponded to a presumably more “direct” parallel pathway inferred in a recent companion study (see Fig. 9 in Segers et al. 2008). In the present data set, we identified 26 PRG→raphé→VRC and 26 VRC→raphé→PRG offset feature correlational linkage chains that included one intermediate raphé neuron. We found, however, that only one of the 52 putative pairs of VRC–PRG “chain-terminating” neurons had an offset correlogram feature. Under the model-based interpretation that offset features reflect synaptic actions between reference and target neurons, this result would suggest that pre-synaptic synchronization effects (Kirkwood and Sears 1991; Kirkwood et al. 1982) and connection...
strengths were not sufficient to generate detectable correlations between indirectly coupled neurons. An alternative but more complex model that would account for the lack of end-of-chain neuron correlations incorporates shared inputs to raphé–VRC and/or raphé–PRG neuron pairs that influence firing probabilities of the target cells with different time lags. The significant difference in the half-widths of offset vs. central correlogram features in this study support the first, simpler model.

We do note that the one set of putative VRC–PRG chain-terminating neurons that was correlated had an offset feature consistent with inhibition (Fig. 6A; CCH 18), while the other branch had serial offset-peak-featured correlational linkages (Fig. 6; CCHs 19 and 20). This arrangement could provide a parallel circuit mechanism to limit the amplitude or duration of changes in firing probability in the common PRG target neuron.

In addition to supporting the role of raphé neurons as relays in VRC–PRG interactions (Bianchi et al. 1995), the present results also extend other prior studies (Lindsey et al. 1994, 1998), demonstrating, to our knowledge for the first time, raphé–PRG correlational linkages and associations of raphé cells with both PRG and VRC neurons. These new results support additional features of a network model, with raphé neurons shaping the motor pattern for breathing through actions upon similar and dissimilar target populations in the PRG as well as in the VRC.

Similar percentages of raphé respiratory-modulated reference neurons had features consistent with projections to the PRG and to the VRC. Populations of PRG neurons apparently received inhibitory and excitatory influences from most respiratory categories of raphé neurons (I, E, IE, and EI); however, not all PRG populations received excitatory functional influences from raphé cells of similar respiratory type. Similarly, midline neurons
in each major category of respiratory modulation had offset correlogram features suggestive of excitatory inputs to ventral respiratory column neurons with similar discharge profiles, as well as inhibitory and excitatory influences to VRC neurons of differing respiratory types. We also note that eight respiratory-modulated raphé neurons had offset correlogram features consistent with divergent functional actions upon both PRG and VRC target cells.

The overall mean detectability index values for offset correlogram peaks and troughs reported here for the midline-PRG pair correlations were 4.6 ± 1.8 and 3.5 ± 0.7, respectively. In comparison, a recent complementary study (Segers et al. 2008) found values in PRG-VRC correlograms of 4.0 ± 1.1 (peaks) and 4.5 ± 2.3 (troughs). The largest mean DI values for offset features were measured in VRC-VRC pair correlograms: peak and trough values were 6.5 ± 5.5 and 5.0 ± 3.3, respectively. Moreover, detectability values for central features in VRC neuron correlograms indicative of strong coupling via shared influences were greater than those found for offset features (Segers et al. 2008). We also note that the mean DI values for offset peaks and troughs in the midline-VRC (4.8 ± 1.9 and 3.8 ± 1.6) and midline-midline (4.5 ± 1.3 and 4.3 ± 1.5) neuron pair correlations in the present study were similar to those previously reported for these types of neuron pairs (e.g., Lindsey et al. 1992a, 1994).

In agreement with previous findings, nonrespiratory-modulated neurons with no preferred phase of maximum activity were found intermingled with the respiratory-modulated cells of the VRC, PRG, and the midline raphé (Bianchi and St. John 1981, 1982; Gang et al. 1990, 1991; Lindsey et al. 1992a; Nuding et al. 2007a; Segers et al. 2008). The recent results of Segers et al. (2008) suggest that medullary peri-columnar and pontine NRM neurons provide modulatory influences to multiple types of respiratory neurons located within both the PRG
and VRC. That study also demonstrated NRM neurons with presumptive excitatory and inhibitory inputs from other cells with diverse patterns of respiratory-modulated firing rates. Other data (Nuding et al. 2003, 2007b) suggest that one function of these neurons involves the transmission and transformation of central and peripheral chemosensory information.

Comparison with anatomical studies

Anatomical evidence for axonal projections between the brain stem regions investigated is consistent with the functional connectivity we describe. Pontine respiratory group neurons are distributed bilaterally in the ventral parabrachial and Kölliker-Fuse nuclei in the dorsolateral rostral pons/mesencephalic region (e.g., Bianchi et al. 1995; Dick et al. 1994), the site of origin for dense projections to medullary raphé nuclei as well as to the ventral respiratory column (Gang et al. 1990; Holstege 1988; Smith et al. 1989). Reciprocal projections between the raphé and PRG (Gang et al. 1990, 1991; Hermann et al. 1997; Holstege 1988), as well as between the VRC and raphé nuclei (Smith et al. 1989; Zagon 1993) have also been reported.

A new perspective on respiratory network architecture

The present results together with the evidence for more “direct” VRC-to-PRG and PRG-to-VRC interactions (Segers et al. 2008) support a model of respiratory network architecture with parallel paths for breathing-related signaling between the pons and medulla (Fig. 8A, B). Such an organization offers multiple sites for the regulation of communications between these rostral and caudal brain stem regions which, given current knowledge (Lindsey et al. 1992a,b,c, 1994; Richerson et al. 2005; Smith et al. 2007), could include raphé circuits that
promote, limit, and balance influences of peripheral and central chemoreceptors (Bernard et al. 1996; Morris et al. 1996a,b; Nattie 2000, 2001; Nattie and Li 2008), baroreceptors (Arata et al. 2000; Li et al. 1999a,b; Lindsey et al. 1998; Morrison and Gebber 1984), and other influences that modulate breathing or reconfigure the respiratory network for engagement in breathing-related behaviors such as cough (Bolser et al. 2003; Shannon et al. 2004).

The new suggested architecture expands the list of potential circuit paths that may be affected by experimental perturbations of the brain stem midline that alter respiratory phase durations. For example, stimulus protocols which alter the rate of breathing (Cao et al. 2006; Holtman et al. 1986; Lalley 1986) could not only act directly through modulatory projections to VRC E-Dec neurons (e.g., CCHs 15-17 in Fig. 5D and CCH 23 in Fig. 6D), which have a key role in the regulation of expiratory duration in current network models (Rybak et al. 2008), but also by removing or changing the gain of intermediary midline neurons in PRG→raphé→VRC circuits that modulate VRC E-Dec neurons (e.g. Fig. 5A), or even by influencing the discharge of pontine IE neurons (e.g., CCH 22 in Fig. 6) that, in turn, exert a functional effect on VRC E-Dec cells (see Fig. 10C of Segers et al. 2008). Future studies should consider these additional possible circuit paths when interpreting the effects of raphé perturbations on the respiratory motor pattern.
ACKNOWLEDGEMENTS

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GRANTS

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REFERENCES


Lindsey BG, Segers LS, Morris KF, Hernandez YM, Saporta S, Shannon R. Distributed actions and dynamic associations in respiratory-related neuronal assemblies of the


FIGURE LEGENDS

Figure 1. Recording sites of neurons evaluated in this study and representative cycle-triggered histograms from a set of simultaneously recorded spike trains. A. Spheres mark coordinates at which signals from single neurons were recorded in the brain stem midline; neurons monitored at the same stereotaxic coordinates are represented by adjacent vertically “stacked” spheres. Spheres are color-coded to indicate the presence (red; \( n = 138 \)) or absence (blue; \( n = 202 \)) of respiratory-modulated impulse activity. B. Spheres mark all AP and RL coordinates at which signals from single neurons were recorded during this study. The stereotaxic coordinates of the raphé recording sites were within 0.2 mm of the midline and extended from the obex to 11.6 mm rostral, and from 0.5 to 6.0 mm below the dorsal surface of the medulla. The recording sites in the pontine respiratory group (PRG; \( n = 145 \)) 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 ventrolateral respiratory column (VRC; \( n = 282 \)) 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. C. Stacks of non-normalized cycle-triggered histograms show average firing rates of the neurons during the respiratory cycle for PRG, raphé (top) and VRC (bottom) cells. 574 cycles averaged. Rate scale does not apply to averaged integrated phrenic multineuron efferent activity (back trace, top).

Figure 2. Functional connectivity between midline and PRG neurons. A and B: Examples of Midline-to-PRG (A) and PRG-to-Midline (B) cross-correlograms with positive time-lag
offset primary features. In CCH 1, the two peaks with lower amplitudes than the central peak reflect the periodicity of the reference raphé trigger spike train (autocorrelation histogram not shown), further supporting the interpretation of raphé-to-PRG excitation (Moore et al. 1970). Circled numbers allow cross-referencing of representative CCHs with linkages shown in the summary figures. The detectability index (DI) is shown in the lower right corner of the CCH. 


C. Cross-correlation feature summary diagram (CFSD) represents the offset peaks (+) or troughs (−) at positive time-lags in correlograms for midline reference neuron-to-PRG target neuron pairs with the indicated respiratory-modulated firing patterns. 

D. PRG reference neuron-to-midline target neuron correlation feature summary diagram.

**Figure 3.** Correlation feature summary diagrams. 

A. Midline reference neuron-to-midline target neuron. 

B. VRC reference neuron-to-midline target neuron. 

C. Midline reference neuron-to-VRC target neuron. VRC E-Dec neurons are further distinguished as phasic (P; zero firing probability during part of the respiratory cycle as assessed in the cycle-triggered histogram average) or tonic (T).

**Figure 4.** Extensive functional linkages in one multi-array recording. 

A. Correlation linkage map shows feature and strength of extended correlation linkages among simultaneously monitored neurons in VRC, PRG, and brain stem midline (see Key). 

B. Cross-correlograms trace a functional connectivity chain from VRC-to-midline-to-midline-to-PRG (heavy black


Figure 6. Parallel functional connectivity pathways in one multi-array recording. A. Correlation linkage map generated from cross-correlograms that suggested three parallel functional connectivity chains from VRC to PRG. B. VRC-to-PRG 18: trough, 14,739 reference and 79,330 target spikes. C. VRC-to-midline 19: peak, 739 reference and 2,476

Figure 7. Detailed functional circuit diagram of detected midline interactions and raphé influences upon the pontomedullary respiratory network. To simplify the diagram, each “output” line from a neuron type divides as necessary to provide input(s) to other cell populations. Divisions close to the neuron type of origin may be marked with a white triangle to make it easier to identify the originating cell type; all other network junctions are labeled with a black circle. These connection lines are not meant to imply, for example, that a single midline I-Dec neuron is functionally connected to nine separate target neurons, but, rather, that there is evidence for connections of neurons of the midline I-Dec respiratory type to nine other types of brain stem neurons (PRG I; VRC NRM, E-Dec-P, and E-Dec-T; and midline I-Other, NRM, E-Dec, and EI neurons as well as other midline I-Dec 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 involving medullary midline neurons inferred from previously published studies that used spike train cross-correlation (Arata et al. 2000; Li et al. 1999a; Lindsey et al. 1992b,c, 1994, 1998; Morris et al. 1996b, 2001). Yellow stars indicate inferred functional connections that are documented for the first time (star alone) or confirmed in this study (star enclosed in green square). Neurons with characteristics of
proposed I-driver cells include neurons with the “inspiratory constant” (I-CON) pattern (Ezure 1990).

**Figure 8.** Overview of raphé–pontomedullary respiratory network interactions. See text in DISCUSSION for details.
Table 1. Summary of results of cross-correlation analysis of 13,843 neuron pairs.

<table>
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<tr>
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<tbody>
<tr>
<td>Significant correlations / Total pairs</td>
<td>124 / 3,067 (4.0%)</td>
<td>292 / 6,755 (4.3%)</td>
<td>291 / 4,021 (7.2%)</td>
</tr>
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<td></td>
<td>Central M→PRG Offset PRG→M Offset</td>
<td>Central M→VRC Offset VRC→M Offset</td>
<td>Central Offset Offset</td>
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<tr>
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Offset features involving PRG–midline and VRC–midline pairs are separated according to the simplest interpretation of their CCHs. Mean DI, half-width, and time-lag from origin (mean ± SD) for all offset features: 4.4 ± 1.6, 25.3 ± 49.5 ms, 28.4 ± 33.9 ms; all offset peaks: 4.6 ± 1.7, 23.4 ± 40.5 ms, 26.7 ± 29.6 ms; all offset troughs: 3.9 ± 1.5, 30.7 ± 69.0 ms, 33.3 ± 43.8 ms. Mean DI and half-width for all central features: 7.3 ± 6.4, 52.5 ± 55.8 ms; all central peaks: 7.6 ± 6.7, 50.4 ± 51.9 ms; all central troughs: 5.7 ± 4.4, 63.6 ± 72.4 ms.
Table 2. PRG-to-midline and midline-to-PRG significant offset feature correlations detected in the analysis of 3,067 midline–PRG neuron pairs.

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Detected peaks and troughs simply interpreted as evidence for a functional connection from the PRG to a midline neuron (left) or from a midline neuron to the PRG (right). Mean DI, half-width, and time-lag from origin (mean ± SD) for **PRG-to-midline offset peaks**: 4.2 ± 0.8, 10.8 ± 13.1 ms, 35.6 ± 30.4 ms; **offset troughs**: 3.4 ± 0.6, 17.2 ± 14.4 ms, 40.0 ± 31.8 ms; for **midline-to-PRG offset peaks**: 4.9 ± 2.2, 21.9 ± 32.6 ms, 37.0 ± 26.6 ms; **offset troughs**: 3.6 ± 0.9, 10.9 ± 7.6 ms, 23.6 ± 24.2 ms. In this and subsequent tables, **P** = Peak and **T** = Trough. The total number of such pairs evaluated for evidence of correlation is shown in italics immediately below the incidences of peaks and troughs detected for that pairing of neuron types.
Table 3. Midline-to-midline significant offset feature correlations detected in the analysis of 4,021 midline–midline neuron pairs.

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<th></th>
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Detected peaks and troughs simply interpreted as evidence for a functional connection from one midline 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 562 pairs composed of an E-Dec and an NRM neuron, an E-Dec $\rightarrow$ NRM connection may be inferred for 10 pairs (1.8%; 6 peaks and 4 troughs) and an NRM $\rightarrow$ E-Dec connection for 3 pairs (0.5%; 3 peaks)). The shaded numbers were summed to calculate the total number of midline–midline pairs analyzed. Mean DI, half-width, and time-lag from origin (mean ± SD) for offset peaks: 4.5 ± 1.3, 21.3 ± 23.9 ms, 21.5 ± 21.0 ms; offset troughs: 4.3 ± 1.5, 51.6 ± 86.4 ms, 20.0 ± 19.1 ms.
Table 4. VRC-to-midline and midline-to-VRC significant offset feature correlations detected in the analysis of 6,755 midline–VRC neuron pairs.

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Detected peaks and troughs simply interpreted as evidence for a functional connection from the VRC to a midline neuron (left) or from a midline neuron to the VRC (right). Mean DI, half-width, and time-lag from origin (mean ± SD) for VRC-to-midline offset peaks: 4.8 ± 2.0, 30.9 ± 66.0 ms, 31.7 ± 44.3 ms; offset troughs: 4.0 ± 1.9, 28.0 ± 80.9 ms, 48.7 ± 66.7 ms; for midline-to-VRC offset peaks: 4.7 ± 1.8, 26.5 ± 39.9 ms, 19.1 ± 18.7 ms; offset troughs: 3.2 ± 0.4, 12.5 ± 13.7 ms, 30.5 ± 27.9 ms.
Table 5. Significant central feature correlations detected in the analysis of 4,021 midline–midline neuron pairs, 3,067 midline–PRG neuron pairs, and 6,755 midline–VRC neuron pairs (top to bottom).

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|        | PRG     |        |        |        |        |        |        |        |        |        |
|        | I-Other | IE     | E-Dec  | E-Aug  | E-Other | EI     | NRM    |        |        |        |
|        | P T     | P T    | P T    | P T    | P T    | P T    | P T    |        |        |        |
| I-Other| 1 -     | 36 -   | 39 -   | 12 -   | 50 -   | 123 -  | 12 -   | 4 -    | 47 -   | 300 -  |
| IE     | - -     | 21 -   | 28 -   | 0 -    | 36 -   | 132 -  | 17 -   | 5 -    | 39 -   | 238 -  |
| E-Dec  | - -     | 1 -    | 2 -    | - -    | - -    | 1 -    | 2 -    | - -    | 3 -    | 4 -    |
| E-Aug  | - -     | 22 -   | 13 -   | 0 -    | 17 -   | 51 -   | 7 -    | 3 -    | 21 -   | 114 -  |
| E-Other| - -     | - -    | - -    | - -    | - -    | - -    | - -    | - -    | - -    | - -    |
| EI     | - -     | 14 -   | 21 -   | 0 -    | 23 -   | 40 -   | 4 -    | 0 -    | 28 -   | 86 -   |
| NRM    | 48 -    | 61 -   | 33 -   | 41 -   | 162 -  | 45 -   | 26 -   | 69 -   | 994 -  |

|        | V-C     |        |        |        |        |        |        |        |        |        |
|        | I-Other | EI     | E-Dec  | E-Aug  | E-Other | EI     | NRM    |        |        |        |
|        | P T     | P T    | P T    | P T    | P T    | P T    | P T    |        |        |        |
| I-Other| - -     | 24 -   | 6 -    | 3 -    | 2 -    | 13 -   | 4 -    | 5 -    | 10 -   | 140 -  |
| EI     | - -     | 29 -   | 23 -   | 4 -    | 51 -   | 102 -  | 16 -   | 28 -   | 34 -   | 585 -  |
| E-Dec  | 1 -     | 1 -    | 1 -    | 1 -    | 1 -    | 6 -    | 2 -    | 3 -    | 4 -    | 2 -    |
| E-Aug  | 41 -    | 96 -   | 8 -    | 102 -  | 238 -  | 10 -   | 30 -   | 88 -   | 659 -  |
Detected peaks and troughs simply interpreted as evidence for the influence of a shared input upon the activity of both neurons of a pair. Midline–midline neuron pairs (top): Mean DI and half-width of the features (mean ± SD) for central peaks: 8.9 ± 8.9, 52.2 ± 52.5 ms; central troughs: 6.7 ± 5.5, 83.4 ± 83.7 ms. PRG–midline pairs (middle): Mean DI and half-width of the features (mean ± SD) for central peaks: 6.0 ± 2.6, 28.2 ± 25.0 ms; central trough (n=1): 6.1, 60 ms. VRC–midline pairs (bottom): Mean DI and half-width of the features (mean ± SD) for central peaks: 6.7 ± 3.9, 56.2 ± 56.4 ms; central troughs: 4.3 ± 1.4, 37.1 ± 45.2 ms.
Sagittal view: Midline recording sites

Recording sites

A / P

Caudal
Rostral

level of obex

Simultaneously recorded spike trains

Cycle-triggered histograms

PRG & Midline

VRC

Non-Respiratory Modulated neurons

Respiratory Modulated neurons
A
Midline-to-PRG

Midline-to-PRG offset correlation features

B
PRG-to-Midline

PRG-to-Midline offset correlation features

C
Midline-to-PRG offset correlation features

D
PRG-to-Midline offset correlation features
Divergent functional connections
Brainstem Midline

OFFSET FEATURES
peak
trough

PRG → VRC

B PRG-to-Midline

0.8 spikes s⁻¹

NRM → E-Dec

10

DI = 5.2

- 775 775 ms

Scaled up 2.44x

14.4 spikes s⁻¹

E → I-Dec

11

DI = 4.2

- 775 775 ms

Midline-to-Midline

5.0 spikes s⁻¹

NRM → I-Dec

12

DI = 4.0

- 775 775 ms

Midline-to-VRC

1.0 spikes s⁻¹

Scaled up 2.45x

I-Dec → E-Dec-T

15

DI = 3.0

- 775 775 ms

2.7 spikes s⁻¹

NRM → E-Dec-T

16

DI = 6.0

- 625 625 ms

3.7 spikes s⁻¹

NRM → E-Dec-T

17

DI = 6.7

- 625 625 ms
Functional Connectivity Key

- Excitatory synapse: Blue
- Inhibitory synapse: Red
- Junction closest to source cell: Triangle
- Junction closest to target cell: Diamond
- Inferred from previous studies: Green
- Current study: Yellow
- Correlation feature confirmed by current study: Orange
- Proposed presynaptic modulation: Purple

Figure 7 data: SN_3

Link to data used to construct this figure:

I-Driver

Proposed presynaptic modulation

Excitatory synapse

Inhibitory synapse

Midline

Lungs

Diaphragm

Exp. Muscles