Subtype Composition and Responses of Respiratory Neurons in the Pre-Bötzinger Region to Pulmonary Afferent Inputs in Dogs

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Krolo, M., V. Tonkovic-Capin, A. G. Stucke, E. A. Stuth, F. A. Hopp, C. Dean, and E. J. Zuperku. Subtype composition and responses of respiratory neurons in the pre-Bötzinger region to pulmonary afferent inputs in dogs. J Neurophysiol 93: 2674–2687, 2005. First published December 15, 2004; doi:10.1152/jn.01206.2003. The brain stem pre-Bötzinger complex (pre-BC) plays an important role in respiratory rhythm generation. However, it is not clear what function each subpopulation of neurons in the pre-BC serves. The purpose of the present studies was to identify neuronal subpopulations of the canine pre-BC and to characterize the neuronal responses of subpopulations to experimentally imposed changes in inspiratory (I) and expiratory (E) phase durations. Lung inflations and electrical stimulation of the cervical vagus nerve were used to produce changes in respiratory phase timing via the Hering-Breuer reflex. Multibarrel micropipettes were used to record neuronal activity and for pressure microejection in decerebrate, paralyzed, ventilated dogs. The pre-BC region was functionally identified by eliciting tachypneic phrenic unit activity in response to ipsilateral inputs. This region was shown to consist of heterogeneous subpopulations of interneurons and motoneurons that are excited or inhibited by ipsilateral inputs but excited by contralateral inputs. The results suggest that only a limited number of neuron subpopulations may be involved in rhythmogenesis, whereas many neuron types may be involved in motor pattern generation.

INTRODUCTION

The role of the pre-Bötzinger Complex (pre-BC) as the possible site for respiratory rhythm generation has been the focus of many studies since its anatomical location was delineated (Gray et al. 1999; Schwarzacher et al. 1995; Smith et al. 1991). In vitro studies show that this site contains a group of inspiratory (I) interneurons with intrinsic bursting properties (Butera et al. 1999a; Johnson et al. 1994; Lieske et al. 2000). Excitatory synaptic connections among these neurons suggest that these neurons constitute the core of a bilaterally distributed pacemaker network that is the origin of respiratory rhythm generation demonstrated in vitro (Butera et al. 1999b; Koshiya and Smith 1999). In in vivo models, microinjections of D,L-homocysteic acid (DLH) into pre-BC produce a marked tachypnea characterized by a decrease in both inspiratory and expiratory durations (TI and TE) (Chitravanshi and Sapru 1999; Monnier et al. 2003; Solomon et al. 1999; Wang et al. 2002). Selective unilateral ablation of neurokinin-1 receptor-expressing (NK1R) neurons in the pre-BC abolishes the tachypneic response to DLH on the ablated side. In awake adult rats, selective and nearly complete bilateral destruction of pre-BC NK1R neurons results in an ataxic breathing pattern with hypercapnic and hypoxic blood gases and inadequate or paradoxical responses to challenges such as hyperoxia, hypoxia, and anesthesia (Gray et al. 2001). Accordingly, it appears that the pre-BC plays a key role in respiratory rhythm generation.

Several models have been proposed to explain the mechanisms underlying rhythm generation. These include neural networks, (Balis et al. 1994; Duffin et al. 1995; Ogilvie et al. 1992; Richter et al. 1986; Rybak et al. 1997; Zuperku et al. 1982b), interconnected pacemaker neurons (Butera et al. 1999b), a hybrid-pacemaker-network system (Feldman et al. 1990; Smith et al. 2000), and recently a maturational network-burst model (Richter and Spyer 2001). However, new findings appear to question some of the basic tenets of these models (Feldman et al. 2003), and the issue of the neural substrates that underlie rhythmogenesis remains unresolved.

In the mature mammal, respiratory-related neurons with several types of discharge patterns can be recorded in the pre-BC in vivo. These include neurons that discharge during the I phase, the E phase, and across the two phases (Connelly et al. 1992; McRimmon et al. 2001; Schwarzacher et al. 1995; Sun et al. 1998). The neuron types can be classified further according to the time course of their patterns, for example augmenting, decrementing, and E-I and I-E phase spanning. The various models have attempted to assign a role in rhythm generation to each neuron type, but these roles have yet to be verified.

The response characteristics of pre-BC neurons to systematic changes in phase timing produced by pulmonary afferent inputs have received limited attention (Hayashi et al. 1996), although reflexly induced changes in phase timing and neuronal responses to peripheral chemoreceptor (Morris et al. 1996), baroreceptor (Lindsey et al. 1998), and airway defense receptor (Shannon et al. 2000) stimulation have been studied. The discharge patterns of those neurons that play a key role in generation and control of phase timing should exhibit some

Address for reprint requests and other correspondence: E. J. Zuperku, Research Service/151, Zablocki VA Medical Center, Milwaukee, WI 53295 (E-mail: ezuperku@mcw.edu). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
aspect of their pattern that is consistently related to TI or TE. This relationship can be revealed when these phase durations are systematically altered. Only neurons the patterns of which tightly correlate with changes in phase timing are likely to play a primary role in rhythmogenesis. The objectives of the present studies were to characterize the types of neurons, their relative frequency of occurrence, their axon projections, and their responses to changes in phase timing produced by pulmonary afferent inputs in an in vivo canine model.

METHODS

This research was approved by the Subcommittee on Animal Studies of the Zablocki VA Medical Center, Milwaukee, WI, in accordance with provisions of the Animal Welfare Act, the PHS Guide for the Care and Use of Laboratory Animals, and Veterans Affairs policy. Data were obtained from mongrel dogs of either sex weighing from 8 to 15 kg. Mask induction with a volatile anesthetic (isoflurane or halothane) was used, and anesthesia was maintained during the surgical procedure with isoflurane (1.4–2.0% end-tidal concentration). Airway concentrations of isoflurane, CO2, and O2 were continuously monitored with an infrared analyzer (POET II, Criticare Systems, Waukesha, WI). The animals were monitored for signs of inadequate anesthesia (e.g., salivation, lacrimation, and/or increases in blood pressure and heart rate), and if required, the depth of anesthesia was increased immediately.

Surgical procedure

Dogs were intubated with a cuffed endotracheal tube and mechanically ventilated with an air-O2-isoflurane mixture. The surgical procedures, monitoring, and maintenance of body homeostasis have been previously described in detail elsewhere (Dogas et al. 1998). Briefly, after cannulating the femoral artery (for blood pressure recording and blood-gas sampling) and vein (for continuous infusion of maintenance fluids and drugs), a bilateral pneumothorax was performed to reduce motion artifacts. The animals were placed in a Kopf (model 1530) stereotaxic apparatus and then decerebrated (Tonkovic-Capin et al. 2002). This procedure leads to an anatomically well-defined, midcol- sterilization positive. A primary role in rhythmogenesis. The objectives of the present studies were to characterize the types of neurons, their relative frequency of occurrence, their axon projections, and their responses to changes in phase timing produced by pulmonary afferent inputs in an in vivo canine model.

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Phrenic nerve activity was recorded from the central end of the desheathed right C3 rootlet. The phrenic neurogram (PNG) was obtained from the moving-time average (100 ms) of the amplified phrenic nerve activity and was used to produce timing pulses corresponding to the beginning and end of the inspiratory phase. The spike-triggered averaging technique was used to test for the presence of laryngeal motoneurons within the region of the pre-BC. The superior and inferior laryngeal nerves were carefully isolated, and the central ends were desheathed and prepared for recording of efferent activity. The laryngeal nerves were placed on bipolar platinum hook electrodes and submerged in warm mineral oil. Spike-triggered averages of amplified efferent nerve activity (0.2- to 3-KHz, bandwidth) were obtained using a Nicolet NIC-370 digital signal averager triggered by the unit activity of single neurons (e.g., Fig. 1A).

To test for neuronal axon projections to the spinal cord, a laminectomy was performed to expose the dorsal surface of the spinal cord at the C3 level. An array of four metal microelectrodes, two per side, was lowered bilaterally into the ventrolateral part of the spinal cord. Optimal electrode placement within the descending bulbospinal tracts was confirmed by the presence of electrically evoked phrenic nerve potentials during the E phase. In addition, further confirmation was obtained from the collision of orthodromic and antidromic unit activity from caudal VRG bulbospinal neurons (e.g., Fig. 1B).

Multi-barrel micropipettes, (10–30 μm composite tip diameter), consisting of one recording barrel containing a carbon filament and drug barrels, were used for extracellular neuronal recordings and pressure ejections. The ejected volumes were measured via changes in meniscus height with a ×100 microscope equipped with a reticle. The location of each recorded neuron was registered in coordinates relative to the obex (rostral), midline (lateral), and dorsal surface at point of micropipette entry (depth). Micropipette penetrations were started at a predetermined position based on maximum tachypneic responses to DLH injections that had been obtained in dogs of similar size in a preliminary study (e.g., Fig. 2). Because of potential toxic effects of DLH, due to relative large injection volumes (~20 nl) and concentration (20 mM) required to produce such responses, confirmation of the tachypneic response was only done after all neuronal recordings were completed. With respect to the initial position, penetration locations for the current study of single neurons were
incremented in a grid-like manner using 250-μm steps in the rostral-caudal and medial-lateral directions. After all neuronal recordings were completed, a DLH injection was made near the center of the region from which the recordings were made. This was then followed by picoejection of Pontamine Sky Blue dye (1% solution, 50–100 nl) to mark the location. After the animals were killed, each medulla was removed and placed in 4% paraformaldehyde for 24 h before being frozen (−70°C). Frozen transverse sections (30 μm) were cut, stained with neutral red, and cover slipped. Sections were examined light microscopically to identify the dye marker, and subsequent photomicrographs were made to establish the coordinates relative to obex (e.g., Fig. 3).

Single-cell neuronal activity, phrenic, superior and inferior laryngeal nerve activities, airway pressure and CO₂ concentration, vagal afferent stimulus frequency patterns, and systemic blood pressure were recorded on a digital tape system (model 3000A; A.R. Vetter, Rebersburg, PA). These variables or their time averages were also continuously displayed on a computerized chart recorder (Powerlab/16SP, ADInstruments, Castle Hill, Australia). The tape-recorded data were digitized and analyzed off-line. A time-amplitude window discriminator was used to generate a standardized pulse for each action potential of given neuron. Cycle-triggered histograms (CTHs; 50-ms bins), triggered by I or E timing signals derived from the PNG, were used to quantify the discharge frequency patterns of the recorded pre-BC neurons. Visual inspection of CTH patterns was used to determine neuron subtype. Discharge frequency $F_n$ was calculated as the average number of spikes/bin/bin duration.

Statistical analyses

Neuron location coordinate values were compared with each other with a one-way ANOVA procedure (StatView, SAS Institute, Cary, NC). The $χ^2$ test was used to test whether the observed frequency of a given neuron subtype was different from expected, based on the hypothesis that all neuron subtypes are equally likely to be observed. The $χ^2$ test was also used to test whether there was a preferred response direction, assuming that all three outcomes, the same, opposite, or different, were equally likely to be observed.

![FIG. 2. Phrenic neurogram demonstrates tachypneic responses produced by D,L-homocysteic acid (DLH) microejections (60 nL, 20 mM) in the pre-BC region. PNG, phrenic neurogram; $T_i$, inspiratory duration; $T_e$, expiratory duration. The stereotaxic coordinates for micropipette tip are indicated above PNG traces. Top: response to DLH at a depth of 5.7 mm from the dorsal surface showed a pronounced tachypnea with marked decreases in $T_i$ and $T_e$. Bottom: response to DLH produced at a depth of 6.7 mm from dorsal surface (same electrode tract) showed an increase in $T_i$, a decrease in $T_e$, and changes in peak PNG.](http://jn.physiology.org/)

![FIG. 3. Anatomical location of the region from which tachypneic responses to DLH injections were obtained, which is presumed to overlap the pre-BC (PNG response at bottom). The region was marked with blue dye (80 nl, 1%) as indicated by the circles. The indicated anatomic structures were identified on neutral red stained transverse sections of the medulla at the levels indicated rostral to obex. In this preparation, the region of the tachypneic response was located in the ventrolateral medulla caudal to the rostral pole of the inferior olivary nucleus (IO) and overlapping the retrofacial nucleus (RFN). In other dogs, tachypneic responses were also obtained at the level slightly rostral to the rostral pole of the IO. LVN, lateral vestibular nucleus; MVN, medial vestibular nucleus; 5ST, spinal thalamic tract; 5SN, spinal trigeminal nucleus; PYR, pyramidal tract.](http://jn.physiology.org/)

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posite, or no response to vagal stimulation, were equally probable. Differences were considered significant for \( P < 0.05 \). The Fisher’s protected least significant difference (PLSD) was used for multiple comparisons. Values are expressed as means ± SE unless indicated otherwise.

**Protocol 1**

The purpose of protocol 1 was to determine the types and frequency of occurrence of neurons within the pre-BC, based on their spontaneous discharge patterns, and the number of neurons that are cranial motor- or bulbo-spinal neurons. This study was carried out in a group of 15 dogs that were vagotomized to eliminate changes in discharge patterns and phase timing due to slowly adapting pulmonary stretch receptor (PSR) inputs. Both spike-triggered averaging and spinal antidromic activation procedures were performed for each recorded neuron. Those neurons, which gave negative spike-triggered averaging and antidromic activation results, were assumed to be propriobulbar. The coordinates for the location of the pre-BC region were verified at the end of the recording session by the presence of the tachyphoeic response produced by DLH picoejection.

**Protocol 2**

The purpose of protocol 2 was to determine the response characteristics of pre-BC neurons to changes in phase timing produced by PSRs. A second group of 31 dogs were used for this purpose. In some preparations, a programmable ventilator synchronized by the PNG was used to produce various inflation patterns during either the I or E phase of test cycles. These were separated by several control cycles to prevent swings in \( \text{PaCO}_2 \) and blood pressure. In most preparations, a programmable stimulation system (Hopp et al. 1983) was used to deliver pulse train stimuli to the ipsilateral, contralateral and both vagus nerves, throughout the I or E phase. Stimulus strength (20-μs pulse duration, 100–150 μA) was adjusted to mimic the Hering-Breuer reflexes, that is, I-phase stimuli shorten the I phase without altering the PNG time course, and E-phase stimuli prolong the E phase. Stimulus parameters were adjusted so that the magnitude of the reflex change in phase timing was the same for both vagus nerves. In some cases, a perfect match could not be completely achieved. Figure 4 shows a typical stimulus protocol for a late E neuron (\( F_n \)). The test stimulus patterns (traces 3 and 4) were separated by baseline cycles with a ramp frequency input during the I phase, which shortened \( T_r \), and a low-level tonic (5 Hz) input during the E phase. Inserting such baseline input patterns between test and no-stimulus control cycles mimics baseline conditions in preparations with intact vagus nerves, where test lung-inflation and no-inflation cycles are used to alter baseline PSR inputs. These baseline patterns condition the breathing pattern, producing more normal breathing rates, and produce memory effects that can carry over into subsequent breath cycles (Clark and von Euler 1972; Zuperku and Hopp 1985). However, in this protocol, both no-stimulus control cycles and test cycles are preceded by comparable amounts of conditioning, thus allowing comparisons under similar conditions. The test sequence included no vagal input control cycles, contralateral, ipsilateral, and bilateral step-frequency vagal input patterns during the E phase. This sequence was repeated three to five times for each step frequency and used for the CTHs. Paired comparisons between test and control CTH patterns were based on an equal number of cycles for both conditions. Step frequencies of 10, 20, 40, 80, 120, and 160 Hz were used with a quasi-random order.

![Protocol for Electrically-Induced PSR inputs](image_url)
RESULTS

Neuronal subtype composition

The discharge patterns of 211 respiratory related neurons in the region of the pre-BC were classified into 13 categories. The I neurons were classified into 6 subtypes: I decrementing (dec), pre-Idec, pre-Iaug, Iaug, I parabolic (p), and late-I neurons as shown by the examples in Figs. 5 and 8. The E neurons were classified into 5 subtypes: Edec, Late-E, Eaug, Eparabolic (e.g., Fig. 6), and pre-Edec, which begins to discharge late in the I phase (e.g., Fig. 15, left). Five neurons were E bulbospinal neurons within the region of the Bötzingen Complex as indicated by their more rostral and lateral location relative to all other neuron types (e.g., Fig. 6, bottom). These neurons were antidromically activated from the spinal cord and yielded positive collisions. Two I neurons with augmenting patterns (Iaug) neurons were identified as superior laryngeal motoneurons, and one Iaug neuron was identified as an inferior laryngeal motoneuron. The remaining 203 neurons (96%) were designated as propriobulbar. Approximately 56% (115/206) of the pre-BC region neurons were I neurons and 43% (89/206) were E neurons (Fig. 7). Two neurons without spontaneous activity were excited only by lung inflation and designated as pump neurons. The pre-I neurons were the most numerous making up 37% of the I neurons and ~20% (42/206) of all I and E neurons. Based on discharge pattern, the pre-I neurons were divided into pre-Idec and pre-Iaug neurons, where pre-Iaug made up ~75% of the pre-I neurons. The Edec and late-E neurons were the most numerous making up 55% (49/89) of the E neurons.

In this study stereotaxic coordinates relative to the obex, midline, and dorsal medullary surface were used to register the location of each neuron. With the dog’s head ventrally flexed 38° from the horizontal plane, the medulla was tilted an average of 17° from the horizontal plane with the rostral aspect lower than the caudal aspect. The tilt angle was trigonometrically estimated using the stereotaxic coordinates and measurements from the dye injection marks in the transverse sections of the medulla. The stereotaxic and histological data were in agreement for the lateral measurements. The axis of the micropipette was perpendicular to the horizontal plane. Using this information, the stereotaxic rostrocaudal and depth coordinates were corrected giving the average (SD) neuronal location for presumed pre-BC neurons as 5.55 ± 0.60 mm rostral, 4.55 ± 0.44 mm lateral, and 5.59 ± 0.88 mm ventral to the dorsal surface. The coordinates of the various neuron subtypes were

FIG. 5. Examples of inspiratory neuron subtypes found in the pre-BC region. In each set, top: PNG; middle: rate meter output for discharge frequency (Fn: number of spikes/0.1 s ×10); bottom: neuronal activity (NA). dec: decrementing; aug: augmenting. - - -: onset of phrenic nerve activity.

FIG. 6. Examples of expiratory neuron subtypes found in the pre-BC region. In each set, top: PNG; middle: rate meter output for discharge frequency (Fn: number of spikes/0.1 s ×10); bottom: NA. - - -: time of inspiratory off switch or start of the expiratory phase as indicated by the rapid decline in the PNG.
similar. The five bulbospinal Bötzinger E neurons were located significantly more rostral and lateral to the pre-BC region neurons, with average (±SD) rostral, lateral, and depth coordinates of 6.49 ± 0.70, 5.35 ± 0.28, and 6.10 ± 0.52 mm, respectively (Fig. 7).

Neuronal responses to vagal afferent input

The responses of 42 I and 37 E neurons to reflex changes in either T₁ or Tₑ produced by electrically induced PSR inputs or by lung inflation (14 I and 8 E) were analyzed. Three qualitatively different types of response were observed when the CTHs associated with the step frequency inputs were compared with those of the no-input control cycles: excitation, inhibition, and no response. In terms of this study, excitation is defined as a decrease in discharge frequency, which may be due to synaptic excitation or to disinhibition, whereas inhibition is an increase in discharge frequency, which may be due to synaptic inhibition or disinhibition. In all cases, the afferent input during the I phase shortened T₁, while those during the E phase prolonged Tₑ.

I neurons

The decrementing slope of Idec neurons increased with the decrease of T₁. This type of inhibition was also seen in the pre-Idec neurons (Fig. 8). Because the pre-Idec and Idec neurons responded similarly, they were pooled for analysis. All 10 neurons belonging to these two subtypes exclusively exhibited inhibitory responses to all three afferent inputs (Fig. 9, top). The inverse relationship between T₁ and the decrementing slope of Idec and pre-Idec neurons, which leads to the gradual decrease in their discharge frequency and hence a decrease in their presumed inhibitory effect on E neurons, is consistent with their possible role in the control of the I phase duration by promoting or enabling I offswitching.

Unlike the responses of the preIdec neurons, the responses of pre-laug neurons to the three inputs were not consistent. Ipsilateral inputs were mainly inhibitory, whereas contra- and bilateral inputs produced either an inhibitory or no response (e.g., pooled data of Fig. 9). The responses to the contralateral inputs were in the same direction as those to ipsilateral inputs in ~56% of the cases, whereas the response direction of ~86% of the prelaug neurons to the bilateral inputs was the same as the ipsilateral response direction (Fig. 10).

Iaug neurons were either inhibited (~50%) or did not respond (~50%) to either the ipsi- or contralateral inputs (Figs. 9; e.g., and 11). Most Iparabolic neurons did not respond to any of the afferent inputs (Fig. 9). In the only late-I neuron with a complete protocol, all three inputs produced excitation (Fig. 11). Two of four late-I neurons were excited by contralateral inputs. Bilateral inputs inhibited one of three, had no effect on one of three, and excited one of three late-I neurons (Fig. 9).

E neurons

The decrementing slope of the Edec neurons decreased with the increase in Tₑ (e.g., Fig. 12). All Edec neurons studied responded in the same way to all three inputs (Fig. 13) with the exception of one in which the contralateral input had no affect on the time course of the discharge pattern. In the latter case, the neuron continued to discharge at the level of activity that was present at the end of the control cycle. With the PSR-induced increase in Tₑ, the slope of the Eaug neurons decreased, but their resulting peak frequency was equal to or greater than the peak Fₑ of control cycles with the contralateral inputs (e.g., Fig. 12, Eaug). In all cases, ipsilateral inputs produced inhibition, whereas bilateral inputs were excitatory in most cases (Fig. 13). The responses of the Eparabolic neurons were inconsistent; however there was a tendency for these neurons to be excited by the contralateral inputs (Figs. 12 and 13).
The responses of 25 late-onset E (Late-E) neurons were analyzed. The response patterns to the three inputs were fairly consistent: ipsilateral inputs were mainly inhibitory while contralateral inputs were mainly excitatory (Fig. 13, Late-E). The response direction to the bilateral inputs was the same as for ipsilateral inputs (Fig. 10, late-E). In three late-E neurons where lung inflation was used during the E phase, inhibition was produced in two neurons (e.g., Fig. 15, late-E) and excitation in the other. The PSR-induced increase in $T_E$ also resulted in a decrease of the slopes of the discharge patterns (e.g., Fig. 14). Only two pre-Edec neurons were found: one was excited by lung inflation (Fig. 15) and the other was not affected and continued to discharge throughout the E phase at the same level as that at the end of the control pattern.

**DISCUSSION**

While a variety of respiratory neuron subtypes can be found in the pre-BC region of dogs, the most frequently encountered I neurons were the pre-Iaug, Iaug, and Iparabolic neurons, which made up 27, 26, and 22% of all I neurons, respectively. The most frequently encountered E neurons were the late-E, Edec, and Eparabolic neurons, which made up 29, 26, and 21% of all E neurons, respectively.

The changes in the discharge patterns of the Idec and Edec neurons were unresponsive. Late-E neurons, which made up the largest number of E neurons studied with PSR inputs, were mainly inhibited by ipsilateral PSR inputs; however, they were excited by contralateral inputs (Fig. 13). In 90% of the cases, their responses to bilateral inputs were in the same direction as those to the ipsilateral inputs (Fig. 10). The responses of the Eparabolic neurons were not consistently related to the three inputs, although contralateral inputs produced small amounts of excitation in their discharge patterns.

**Location of pre-BC in dogs**

Our main criterion for locating the pre-BC in dogs was the tachypneic response produced by localized microinjections of the excitatory amino acid DLH. This criterion is based on similar responses produced by DLH injections in the pre-BC of other species such as rats and cats (Chitravanshi and Sapru 1999; McCrimmon et al. 2000; Monnier et al. 2003; Solomon 2002; Solomon et al. 1999; Wang et al. 2002). DLH microinjections ~0.5 mm rostral or caudal to the center of the pre-BC produce bradypnea, whereas those approximately 0.5 mm more dorsal or ventral or ~0.5 mm more medial or lateral are without effect (Monnier et al. 2003). Based on preliminary DLH mapping studies in our dog model, the “tachypneic” region appears to be comprised of a 1- to 2.5-mm section of the ventral respiratory neuron column, located from ~1–1.5 mm caudal to 1–1.5 mm rostral to the rostral pole of the inferior olive (~4.2–6.5 mm rostral to obex), and includes the ventral portion of the retrofacial nucleus, especially at the more rostral extent of this response region (e.g., Fig. 3).

In dogs with weights similar to the ones in our study (7–11 kg), Fukuda and Koga (1997b) defined the pre-BC on the basis of the location of the pre-I neurons, which made up only 8.6%
(14/163) of the total neurons they recorded from in the region of pre-BC. They estimated that the pre-BC extended from 4.2 to 5.2 mm rostral to obex. However, they also showed that the more numerous Idec neurons (35.6% or 58/163) were distributed from 3.5 to 5.5 mm rostral to obex. Thus it is possible that the pre-BC extends over a greater rostral-caudal extent, including the region from which we evoke DLH-induced tachypneic responses. In other studies by the same investigators (Fukada and Koga 1997a; Koga et al. 1998), they suggest that the Bo¨tzinger complex extends from 6 to 7 mm rostral to obex. Because the average rostral coordinate for our neuronal study was 5.5 mm rostral, our recordings are likely to have been from the rostral portion of the pre-BC. It should also be noted that dog size can influence the location and extent of intramedullary nuclei. For example, Wallach et al. (1983), using 15–25 kg dogs, showed the rostral pole of the inferior olivary nucleus at >7.0 mm rostral to the obex. The subtype composition of our recorded neurons and their propriobulbar nature are consistent with the phenotype of pre-BC neurons or at least, neurons located in a region that contains neuronal circuitry capable of controlling I and E phase timing.

Neuronal axon projections

Other studies have shown that the pre-BC consists mainly of propriobulbar neurons (Dobbins and Feldman 1994; Ellenberger and Feldman 1990). Our results are in agreement with these findings. Only 5 of 211 neurons were found to be bulbspinal neurons. These neurons were located more rostral and lateral to the region that produced tachypnea after DLH injections. Three other neurons were found to be laryngeal motoneurons. Most neurons (203 of 216) could neither be antidromically activated from the spinal cord nor did they register responses with spike-triggered averaging of the superior and inferior laryngeal nerve activities. We conclude that most of these neurons are propriobulbar, although some may have been cranial motoneurons with axons in the pharyngeal branch of the vagus nerve (Bianchi et al. 1995) or possibly within the glossopharyngeal nerve. The pharyngeal branch of the vagus nerve is inaccessible in the dog and could not be isolated. We also did not record from the glossopharyngeal nerve as it only contains very few motoneurons that innervate the stylopharyngeus muscle deep within the pharynx (Martin 1996).

Neuronal discharge patterns

This study found that the canine pre-BC region contains a wide variety of I, E, and phase spanning neurons with discharge patterns similar to those previously reported by others in rats (McCrimmon et al. 2001; Sun et al. 1998), cats (Connelly et al. 1992; Schwarzacher et al. 1995) and dogs (Fukuda and Koga 1997b).

Pre-I neurons

The pre-I neurons were the most numerous (42 of 115 = 37%) of the recorded I neurons. The onset of activity of the pre-I neurons preceded the onset of phrenic activity by >100 ms, and they continued to discharge throughout the I phase. About 74% had augmenting patterns while the remaining 26%

![Fig. 9. Pooled response data to ipsi-, contra-, and bilateral vagal afferent inputs for each subtype of inspiratory neuron. Three types of responses are shown: inhibitory, no effect, and excitatory for each input. Data are normalized with respect to the total number of neurons within each of the input categories. Numbers in parentheses: total number of neurons studied.](image_url)
had decrementing patterns. The discharge patterns of preI neurons with a decrementing pattern are somewhat similar to the type I neurons reported in other species (Connelly et al. 1992; Guyenet and Wang 2001; McCrimmon et al. 2001; Schwarzacher et al. 1995; Sun et al. 1998) as well as in dogs (Fukuda and Koga 1997b). We did not encounter any biphasic-

**FIG. 11.** Examples of the responses of an I augmenting and late-onset I neurons to contra-, ipsi-, and bilateral vagal pulmonary afferent inputs. Horizontal bar: duration of stimulus; thick line cycle-triggered histograms (CTHs) and PNG are for the nonstimulated control (CON) cycles; thin line CTHs are for the stimulated test cycles. PNG response to the bilateral test stimulus is shown. Vertical dashed line indicates the onset of the PNG. CTH: 50-ms bins.

**FIG. 12.** Examples of the responses of an E-decrementing (Edec), E-augmenting (Eaug), and E-parabolic neurons to contra-, ipsi-, and bilateral vagal pulmonary afferent inputs. Horizontal bar: duration of stimulus; thick line cycle-triggered histograms (CTHs) and PNG are for the nonstimulated control (CON) cycles; thin line CTHs are for the stimulated test cycles. Partial PNG response (bottom trace) to the bilateral test stimulus is shown for timing purposes. Vertical dashed line indicates the onset of the E phase. CTH: 50-ms bins.
was a sufficient number of these neurons \((n > 0.05)\). This analysis was only possible for the Late-E neurons because there were some differences between our study results and those of the latter study. The site that resulted in a maximum tachypneic response to DLH microinjections defined the coordinates used for this study.

**Comparisons of neuron subtype composition among species**

The results of this study suggest that differences exist among dogs, cats, rats, and mice in the proportion of neuron subtypes that comprise the pre-BC. Not all subtypes have been reported for each species, but some of the more common subtypes have been reported for all four species (Chitravanshi and Sapru 1999; Connelly et al. 1992; Fukuda and Koga 1997b; Guyenet and Wang 2001; Hayashi et al. 1996; McCremon et al. 2001; Paton 1996; Schwarzacher et al. 1995; Sun et al. 1998). The frequency distribution is: rat \(>\) dog \(>\) mouse \(>\) cat for pre-I neurons, cat \(\approx\) dog \(\approx\) rat for Iaug neurons, dog \(>\) cat \(>\) rat for Idec neurons, dog \(\approx\) cat \(\approx\) mouse for late-I neurons, rat \(>\) mouse \(>\) cat \(\approx\) dog for Edec neurons, rat \(>\) cat \(\approx\) dog for pre-E neurons, mouse \(>\) dog \(>\) cat \(>\) rat for Eaug/Parabolic neurons, and cat \(\approx\) dog for late-E neurons. A notable difference was rat \(\gg\) cat for pre-I neurons. In rats, \(\approx\) 37\% of neurons were Edec and no Eaug neurons were found.

There were some differences in the subtype composition between our study and another canine study where a substantial number of pre-BC neurons \((n = 183)\) were recorded (Fukuda and Koga 1997b). We found more preI neurons \((37 vs. 10\%)\) but few Idec neurons \((4 vs. 34\%)\). We also found more E neurons of all subtypes \((44 vs. 18\%)\). These differences may be due to our sampling site being \(\approx 0.6\) mm more rostral than that of the other canine study.

**Direct and indirect effects of vagal afferent inputs**

Electrically evoked pulmonary vagal input patterns elicit highly reproducible changes in phase timing that mimic those of the Hering-Breuer reflexes (Feldman and Gautier 1979) and have unique advantages (D'angelo 1985; Trenchard 1977; Zuperku et al. 1982a). Because the vagi are transected, ventilation remains constant despite the induced changes in central respiratory phase timing, preventing oscillations in blood gases. Compared with lung inflation mediated inputs, highly controllable, graded input patterns can be produced, and the effects of ipsi-, contra-, and bilateral inputs can be isolated and compared.

The central processing of the vagal afferent inputs, as seen by its effects on the neuronal discharge patterns, appears to occur in at least two different modes. In one mode, the neuronal discharge patterns are either compressed or expanded along the time axis with the changes in phase duration, but the morphology of the pattern is preserved. Examples of this are shown by the responses of the Idec and Edec neurons of Figs. 8 and 12, respectively, and the Eaug pattern in response to the contralateral input (Fig. 12). Mechanistically, this may be due to the neuronal patterns being sculptured by elements of the phase timing circuitry. In addition, this may be combined with presynaptic, long time-constant processing of the afferent input (Bajic et al. 1989; Zuperku and Hopp 1985, 1987; Zuperku et al. 1982a). The second mode of vagal afferent processing...
appears to be mediated by rapid mechanisms and is more directly transmitted to the neurons, possibly over oligosynaptic pathways. The effect of this mechanism, which appears to occur in combination with the more indirect slower processing, manifests itself as a parallel upward or downward shift in the neuronal discharge patterns. For example, this effect can be seen by comparing the contra- and ipsilateral responses of the Eaug neuron of Fig. 12 and the late-E neurons of Fig. 14.

Responses of neurons with decrementing patterns

The response characteristics of the Idec and Edec neurons were consistently related to the changes in phase timing produced by ipsi-, contra-, or bilateral afferent PSR inputs (Figs. 9 and 13). The negative slope of the Idec and preIdec neurons increased in proportion to the decrease in TI, while the negative slope of the Edec neurons decreased in proportion to the...
increase in TE. These findings are consistent with the responses of Idec and Edec neurons previously reported for other species, even though such neurons may not have been within the pre-BC, and they are also consistent with their proposed role in phase timing (Cohen 1979; Cohen and Feldman 1977; Ezure 1990; Feldman and Cohen 1978; Hayashi et al. 1996; Zuperku and Hopp 1987). In rat Edec neurons, excitatory postsynaptic potentials are produced by electrically evoked vagal afferent volleys acting via a paucisynaptic pathway, suggesting that increases in Edec activity produced by lung inflation are mediated by excitation rather than disinhibition (Hayashi et al. 1996). Alternatively, there is evidence for reciprocal inhibition between Edec and Aaug neurons (Shen et al. 2003), which suggests the possibility that an inflation mediated inhibition of Aaug activity (Feldman and Cohen 1978; Manabe and Ezure 1988) could contribute to the increase in Edec activity.

In most network models, it has been assumed that reciprocal inhibition between pools of Idec and Edec neurons provides the main substrate for rhythm generation (Balas et al. 1994; Duffin et al. 1995; Ogilvie et al. 1992; Richter et al. 1986; Rybak et al. 1997; Zuperku et al. 1982). Recent studies have shown that these neurons are either GABAergic or glycinerergic (Ezure et al. 2003; Okazaki et al. 2001) supporting their inhibitory role (Ezure and Manabe 1988; Lindsey et al. 1987; Shen et al. 2003). The results of this study support a control-of-timing role for these neurons.

Responses of Eaug and late-E neurons

Late-E neurons (29% of pre-BC region E neurons) may be similar to the E2 augmenting neurons reported in other studies (Cohen 1969; Cohen et al. 1985; Hayashi et al. 1996; Schwarzacher et al. 1995; Shen et al. 2003). However, their discharge pattern is different from the E bulbospinal neurons of the canine caudal VRG, which begin to discharge immediately at the end of the I phase and continue to discharge throughout the E phase. Most of the canine E bulbospinal neurons have decrementing patterns, and they are excited at low transpulmonary pressures and strongly inhibited at higher transpulmonary pressures (Bajic et al. 1992; Tonkovic-Capin et al. 2000). The Eaug and late-E neurons of the pre-BC do not exhibit an inflation pressure-dependent bidirectional response. There are, however, similarities among the pre-BC region and bulbospinal E neurons in terms of the laterality of the responses. In both cases, the ipsilateral vagal input produces mainly inhibition, whereas the contralateral vagal input either has no effect or produces excitation (e.g., Figs. 12 and 14) (Tonkovic-Capin et al. 1991).

A consistent finding for most late-E neurons was that, within the same neuron, ipsilateral inputs produced inhibition while contralateral inputs produced excitation, however the response to bilateral inputs was inhibitory (Figs. 10 and 13). This suggests a dominant role for the ipsilateral inputs. With intact vagal nerves, lung inflation also produced inhibition (e.g., Fig. 15), which is consistent with previous reports of Eaug neurons in the VRG and Bötzingern complex (Feldman and Cohen 1978; Manabe and Ezure 1988). Because an ipsilateral input inhibits ipsilateral late-E neurons while at the same time exciting contralateral late-E neurons, it is possible that this mechanism adjusts E muscle activities in response to uneven inflation of the lung. If the control of these neurons serves a compensatory function, then perhaps the late-E neurons function in a pattern-generating capacity rather than in the control of phase timing. The reflex effects on the discharge patterns of these propriobulbar neurons may be relayed to other postsynaptic brain stem respiratory neurons controlling, in turn, their discharge patterns. Because both ipsi- and contralateral inputs delay the onset of late-E activity, they most likely have their maximum effect in the second half of the E phase.

Responses of other pre-BC region neurons

About 50% of the Iaug neurons were inhibited to a small degree near the end of the I phase with all three afferent inputs, whereas the time courses of the other 50% were unaltered but terminated with the ending of the I phase. These responses are similar to those previously reported for Iα or I(0)/I(−) neurons but unlike the Iβ or I(+) neurons (Cohen and Feldman 1984). The time courses of I neurons with parabolic patterns were mainly unaffected by the vagal afferent inputs. Thus it appears that Iaug and Iparabolic neurons are not involved with phase timing.

The only I neurons to be excited by vagal afferent inputs were the late-I neurons, although this did not happen in every case (Figs. 9 and 11). It has been previously suggested that the late-I neurons may play a role in the graded I inhibition and irreversible termination of the I phase (Baker and Remmers 1980; Cohen and Feldman 1984; Cohen et al. 1993; Haji et al. 2002).

Summary

The canine pre-BC region consists of a heterogeneous mixture of I and E neuron subpopulations similar to those reported in other species. In general, the neuronal responses to pulmonary vagally mediated afferent inputs were similar to those of other species, even though those neurons were recorded in regions outside of the pre-BC. Thus it appears that the response properties of the pre-BC neurons are not unique. However, the late-E neurons of the canine pre-BC region are not found in the caudal VRG. The time courses of the discharge patterns of I and E neurons with decrementing patterns were consistently related to the duration of I and E phases, respectively. These were the only neuron subpopulations to have this property, which suggests an important role in phase timing control.

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References


