Neurokinin1 Receptors Modulate the Excitability of Expiratory Neurons in the Ventral Respiratory Group

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Running title: NK1-Rs excite expiratory neurons in VRG

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ABSTRACT:

We studied the role of neurokinin-1 receptors (NK1-R) on the excitability of expiratory (E) neurons (tonic discharge, $\text{E}_{\text{TONIC}}$; augmenting, $\text{E}_{\text{AUG}}$; decrementing, $\text{E}_{\text{DEC}}$) throughout the ventral respiratory group (VRG), including Bötzinger Complex (BötC) using extracellular single-unit recording combined with pressurized picoejection in decerebrate, arterially-perfused juvenile rats. Responses evoked by picoejection of the NK1-R agonist, [Sar$^9$-Met(O$_2$)$_{11}$]-substance P (SSP) were determined before and after the selective NK1-R antagonist, CP99,994. SSP excited 20 of 35 expiratory neurons by increasing the number of action potentials per burst (+33.7 ± 6.5% of control), burst duration (+20.6 ± 7.9% of control) and peak firing frequency (+16.2 ± 4.8% of control). Pretreatment with CP99,994 completely blocked SSP-evoked excitation in a subset of neurons tested, supporting the notion that SSP excitation was mediated through NK1-R activation.

Since we had previously shown that $\text{E}_{\text{AUG}}$ neurons were crucial to locomotor-respiratory coupling (LRC) (Potts et al. 2005), we reasoned that blockade of NK1-R would alter LRC by preventing somatic-evoked excitation of $\text{E}_{\text{AUG}}$ neurons. Blockade of NK1-Rs by CP99,994 in the BötC severely disrupted LRC and prevented somatic-evoked excitation of $\text{E}_{\text{AUG}}$ neurons. These findings demonstrate that LRC is dependent on endogenous SP release acting via NK1-Rs on $\text{E}_{\text{AUG}}$ neurons of the BötC. Taken together with our earlier finding that inspiratory off-switching by the Hering-Breuer Reflex requires endogenous activation of NK1-Rs through activation of NK1-Rs on $\text{E}_{\text{DEC}}$ neurons (Fong and Potts 2006), we suggest that endogenous release of substance P in the BötC provides a reflex pathway-dependent mechanism to selectively modulate respiratory rhythm.
INTRODUCTION:

Respiratory rhythm is characterized by a coordinated pattern of motor outflow directed to the upper airway and diaphragm. This pattern of activity in vivo is characterized by a three-phase rhythm delineated as inspiration followed by a period of expiration that can be further subdivided into two phases: post-inspiration and late expiration (Richter and Spyer 2001). Respiratory rhythm is continually modulated by a number of inputs including chemosensory drive from central and peripheral chemoreceptors, dynamic feedback from pulmonary and somatosensory afferents, as well as local release of neuropeptides such as substance P (SP). Considerable attention has focused on the effect of SP on breathing in brainstem regions that predominately contain inspiratory neurons (Gray et al. 2001; Gray et al. 1999; Morgado-Valle and Feldman 2004; Ptak et al. 2000a). Neurons in pre-Bötzinger Complex (pre-BötC) of the ventral respiratory group (VRG) express high levels of the SP receptor, neurokinin-1 (NK1-R) (Gray et al. 1999; Guyenet et al. 2002; Nakaya et al. 1994; Wang et al. 2001). Ablation of NK1-R expressing neurons in pre-BötC, the putative kernel for respiratory rhythmogenesis (Smith et al. 1991), severely disrupts breathing and markedly depresses the ventilatory response to inspired CO₂ (Gray et al. 2001; McKay et al. 2005; Wenninger et al. 2004). Earlier studies have shown that exogenous SP excites respiratory activity by increasing breathing frequency and tidal volume (Chen et al. 1990a; Gray et al. 1999; Hedner et al. 1984; Johnson et al. 1996; Monteau et al. 1996; Morgado-Valle and Feldman 2004; Ptak et al. 2000a). However, in a recent study, we showed that NK1-R activation could excite both inspiratory and expiratory activity depending on the VRG neuronal subtype activated (Fong and Potts 2006). Despite the central effect of SP on breathing, NK1-R do not appear to be essential for the generation of respiratory rhythm since genetic deletion of NK1-R or SP synthesizing neurons failed to alter basal breathing (Cao et al. 1998; De Felipe C. et al. 1998; Ptak et al. 1999; Telgkamp et al. 2002). These findings suggest that SP may be involved in modulating respiratory rhythm, rather than in the generation of respiratory rhythm.
To date, no study has directly examined the role of NK1-R on expiratory (E) neurons in the VRG. Given that NK1-R are expressed in regions containing E neurons (Guyenet et al. 2002; Wang et al. 2001) it is reasonable to propose that SP evoked excitation of E neurons may provide a mechanism to modulate breathing. The present study was designed to test this prediction and to demonstrate that SP-induced excitation of E neurons in the VRG selectively modulates respiratory rhythm in a reflex pathway-dependent manner. Since reflex activation of E neurons contributes to changes in respiratory rhythm (Ezure et al. 2002; Fong and Potts 2006; Hayashi et al. 1996; Kubin et al. 2006; Potts et al. 2005), and respiratory rhythm can be coupled to locomotor activity via somatosensory-evoked excitation of expiratory neurons (Funk et al. 1992; Iscoe 1981; Potts et al. 2005), we hypothesized that locomotor-respiratory coupling (LRC) would require endogenous SP release in the VRG.

**METHODS:**

All animals were handled in accordance with National Institute of Health and University of Missouri Animal Care and Use Committee guidelines.

*In situ working heart-brainstem preparation*

Experiments were performed on 39 juvenile male Wistar rats (55-120g, Harlan), using the *in situ* arterial-perfused juvenile rat preparation. The surgical procedures and the extracorporeal circuit for this preparation have been described in details previously (Fong and Potts 2006; Paton 1996; Potts et al. 2000). In brief, rats were deeply anaesthetized with isoflurane via spontaneous inhalation and the depth of anaesthesia was gauged by the absence of limb withdrawal to noxious pinch and the lack of corneal reflex. The rat was transected subdiaphragmatically and the upper torso immediately submerged into ice-cold ringer solution bubbled with carbogen gas (95% O₂-5%
CO₂), decerebrated precocically and the cerebellum removed to expose the dorsal surface of the brainstem. The preparation was skinned and a portion of the posterior thoracic wall was removed to expose the heart and lungs. The descending aorta and the left phrenic nerve (PN) were blunt dissected, isolated and cut. After the surgery, the preparation was transferred to an acrylic chamber and the thoracic aorta cannulated with a double lumen catheter (16 and 18ga; Braintree Scientific) and secured. Peristaltic pump perfusion of Ringer’s solution bubbled with 95% O₂-5% CO₂ was started immediately and the perfusate was warmed to 32-33°C using an in-line heat exchanger, pumped through two in-line bubble traps and a filter (polypropylene mesh; pore size 40µm, Millipore). Perfusion pressure was measured via one of the lumens of the double-lumen catheter using a pressure transducer (model PT300, Grass Instruments), connected to an amplifier (model 13-6615-50, Gould). Pump flow rate was calibrated at the beginning of each experiment and flow rates between 25-36ml/min were used in this study. Whole nerve activity was recorded from the PN via suction electrodes (tip diameter, 0.2-0.3mm), amplified (20k -50k) and filtered (100 Hz-3 kHz, model P511, Grass Astro-Med), and sampled at 2.5kHz. The electrocardiogram (ECG) was measured via silver wires placed directly on the heart, amplified (10k - 20k), filtered (100Hz to 3kHz, model P511; Grass Astro-Med), displayed on an oscilloscope and a discriminator circuit (model N-750, Mentor) was used to generate transistor-transistor logic (TTL) pulses triggered from the upstroke of the R wave. Instantaneous heart rate was derived from measuring the interbeat interval and displayed as beats per minute (bpm).

The elapsed time from induction of anesthesia to the start of systemic perfusion was generally 10-15 mins. Neuromuscular paralysis was produced by addition of vecuronium bromide (50µg) directly to the perfusate. Only preparations that exhibited ramping phrenic discharge with regular burst intervals, an index of adequate brainstem perfusion (Paton 1996), were included in this study.
**Extracellular recording of VRG neurons**

Multibarrel glass micropipettes containing 3 to 5 barrels (1.2mm o.d., 0.68mm i.d., World Precision Instruments) were pulled and broken back to yield a total tip diameter of <10 µm for single unit recording and picoejection, or <30 µm for multiunit recording and microinjection. One barrel was filled with 3M NaCl and a silver wire introduced into the NaCl solution for recording neuronal activity (electrode tip impedance: 12 -28MΩ). Extracellular recordings were amplified using an AC amplifier (5,000-10,000X; NeuroLog NL104, Digitimer Ltd, Welwyn Garden City, UK) and filtered (300 Hz– 3kHz, Neurolog NL126, Digitimer). Action potentials (AP) were continuously monitored using a window discriminator (Neurolog NL201, Digitimer) using dual threshold windowing to capture both the trajectory and amplitude of each unit and displayed on a storage oscilloscope (20mHz, Kikisui). For picoejection studies, only neurons whose action potentials could be clearly discriminated were studied. Single unit activity was digitized using 12 bit analog-to-digital conversion (micro 1401, Cambridge Electonics Design, Cambridge, UK) at 25kHz and recorded using Spike2 software (Cambridge Electonics Design, Cambridge, UK). The system recorded and displayed discriminated unit activity in real time. Wave-shape template matching was used off-line to confirm the waveshape of all recorded units as single units.

**Drug preparation for picoejection or microinjection**

The remaining drug barrels of the combined recording/picoejection microelectrode array were back-filled with one of the following solutions: 1mM of the selective NK1-R agonist, [Sar⁹-Met(O²)¹¹]-substance P (SSP); 10mM of the selective non-peptide NK1-R antagonist, CP99,994; vehicle (Ringer solution). In experiments designed to characterize the role of NK1-R in LRC, each barrel of a triple-barrel pipette was back-filled with 3M NaCl (extracellular recording), CP99,994 (NK1-R antagonist) and D-L-homocysteric acid (DLH). DLH was used to functionally identify the
Bötz region as previously described (Fong and Potts 2006; Monnier et al. 2003; Wang et al. 2002). All drugs were dissolved in the Ringer solution and pH adjusted to 7.4. The ends of the microinjection barrels were sealed with silicone tubing connected to a pneumatic pressure injection system (Picospritzer II, General Valve) via polyethylene tubing.

**Experimental procedure**

The micropipette was secured to a pipette holder attached to a piezoelectric stepper motor (Inch Worm, model IW-711-01; Burleigh Instruments) driven by a low-noise controller (model 6200ULN-1-1; Burleigh Instruments) mounted on a stereotaxic frame. The tip of the micropipette was placed at calamus scriptorius (CS) using a stereomicroscope (Total magnification=66X, Stemi SV11, Carl Zeiss) and this landmark was used as relative zero for the rostrocaudal and lateral displacement of the micropipette array. The cranium was tilted nose down at an angle of approximately 30° from the horizontal such that the brainstem was horizontal. The pipette was positioned into the VRG using the following co-ordinates: AP: +0.3 - 1.8mm, ML: ± 1.4 - 2.0mm from CS, DV: 1800 – 2700µm below the dorsal surface of the medulla. Extracellular unit activity was continuously monitored as the micropipette was advanced ventrally into the medulla and the locations of respiratory neurons were noted. Once a respiratory neuron was located, the neuron was classified as either decrementing-expiratory (E<sub>DEC</sub>), augmenting expiratory (E<sub>AUG</sub>) or tonic expiratory (E<sub>TONIC</sub>) based on its discharge pattern relative to phrenic nerve discharge (PND) (Ezure 1990). Following characterization of bursting profile of the expiratory neuron, the chosen protocol was used as described below. The final stereotaxic location of recorded neurons was documented and subsequently plotted on a reconstruction of a representative rat brainstem of the weight range used in this study.
Protocol 1: Effect of SSP picoejection on expiratory neurons

The first series of experiments determined the effect of SSP on excitability of single unit expiratory neurons in the VRG. To facilitate recording of single units during drug delivery to the recorded cell, picoejection pressure was maintained as low as possible (pressures range between 10-25 PSI). Ejection pressure was slowly adjusted to the drug barrel until a minimum pressure that produced approximately 10% change in AP amplitude was achieved. In the majority of cases, this resulted in a decrease in AP amplitude. However, in two cases AP amplitude increased during pressure application. The drugs were pressure-ejected over a period of 20-30 seconds which typically coincided with 6-7 respiratory cycles while the amplitude of the recorded unit was constantly monitored. The ejection volumes could not be determined as the ejected volume was below the visual resolution of the microscope (3 nl).

To verify that the SSP-evoked response was, in fact, due to activation of NK1-R, the picoejection was repeated following delivery of CP99,994 (CP) from an adjacent barrel. Pressure transfer between picoejection barrels was facilitated by a series of 4-way stopcocks that were connected in-series with the external pressure source. In preliminary studies, we determined that a minimum period of 1 min was required between repeat applications of SSP to prevent receptor desensitization and for the response to SSP to be reproducible (see Figure 1A). Therefore, to ensure sufficient time for recovery, at least 1 min was given between sequential SSP applications. In a subset of E neurons excited by SSP (n = 5), the non-peptide NK1-R antagonist CP was picoejected onto the same neuron. Immediately following CP, SSP picoejection was repeated. Finally, SSP was picoejected serially over the next 10 mins to examine recovery of the SSP response.

Vehicle effects and pressure ejection artifacts were examined by pressure-ejection of Ringer’s solution onto a subset of respiratory neurons (n = 9) using pressures that produced a similar reduction (ie. 10%) in AP amplitude. No change was detected in any of the measurements.
**Protocol 2: Effect of NK1-R antagonism on locomotor respiratory coupling (LRC)**

Somatic afferents innervating the forelimb were activated by electrical stimulation of the forelimb flexors, as previously described (Potts et al. 2000; Potts et al. 2005). The ipsilateral scapula was stabilized using a vertebral clamp and the distal end of the forelimb attached to a force displacement transducer (FT-100, CB Science) with developed tension measured using a recorder amplifier (Neurolog NL107, Digitimer, Ltd, Welwyn Garden City, UK). Stimuli were delivered using 2 fine silver wires (0.005 inch O.D., A-M Systems, Carlsborg, WA) introduced into the biceps brachii and coracobrachialis muscle groups parallel to the humerus. Pulse-train stimuli (0.4 – 1.3Hz, 50-75ms train duration, 1-3 ms pulse duration, 250µA to 1mA) were generated by a stimulator (model S88, Astro-Med Grass) and a stimulation isolation unit (model PSIU6, Astro-Med Grass). This stimulus activated somatic afferents as evidenced by tachypnea and pressor/tachycardic responses (Potts et al. 2000; Potts et al. 2005).

Once the forelimb was stabilized, the multibarrel microelectrode (3 barrels, total O.D. <30µm) was introduced into the VRG ipsilateral to the stimulated forelimb (CS: AP +1.6-1.8mm, ML ± 1.6-2.0mm, depth from surface 1800-2600µm). Multiunit neuronal activity was recorded as the electrode was advanced ventrally. Once a region containing expiratory activity was located, the electrode was left in place for 10mins prior to microinjection of DLH (6pmol in 6nl) to functionally confirm that the electrode tip was in the BötC region, as evidenced by an evoked bradypnea represented by an increase in expiratory duration of >1.5 fold of baseline for over 5 respiratory cycles (Fong and Potts 2006; Monnier et al. 2003; Wang et al. 2002). Following functional identification of the bradypneic region, the effect of somatic afferent stimulation on LRC was determined before and after blockade of NK1-R using CP99,994 (500pmol in 50nl).
Protocol 3: Effect of NK1-R antagonism on somatic afferent stimulation evoked excitation of expiratory neurons

A multibarrel microelectrode for single unit recording and picoejection of SSP and CP as described in Protocol 1 was positioned into the VRG. Additionally, the forelimb was stabilized and stimulation electrodes were placed in the forelimb flexors as described for protocol 2. E neurons were examined for responsiveness to both picoejection of SSP and somatic afferent stimulation. Only neurons that were excited by both exogenous SSP and somatic afferent stimulation were used in this series of experiments. Once an E neuron that fitted the above profile was localized, CP was picoejected onto the neuron and the effect of somatic afferent stimulation was immediately repeated.

Data and statistical analyses

All data were collected on-line using commercially available data acquisition hardware and software (micro1401 analog-to-digital converter, Spike 2 software, ver. 5.16; Cambridge Electronics Design). Wave-shape template matching algorithms (Spike2; Cambridge Electronic Design) was used off-line to confirm the shape of action potentials belonging to a single unit or to discriminate different units prior to analysis; all other analyses were performed off-line using custom written scripts.

The following variables were measured from the phrenic neurogram: inspiratory duration (T_I), determined as duration of phrenic nerve discharge (PND); expiratory duration (T_E) determined as the duration from the end of one PND to the onset of the following burst; respiratory cycle length (T_Tot) was calculated by summing T_I and the subsequent T_E; respiratory frequency was calculated from T_Tot and expressed in hertz (Hz). The latency between somatic afferent stimulation and the following PND was measured using a event correlation algorithm (Spike 2, V 5.16) over a series of somatic afferent stimulation events (generally 9 to 19 events).
For extracellular unit activity, the duration of each burst and the number of action potentials per burst was recorded and used to calculate the mean discharge frequency. In addition, the peak discharge frequency (determined from instantaneous firing frequency) was also recorded. Changes in neuronal activity in response to SSP or CP picoejection were expressed as percent of baseline. Baseline was determined by averaging the variables measured for 10-15 respiratory cycles immediately before drug ejection. Effect of the drug on extracellular activity was determined by averaging over all the respiratory cycles during picoejection (generally 6 to 7 respiratory cycles). Following drug ejection, the variables were measured over 10-15 respiratory cycles to determine recovery of neuronal activity towards baseline levels. This time frame was chosen based on preliminary experiments showing that this was sufficient for extracellular neuronal activity to recover to control values. Drug application was determined to have elicited an effect on the recorded neuron if the parameters measured during picoejection increased ≥15% above baseline.

All graphs were plotted using the software package SigmaPlot (Ver 9). All data are presented as mean ± SEM, n is the number of neurons, unless otherwise indicated. All statistical analysis was performed on raw data using the software program SAS (Ver. 9.1, SAS Institute, Cary, NC). Data was tested for normality using the Kolmogorov-Smirnov test. Data that were normally distributed was test for statistical significance on the effect of drug on neuronal activity or PND parameters using one-way repeated measures ANOVA followed by Student Newman-Keul (SNK) post-hoc test. Data that were not normally distributed were evaluated using repeated-measures ANOVA on Ranks followed by SNK post-hoc test. The effect of somatic afferent stimulation on E neurons in the absence and presence of NK1-R blockade was determined using paired t-test. The effect of NK1-R blockade on respiratory rhythm entrainment was determined using Student t-test. P <0.05 was considered significant in all cases.
**Solutions and drugs**

The Ringer’s solution, containing 125 mM NaCl, 2.4 mM NaHCO₃, 5 mM KCl, 2.5 mM CaCl₂, 1.25 mM MgSO₄, 1.25 mM KH₂PO₄ and 1-mM D-glucose, was made fresh for each experiment. A high molecular weight compound (1.25%, Ficoll, type 70, 70 kDa, Sigma) was added to the Ringer’s solution as an oncotic agent, with the resulting oncotic pressure of the perfusate of approximately 310 mOsm/KgH₂O. The neuromuscular blocking agent, vecuronium bromide (0.04 µg/ml, Sicor Pharmaceuticals), was also applied to the perfusate.

**RESULTS:**

*Effect of SSP picoejection on expiratory neurons*

Thirty-five E neurons located within the confines of the VRG were successfully recorded and included in the study. Picoejection of SSP excited 57% (20 / 35 cells) of E neurons, while the remaining 43% failed to alter their firing pattern. Table 1 summarizes the classification of E cells based on neuronal discharge subtype and summarizes their response to exogenous SSP. ETONIC neurons were the most commonly recorded and SSP excited 71% of these neurons. Fifty-five percent of augmenting expiratory (E_AUD) neurons and 50% of decrementing-expiratory (E_DEC) neurons were also excited by SSP. One neuron whose activity spanned from late expiration to early inspiration was also excited by SSP. Exogenous application of SSP significantly increased the total number of APs per burst (15/20 cells), burst duration (8/20 cells), as well as the mean (9/20 cells) and peak (11/20 cells) firing frequency (see Table 2). We also evaluated the effect of SSP on respiratory rhythm and pattern and found that picoejection of SSP had no significant effect on inspiratory duration (Tᵢ), expiratory duration (Tₑ), total respiratory cycle time (T_{tot}), or phrenic burst frequency (Table 3). These results suggest that the delivery of exogenous SSP was highly...
localized and predominately affected the recorded neuron, while having little to no effect on other neighboring respiratory neurons.

An example of the effect of SSP and CP on the excitability of an E_{TONIC} is shown in Figure 1. In this experiment, picoejection of SSP excited an extracellularly-recorded single-unit neuron. However, the basal firing pattern was restored within 2 – 3 respiratory cycles following termination of SSP application. This excitatory pattern was repeated when SSP was reapplied 60 sec later. CP99,994 completely blocked the excitation which was restored within 2 min following washout of the antagonist (Fig 1A). In five E_{TONIC} neurons where SSP produced a brisk excitation by increase in the mean and peak firing frequency and the total number of action potentials per burst (Fig 1C), we examined the effect of NK1-R blockade. SSP evoked excitation was completely eliminated by pretreatment with CP. NK1-R blockade alone had no effect on the mean or peak firing frequency, number of APs per burst or burst duration of on these five E_{TONIC} neurons (Table 4). The excitation by SSP was reproducible, prevented by selective blockade of NK1-Rs and was completely reversible following drug washout.

An example of the effect of SSP on an E_{TONIC} neuron is shown in Figure 2. This neuron had activity throughout the expiratory phase with no activity during the inspiratory phase (Fig 2A, 2B). Picoejection of SSP increased peak firing frequency that recovered rapidly following cessation of SSP picoejection (Fig 2A). SSP picoejection increased the number of APs generated per burst with the burst duration of each burst occurring later in the expiratory cycle (Fig 2D). SSP significantly increased mean and peak firing frequency, number of APs per burst and burst duration on this subpopulation of E neurons (Fig 2E).

The effect of SSP on an E_{AUG} neuron is shown in Figure 3. This neuron was characterized by activity during expiratory phase with a progressive increase in its frequency of AP discharge that terminated abruptly with the onset of inspiration (Fig 3A, B). Picoejection of SSP produced an increase in the peak instantaneous firing frequency over the duration of the picoejection which returned to baseline shortly following termination of the picoejection (Fig 3A). In addition to
increasing its peak discharge rate, SSP also increased the mean discharge rate, the number of AP generated during the early bursting phase and SSP also increased the burst duration of each burst with activity occurring earlier in expiration, compared to baseline conditions as seen in figure 3D.

Similar results were found when SSP was applied to an $E_{DEC}$ neuron (Fig 4). This neuron displayed a decrementing pattern of action potential discharge that began abruptly when phrenic nerve activity ceased and terminated near the end of expiration. SSP the total number of APs generated per burst, and also prolonging the duration of each burst with action potentials generated later in the expiratory phase (Fig 4C, D). However, SSP had no effect on the mean or peak firing frequency in this subpopulation of neurons.

**Effect of NK1-R antagonism on locomotor respiratory coupling by somatic afferent stimulation**

To determine whether phasic coupling between locomotor and respiratory rhythms depended upon SPergic signaling in the VRG, we unilaterally blocked NK1-R by microinjecting CP into a region that contained expiratory neurons, verified by extracellular recording (Fig 5A) and functionally identified as the BötC region by DLH microinjection evoked bradypnea (Fig 5B). Prior to blockade of NK1-Rs, somatic stimulation increased the PND frequency and phase-locked PND with the onset of muscle contraction (Fig 6A), indicated by constant latency between contraction and subsequent phrenic burst (Fig 6C). Unilateral microinjection of CP, ipsilateral to somatic afferent stimulation, did not affect basal respiratory rhythm but eliminated the phasic coupling of locomotor and respiratory rhythms (Fig 6B, C). However, it should be noted that somatic stimulation increased overall PND frequency to the same extent as during the control condition (Fig 6A and 6B) which was in agreement with previous work (Potts et al. 2005). A peristimulus-timed histogram (PSTH) between the onset of somatic stimulation (ie. forelimb tension development) and PND demonstrated that phase-coupling was completely eliminated following
NK1-R blockade (Fig 6C). In addition, CP significantly increased the average latency and its variability between the onset of locomotor and respiratory activity (Fig 6D).

**Effect of somatic afferent stimulation on expiratory neurons**

Based on the above finding that blockade of NK1-R abolished LRC and that activation of somatic afferents selectively augments the discharge frequency of $E_{AUG}$ neurons (Potts et al. 2005), we hypothesized that somatic-evoked excitation of $E_{AUG}$ neurons require local SP release. In recorded $E_{AUG}$ neurons that were excited by exogenous SSP (n=4), somatic stimulation increased mean and peak instantaneous firing frequency (Fig 7A). This was accompanied by a shortening of expiratory duration and a premature onset of phrenic nerve discharge. Picoejection of CP completely abolished somatic-evoked excitation of $E_{AUG}$ neurons (Fig 7B). However, CP alone failed to alter the cell’s overall discharge profile (Fig 7B). Group results found that NK1-R blockade significantly prevented somatic-evoked excitation of $E_{AUG}$ (Fig 7C).

Since somatic stimulation also inhibits $E_{DEC}$ neurons (Potts et al., 2005), we tested whether blockade of NK1-Rs would alter this inhibition. As we have previously shown, somatic afferent stimulation reduced the burst duration of $E_{DEC}$ neurons, accompanied by a reduction in expiratory duration (Fig 8A). NK1-R antagonism had no effect on somatic evoked reduction in either $E_{DEC}$ burst duration or expiratory duration (Fig 8B). Table 5 contains group data showing that blockade of NK1-R had no effect on somatic evoked inhibition of $E_{DEC}$ burst duration, the number of action potentials per burst, or the reduction in expiratory duration (n=7). In addition, the latency for inhibition of $E_{DEC}$ neurons was not altered by NK1-R blockade (89.7 ± 17.8ms vs. 109 ± 30.8 ms, control vs. NK1-R blockade, P = 0.372, Paired t-test, n = 7).
Localization of recording sites

The stereotaxic coordinates where E neurons were recorded in the VRG (n=35) is illustrated in Figure 9. Recorded neurons were distributed in a longitudinal column ventral of nucleus ambiguus extending rostrocaudally from the caudal pole of the facial nucleus to calamus scriptorius (CS). These neurons were concentrated in two regions, 300-600 µm caudal to the caudal pole of the facial nucleus and 300-600µm rostral of CS corresponding to the Bötc and rVRG, respectively.

Discussion:

The current study provides the first direct evidence that E neurons in the VRG can be excited by exogenous substance P. This excitation was eliminated by pharmacological blockade of NK1-Rs, suggesting that SP-evoked excitation of E neurons was mediated through activation of the NK1-R. Furthermore, baseline activity of E neurons was unaffected by NK1-R blockade indicating that SP in Bötc is likely not involved in establishing the baseline activity of E neurons during eupnea. These data demonstrate that E neurons in the VRG express functional NK1-Rs, and thus, suggest that SPergic signaling may be involved in modulating respiratory rhythm under specific physiological conditions. We also found that LRC requires SPergic modulation of E neurons. We reported that unilateral blockade of NK1-Rs in the Bötc eliminated the phasic coupling between locomotor and respiratory rhythms. However, it failed to alter the overall excitation of respiratory frequency during skeletal muscle contraction. Furthermore, muscle contraction excited a population of $E_{AUG}$ neurons whose excitation was prevented following blockade of NK1-Rs. Together, these findings suggest that somatic afferent stimulation evokes endogenous release of SP in the Bötc which plays a critical role in LRC by selectively modulating the excitability of $E_{AUG}$ neurons.
Substance P modulates the excitability of expiratory neurons

Substance P is a potent stimulator of respiration and can excite inspiration and prolong expiration by selectively activating separate populations of respiratory neurons in the VRG (Chen et al. 1990a; Chen et al. 1990b; Fong and Potts 2006; Gray et al. 1999; Hedner et al. 1984; Morgado-Valle and Feldman 2004; Ptak et al. 2000a). The role of SP and NK1-R has been the focus of much research as it is involved in mediating a number of respiratory reflex responses, including hypoxic ventilatory response and central chemosensitivity (Chen et al. 1990a; Gray et al. 2001; Mazzone et al. 1997; Nattie and Li 2002; Ptak et al. 2002; Wickstrom et al. 2004). Although NK1-R are localized throughout the VRG (Gray et al. 1999; Guyenet et al. 2002; Nakaya et al. 1994; Wang et al. 2001) with expression confirmed on pre-inspiratory neurons (Guyenet and Wang 2001), the effect of direct activation of NK1-R expressing expiratory neurons on respiratory rhythm has not been thoroughly explored. Although substance P can decrease respiratory frequency by prolonging expiratory duration (Chen et al. 1990b; Fong and Potts 2006; Makeham et al. 2005), the functional expression of NK1-R on individual expiratory neurons has not been demonstrated. We now provide evidence that exogenous SP modulates the excitability of a subpopulation of E neurons suggesting a possible functional role for NK1-Rs in an expiratory region of the VRG. The ability of the selective NK1-R antagonist, CP99,994, to block the effects of exogenous SSP further strengthens the proposal that excitation of E neurons by SSP was mediated by selective activation of NK1-Rs.

The increase in excitability of E neurons by SSP agrees with previous data demonstrating that exogenous SP can increase the discharge rate of several neuronal populations including, phrenic motor neurons (Ptak et al. 2000b), C1 bulbospinal neurons in the rostroventral lateral medulla (Li and Guyenet 1997), midbrain periaqueductal grey neurons (Drew et al. 2005) and NTS neurons (Boscan et al. 2002). While the mechanism by which NK1-R activation leads to an
increase in neuronal excitability remains to be determined, this effect may be mediated through a reduction in leak K⁺ conductance that would result in raising resting membrane potential, possibly via inhibition of TASK channels (Boscan et al. 2002; Li and Guyenet 1997; Ptak et al. 2000b; Washburn et al. 2003). As TASK channels are modulated by receptors coupled to Gαq proteins, such as NK1-R, and these channels are expressed on majority of NK1-R expressing neurons throughout the VRG (Washburn et al. 2003), this is a likely possibility. Alternatively, NK1-R activation could increase the excitability of E neurons through phosphorylation of G-proteins leading to turnover of intracellular inositol triphosphate (IP3) to raise intracellular calcium (Harrison and Geppetti 2001; Washburn et al. 2003). Finally, NK1-R may increase neuronal excitability by modulating R-type Ca²⁺ currents (Meza et al. 2007). Thus, future work is needed to identify the cellular mechanism(s) of NK1-R activation on membrane excitability of E neurons.

**Reflex activation of NK1-R on expiratory neurons modulates respiratory rhythm**

The lack of effect on the basal activity of E neurons following blockade of NK1-R (Table 4) suggests there is no tonic release of endogenous SP onto expiratory neurons of the VRG. Thus, this raises the following question: under what condition(s) is endogenous SP released? Data from the present study found that a heterogeneous population of E neurons, including E_{DEC} and E_{AUG} neurons, was excited by exogenous SP. This suggests that endogenous SP and NK1-R may be involved in modulating the excitability of E_{DEC} and E_{AUG} neurons and these neurons are involved in modulating respiratory rhythm in response to activation of a variety of afferents, including arterial chemoreceptors (Sun and Reis 1996), pulmonary stretch receptors (Hayashi et al. 1996) and somatic afferents (Potts et al. 2005). It has previously been shown that activation of the slowly adapting pulmonary stretch receptors (SARs) by the Hering-Breuer reflex (HB-reflex) excites E_{DEC} neurons in the VRG (Hayashi et al. 1996). Recently, we have demonstrated that extension of expiratory duration by SARs of the Hering-Breuer reflex requires the activation of NK1-R in the
BöttC (Fong and Potts 2006). We, therefore, posited that activation of SARs evokes endogenous release of SP in the BöttC which, in turn, activates NK1-R and excites a subpopulation of $E_{DEC}$ neurons. This excitation leads to prolonged inhibition of $E_{AUG}$ neurons, resulting in extension of expiration, as proposed by current models of respiratory rhythm generation (Fong and Potts 2006; Richter et al. 1986; Rybak et al. 2004) (see also Figure 10). We now show that NK1-Rs are functionally expressed on a subpopulation of propriobulbar $E_{DEC}$ neurons which support our earlier proposal.

In addition to $E_{DEC}$ neurons, we found that a subpopulation of $E_{AUG}$ neurons was also excited by exogenous SSP. It is highly unlikely that this subpopulation of E neurons is involved in the HB-reflex since their excitation strongly inhibits $E_{DEC}$ neurons (Fong and Potts 2006; Potts et al. 2005; Rybak et al. 2004). However, since $E_{AUG}$ neurons are excited by somatic afferent stimulation (Potts et al. 2005), their excitation may require endogenous SP. Substance P has been demonstrated to participate in the somatic afferent reflex at the level of the spinal cord (Wilson et al. 1993b; Wilson et al. 1993a) and the nucleus tractus solitarius (NTS) (Potts et al. 1999). Since spinal dorsal horn neurons project predominately ipsilaterally throughout the VRG (Potts et al. 2002), we hypothesized that excitation of $E_{AUG}$ neurons and LRC evoked by somatic afferent stimulation requires NK1-R activation in the ipsilateral VRG. We determined that NK1-R blockade in the ipsilateral BöttC by microinjection of CP99,994 eliminated the phasic coupling of respiratory and skeletal muscle activity. Additionally, we determined this phasic coupling was mediated by activation of NK1-Rs on $E_{AUG}$ neurons, as the contraction evoked excitation of $E_{AUG}$ neurons was abolished following NK1-R blockade. Together, these findings demonstrate that endogenous SP is released in the VRG during somatic afferent stimulation to excite BöttC $E_{AUG}$ neurons which participate in the coupling of respiratory and locomotor rhythms. However, it is interesting to note that while LRC was blocked by CP, the overall excitation of respiratory activity (ie. PND frequency) was unaltered. This finding was similar to the effect we observed following pharmacological blockade of synaptic transmission in the lateral parabrachial nucleus (LPBN) (Potts et al. 2005).
suggesting that SPergic mechanisms in the BötC contribute to phase-transitions in respiratory rhythm.

Interestingly, both the HB-reflex (Fong and Potts 2006) and LRC require SPergic signaling in the BötC. Activation of these two reflex pathways result in opposing effects on respiratory rhythm: the HB-reflex extends expiration and slows respiratory rhythm; whereas, LRC excites respiration by reducing expiratory duration. These two reflex pathways also have different synaptic targets: the HB-reflex excites E_{DEC} neurons while somatic afferent stimulation excites E_{AUG} neurons (although both of these E populations are excited by exogenous SSP). This finding suggests that multiple afferent pathways innervating different E populations utilize SP as a neuromodulator.

In the present study, we did not directly determine whether the neurons we recorded were bulbospinal neurons or vagal motorneurons. However, our data supports the idea that these neurons were likely propriobulbar neurons based on the finding that a subpopulation of NK1-R expressing E_{DEC} neurons that inhibit E_{AUG} neurons in the BötC following HB-reflex activation (Fong and Potts 2006). Additionally, earlier work from our lab found that E_{AUG} neurons increase respiratory frequency by inhibiting E_{DEC} neurons in the BötC (Potts et al., 2005). Thus, our present data is consistent with the notion that NK1-Rs are expressed on a subpopulation of propriobulbar E_{AUG} neurons. However, we cannot rule out the possibility that NK1-Rs may also be expressed on expiratory premotor neurons, as has been reported on a subpopulation of inspiratory premotor neurons (Guyenet et al. 2002).

Previously, we hypothesized that a possible source of SP for the excitation of E_{DEC} neurons by SARs may be pump neurons in the NTS (Fong and Potts 2006) (see Figure 10). We now propose that LPBN may be an additional source of SP to the BötC that specifically targets E_{AUG} neurons. This is based on the following observations: 1) Inhibition of LPBN (Potts et al. 2005) as well as blockade of NK1-R in the BötC, result in a similar loss of phase coupling of respiration to muscle contraction without a loss in overall excitation of respiration, 2) SPergic neurons are localized in the LPBN (Block and Hoffman 1987; Douglas et al. 1982; Leger et al. 1983; Potts et al. 2005).
2005) and 3) BötC receives direct projections from respiratory neurons in the dorsolateral pons region, including the LPBN (Ezure and Tanaka 2006). We propose that ascending somatosensory input from the spinal dorsal horn transmitting sensory information from contracting muscles excites SPergic neurons in the LPBN that in turn project to and excite $E_{\text{AUG}}$ neurons in the VRG through release of SP (Figure 10), although a direct SPergic projection from the LPBN to the BötC remains to be confirmed. Taking these current findings together with the existing knowledge that NK1-R are vital to the hypoxic ventilatory response and central chemosensitivity (Chen et al. 1990a; Ezure and Tanaka 2006; Gray et al. 2001; Mazzone et al. 1997; Nattie and Li 2002; Ptak et al. 2002; Wickstrom et al. 2004) further establishes a fundamental role for the endogenous release of SP in modulating E neuron excitability and demonstrate that the modulation and formation of respiratory rhythm depends upon reflex specific inputs to BötC.

**Technical considerations:**

The picoejection method employed in the current study has been used by a number of other investigators to examine the effect of discrete drug application onto recorded neurons (Boscan et al. 2002; Boscan et al. 2005; Brandes et al. 2006). This method allows for highly localized application of drugs onto functionally-identified neurons using drug solutions at physiological pH 7.4, compared to iontophoresis which typically requires the solutions to carry an ionic charge that alters solution pH. The ability to use solutions at physiological pH is particularly important as neurons in the ventrolateral medulla, including the VRG neurons which have been shown to express TASK channels, can be inhibited by changing extracellular pH (Washburn et al. 2003). Due to this characteristic of neurons, we felt that the use of drug solutions departing from neutral pH may make it difficult to differentiate between the direct effects of the drug vs. the indirect effect of pH, thus making the results difficult to interpret.
Although we were unable to directly measure the volume of drug ejected, we are confident that each ejection was localized to a finite volume as there was no effect on network output, including no change in the frequency or amplitude of the phrenic motor output (Table 3). An earlier study by Monnier and colleagues (2003) showed that microinjection volumes as small as 3nl into the VRG can affect network output. Similarly, we recently showed in the same preparation that microinjection volumes as small as 4-6nl of SSP at a similar concentration to that used in the current study, could produce profound effects on respiratory frequency (Fong and Potts 2006). Together with the slow ejection rate, the short duration of ejection time (30sec) and the rapid decrease in concentration of the drug relative to the distance from the pipette tip (Stone 1985), we propose that the sphere of influence for the picoejected drug was highly localized and mainly affected the recorded neuron. While we cannot exclude the possibility that the drug may have produced presynaptic effects, NK1-R labeling is predominantly localized to the soma and dendrites of VRG neurons (Liu et al. 2004) suggesting that the likely site of action was post-synaptic.

**Summary**

Data from the present study identify a subpopulation of E neurons that express NK1-R whose activation increases neuronal excitability. Following blockade of NK1-R, the basal activity of E neurons remained unchanged suggesting that SP does not significantly contribute to the basal level of excitability of these neurons nor to the generation of respiratory rhythm during eupnea. However, its endogenous release plays an important role in modulating respiratory rhythm during reflex activation. Indeed, LRC evoked by somatic afferent stimulation requires functional NK1-R expression on $E_{\text{AUG}}$ neurons in the BötC. Together with existing data, the present findings further establish the functional importance of SPergic mechanisms in modulating respiratory rhythm by increasing the excitability of $E_{\text{DEC}}$ and $E_{\text{AUG}}$ neurons in a reflex pathway-specific manner.
Acknowledgements

We are grateful for the funding support of the National Institute of Health (HL059167, JTP). We would also like to thank Pfizer Corp for their general gift of CP99,994. Current address for JTP: Department of Integrative Physiology, University of North Texas Health Science Center, 3500 Camp Bowie Blvd, Fort Worth, Texas 76107, USA.
Reference List


37. **Nattie EE and Li A.** Substance P-saporin lesion of neurons with NK1 receptors in one chemoreceptor site in rats decreases ventilation and chemosensitivity. *J Physiol* 544: 603-616, 2002.


40. **Potts JT, Lee SM and Anguelov PI.** Tracing of projection neurons from the cervical dorsal horn to the medulla with the anterograde tracer biotinylated dextran amine. *Auton Neurosci* 98: 64-69, 2002.


45. **Ptak K, Hunt SP and Monteau R.** Substance P and central respiratory activity: a comparative in vitro study in NK1 receptor knockout and wild-type mice. *Pflugers Arch* 440: 446-451, 2000a.


**FIGURE LEGENDS:**

**Figure 1:**

Picoejection of [Sar⁹-Met(O₂)¹¹]-substance P (SSP) excites expiratory neurons via activation of NK1 receptors (NK1-R).

(A) Picoejection of SSP on to an expiratory neuron elicited a large increase in instantaneous firing frequency that recovers back to pre-ejection level shortly after cessation of picoejection (A1). This excitation was reproducible and was blocked by prior picoejection of the selective NK1-R antagonist, CP99,994 (CP, A2). The response to SSP recovered following wash out of the antagonist (A3-A4). From top to bottom: Instantaneous firing frequency (Inst. Freq), extracellular unit recording (VRG unit), integrated phrenic nerve discharge (IPND).

(B) Waveform analysis of action potential trajectory before (baseline), during (SSP) and after (recovery) SSP picoejection from Panel A1. These data confirm that this recording was from a single unit and that shape of the action potential was not affected by picoejection of SSP. The number of action potential shown in each overlay is indicated.

(C) Quantification of the effect of picoejection of SSP alone, or immediately following NK1-R antagonist, CP99,994. SSP alone resulted in an increase in the number of action potentials per burst (# APs), mean and peak frequency. The effect of SSP was completely blocked in the presence CP99,994. * = p < 0.05, One way repeated measures ANOVA, post-hoc SNK, n = 5. Abbreviations: Burst Dur, Burst duration; Mean freq, Mean frequency, Peak freq, Peak frequency.
Figure 2:

Picoejection of \([\text{Sar}^9-\text{Met(O}_2)^{11}\text{]-substance P (SSP)}\) excites tonically discharging expiratory neurons (ETONIC).

(A) Picoejection of SSP (thick horizontal line) resulted in an increase in peak firing frequency. From top to bottom: Instantaneous firing frequency (Inst. Freq), extracellular unit recording (VRG unit), integrated phrenic nerve discharge (PND). Horizontal dashed line is the peak discharge averaged over 10 respiratory cycles prior to picoejection.

(B) Enlargement of a single respiratory cycle illustrating the activity of this ETONIC neuron throughout expiration.

(C) Waveform analysis of the action potentials trajectories during baseline (blue) and SSP picoejection (red) during periods indicated by the blue and red horizontal lines in panel A. The waveform analysis confirmed that the recording was from the same single unit. The number of action potential shown in each overlay is indicated.

(D) Average number of action potentials (5 respiratory cycles, 50ms bins) during baseline (blue bars) and SSP picoejection (red bars) plotted in relation to phrenic nerve discharge (PND), where time 0 is the onset of a phrenic burst. These data demonstrate the increase in unit discharge rate throughout expiration with prolonged discharge duration.

(E) Quantification of the effect of \([\text{Sar}^9-\text{Met(O}_2)^{11}\text{]-substance P (SSP)}\) on ETONIC neurons (n=9). SSP picoejection significantly increased burst duration (burst dur't), number of action potentials generated during each burst (# APs), mean and peak discharge frequency (Mean freq and Peak freq, respectively) compared to baseline. The activity of these neurons rapidly recovered back to baseline levels (recovery). * = p < 0.05, One way measures ANOVA, post-hoc SNK, n = 9.
Figure 3:

Picoejection of [Sar⁹-Met(O₂)¹¹]-substance P (SSP) excites augmenting expiratory neurons (E_{AUG}).

(A) Picoejection of SSP (thick horizontal line) resulted in an increase in peak firing frequency. From top to bottom: Instantaneous firing frequency (Inst. Freq), extracellular unit recording (VRG unit), integrated phrenic nerve discharge (I\text{PND}). Horizontal dashed line is the peak discharge averaged over 10 respiratory cycles prior to picoejection.

(B) Enlargement of a single respiratory cycle illustrating the incrementing activity of this E_{AUG} neuron during late expiration terminating abruptly at the onset of the following inspiration.

(C) Waveform analysis of the action potentials trajectories during baseline (blue) and SSP picoejection (red) during periods indicated by the blue and red horizontal lines in panel A. The waveform analysis confirmed that the recording was from the same single unit. The number of action potential shown in each overlay is indicated.

(D) Average number of action potentials (5 respiratory cycles, 50ms bins) during baseline (blue bars) and SSP picoejection (red bars) plotted in relation to phrenic nerve discharge (PND), where time 0 is the onset of a phrenic burst. These data demonstrate the increase in unit discharge rate throughout expiration with prolonged discharge duration.

(E) Quantification of the effect of [Sar⁹-Met(O₂)¹¹]-substance P (SSP) on E\text{TONIC} neurons (n=9). SSP picoejection significantly increased burst duration (burst dur't), number of action potentials generated during each burst (# APs), mean and peak discharge frequency (Mean freq and Peak freq, respectively) compared to baseline. The activity of these neurons rapidly recovered back to baseline levels (recovery). * = p < 0.05, One way measures ANOVA, post-hoc SNK, n = 4.
Figure 4:

Picoejection of [Sar⁹-Met(O₂)¹¹]-substance P (SSP) excites decrementing-expiratory (E_{DEC}) neurons.

(A) Picoejection of SSP (thick horizontal line) resulted in an increase in the number of action potentials generated per burst but had no effect on peak firing frequency. From top to bottom: Instantaneous firing frequency (Inst. Freq), extracellular unit recording (VRG unit), integrated phrenic nerve discharge (IPND). Horizontal dashed line is the peak discharge averaged over 10 respiratory cycles prior to picoejection.

(B) Enlargement of a single respiratory cycle illustrating the activity of this E_{DEC} neuron with activity decreasing throughout expiration.

(C) Waveform analysis of the action potentials trajectories during baseline (blue) and SSP picoejection (red) during periods indicated by the blue and red horizontal lines in panel A. The waveform analysis confirmed that the recording was from the same single unit. The number of action potential shown in each overlay is indicated. Average number of action potentials (5 respiratory cycles, 50ms bins) during baseline (blue bars) and SSP picoejection (red bars) plotted in relation to the phrenic nerve discharge (PND), where time 0 is the end of a phrenic burst. This demonstrates that this neuron was excited throughout the duration of the neuronal discharge.

(D) Average number of action potentials (5 respiratory cycles, 50ms bins) during baseline (blue bars) and SSP picoejection (red bars) plotted in relation to phrenic nerve discharge (PND), where time 0 is the end of a phrenic burst. These data demonstrate the increase in unit discharge rate throughout expiration with prolonged discharge duration.
(E) Quantification of the effect of [Sar\textsuperscript{9}-Met(O\textsubscript{2})\textsuperscript{11}]-substance P (SSP) on E\textsubscript{DEC} neurons (n=6).

SSP picoejection significantly increased burst duration (burst dur't), number of action potentials generated during each burst (# APs). However, mean and peak discharge frequency (Mean freq and Peak freq, respectively) was not altered compared to baseline. The activity of these neurons rapidly recovered back to baseline levels (recovery). * = p < 0.05, One way measures ANOVA, post-hoc SNK, n = 6.

Figure 5:

Electrophysiological and functional characterisation of Bötzinger Complex prior to neurokinin1 receptor antagonism.

(A) Multiunit extracellular recording of compound unit activity during the expiratory phase, compared to the phrenic nerve discharge, demonstrating placement of the microelectrode in an expiratory region.

(B) Microinjection of DLH (6 pmol in 6nl) resulted in an extension of expiration duration confirming placement of the microelectrode in the Bötzinger Complex, as previously described (Fong & Potts, 2006; Monnier et al, 2003; Wang et al., 2002).

Figure 6:

Neurokinin1 receptor (NK1-R) antagonism in the Bötzinger Complex (BötC) abolished locomotor-respiratory coupling (LRC).

(A) Electrically induced contraction of the forelimb resulted in excitation of phrenic nerve discharge frequency (top trace) and phasically coupled phrenic nerve discharge (PND) to
forelimb contraction. Inset shows an enlarged section clearly illustrating the phasic coupling of PND onset to electrically evoked contraction.

(B) Following NK1-R blockade in the BötC, the phasic coupling between forelimb contraction and PND was lost, although overall excitation of PND remained.

(C) Post-stimulus time histogram of the latency between the onset of muscle tension and PND calculated from panels A and B. During control (black bars) there was a tight correlation between the onset of muscle tension and PND (average latency = 126ms). This relationship was lost following NK1-R blockade with CP99,994 (white bars) in the BötC (average latency = 606ms).

(D) Box-whisker plot of the latency between contraction onset and PND during control (black box) and following NK1-R blockade (white box). The latency was significantly lengthened following NK1-R blockade in the BötC. The latency between the onset of muscle tension and PND for each contraction is denoted by a gray dot. * p < 0.05, control vs. CP99,994, n = 57 (control) and 67 (CP99,994) stimulated contractions.

Figure 7:

Neurokinin1 receptor (NK1-R) antagonism blocks somatic afferent evoked excitation of augmenting expiratory neurons (E_AUG).

(A) Forelimb contraction excited this E_AUG neuron and reduced expiratory duration. Panel at the right shows an overlay of the respiratory period before (blue) and during (red) somatic afferent stimulation, as indicated by the dashed boxes on the left panel. The peak firing frequency of this neuron was increased by contraction. Inset illustrates waveform analysis of this neuron showing that the recording was obtained from a single unit.

(B) In the same neuron, blockade of NK1-R by CP99,994 prevented contraction-evoked excitation, although electrical stimulation of the forelimb still produced a shortening of expiratory duration. Panel at the right is an overlay of the respiratory period before (blue)
and during (red) forelimb contraction as indicated by the dashed boxes in the left panel. Following blockade of NK1-R there was no discernible change in the firing frequency between these two respiratory cycles. Inset illustrates waveform analysis of this neuron showing it is the same neuron as in Panel A.

(C) Quantified data showing that somatic afferent stimulation increased both the mean and peak discharge frequency of $E_{\text{AUG}}$ neurons. However, contraction evoked excitation was prevented by CP99,994. * $p < 0.05$, Paired $t$-test, Control vs. CP99,994, $n = 4$.

Figure 8:
Neurokinin1 receptor (NK1-R) blockade did not affect inhibition of decrementing-expiratory ($E_{\text{DEC}}$) neurons by somatic afferent stimulation. (A) Somatic afferent stimulation reduced expiratory duration ($T_E$) and the duration of $E_{\text{DEC}}$ discharge. (B) Contraction evoked inhibition of $T_E$ and $E_{\text{DEC}}$ discharge was not altered by CP99,994.

Figure 9:
Locations of recorded expiratory neurons in the ventral respiratory group and their responsiveness to picoejection of [Sar$^9$-Met(O$_2$)$^{11}$]-substance P (SSP), plotted using the stereotaxic co-ordinates documented on sections drawn from a representative brainstem. Red symbols represent neurons that were excited by picoejection of SSP. Gray symbols represent neurons that did not respond to SSP. Abbreviations: CS, calamus scriptorius; $E_{\text{AUG}}$, augmenting expiratory neuron; $E_{\text{TONIC}}$, tonic expiratory neuron; IO, inferior olivary complex; NTS, nucleus tractus solitarius; $E_{\text{DEC}}$, decrementing-expiratory neuron; Sp5, spinal trigeminal nucleus.

Figure 10:
Proposed models of SPergic modulation of respiratory rhythm in the pontomedullary axis.
(A) Parasagittal section of the brainstem illustrating the proposed neural pathways responsible for mediating the Hering-Breuer (HB) Reflex (blue) and locomotor-respiratory coupling (LRC, green). Slowly adapting pulmonary stretch receptors (SAR) project to the NTS where they synapse onto pump cells. These neurons, in turn, project to the Bötzinger Complex (BötC) and activate decrementing-expiratory (E_{DEC}) neurons that inhibit inspiratory (I) and augmenting expiratory (E_{AUG}) cells resulting in early termination of inspiration, prolongation of expiration and the slowing of respiratory frequency (Panel B). Contraction-sensitive somatic afferents, on the other hand, project to the dorsal pons (including the lateral parabrachial complex) via a synapse in the dorsal horn and activate excitatory cells which project to BötC. These neurons selectively target E_{AUG} neurons which, in turn, inhibits E_{DEC} neurons resulting in the disinhibition of I neurons. Release of phasic inhibition by E_{DEC} neurons promotes the early onset of inspiration and increases respiratory frequency (Panel B). Abbreviations: 7, facial nucleus; BötC, Bötzinger complex; CVLM, caudal ventrolateral medulla; E_{AUG}, augmenting expiratory neuron; I, inspiratory neuron, NK1-R, neurokinin1 receptor; NTS, nucleus tractus solitarius; E_{DEC}, decrementing-expiratory neuron; Pre-BötC, Pre-Bötzinger complex; r/cVRG, rostral / caudal ventral respiratory group; SAR, Slowly adapting pulmonary stretch receptors.
Table 1 - Effect of [Sar⁹-Met(O₂)¹¹]-substance P (SSP) picoejection on expiratory neurons by neuronal subtype

<table>
<thead>
<tr>
<th></th>
<th>E_{DEC}</th>
<th>E_{TONIC}</th>
<th>E_{AUG}</th>
<th>Others</th>
<th>TOTALS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Excited by</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSP</td>
<td>6 (6/20 = 30%)</td>
<td>9 (9/20 = 45%)</td>
<td>4 (4/20 = 20%)</td>
<td>1 (1/20 = 5%)</td>
<td>20</td>
</tr>
<tr>
<td><strong>No effect</strong></td>
<td>6 (6/15 = 40%)</td>
<td>4 (4/15 = 27%)</td>
<td>4 (4/15 = 27%)</td>
<td>1 (1/15 = 6%)</td>
<td>15</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>12</td>
<td>14</td>
<td>9</td>
<td>2</td>
<td>35</td>
</tr>
</tbody>
</table>

E_{AUG}, augmenting expiratory neuron; E_{TONIC}, tonically-discharging expiratory neuron; E_{DEC}, decrementing-expiratory neuron
Table 2 - Response of expiratory neurons to picoejection of \([\text{Sar}^9-\text{Met(O}_2)^{11}\text{-]}}\)-substance P (SSP)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>During SSP</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Burst dur’n (sec)</strong></td>
<td>0.74 ± 0.12</td>
<td>0.82 ± 0.11*</td>
<td>0.72 ± 0.10</td>
</tr>
<tr>
<td><strong>Number of APs per burst</strong></td>
<td>17.30 ± 2.36</td>
<td>24.07 ± 3.80*</td>
<td>16.79 ± 2.28</td>
</tr>
<tr>
<td><strong>Mean freq (Hz)</strong></td>
<td>37.37 ± 5.71</td>
<td>41.07 ± 4.85*</td>
<td>35.40 ± 5.41</td>
</tr>
<tr>
<td><strong>Peak freq (Hz)</strong></td>
<td>91.23 ± 11.65</td>
<td>103.13 ± 11.08*</td>
<td>86.35 ± 10.84</td>
</tr>
</tbody>
</table>

Abbreviations: AP, action potential. * = p < 0.05, One way repeated measures ANOVA on ranks, n = 20 neurons. No difference between baseline and recovery states. Mean ± SEM.
Table 3: Phrenic burst parameters before, during and after picoejection of [Sar⁹-Met(O₂)¹¹]-substance P (SSP) onto expiratory neurons

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>During SSP</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tᵢ (sec)</strong></td>
<td>0.45 ± 0.02</td>
<td>0.45 ± 0.02</td>
<td>0.45 ± 0.02</td>
</tr>
<tr>
<td><strong>Tₑ (sec)</strong></td>
<td>1.29 ± 0.13</td>
<td>1.28 ± 0.12</td>
<td>1.26 ± 0.11</td>
</tr>
<tr>
<td><strong>Tₜtot (sec)</strong></td>
<td>1.74 ± 0.12</td>
<td>1.73 ± 0.12</td>
<td>1.71 ± 0.11</td>
</tr>
<tr>
<td><strong>PN freq (Hz)</strong></td>
<td>0.65 ± 0.05</td>
<td>0.65 ± 0.04</td>
<td>0.65 ± 0.04</td>
</tr>
<tr>
<td><strong>PN burst area</strong></td>
<td>1.0 ± 0.10</td>
<td>0.98 ± 1.39</td>
<td>0.97 ± 1.73</td>
</tr>
<tr>
<td><strong>PN amplitude</strong></td>
<td>1.0 ± 0.00</td>
<td>99.4 ± 0.81</td>
<td>0.97 ± 1.28</td>
</tr>
</tbody>
</table>

Abbreviations: Tᵢ, inspiratory duration; Tₑ, expiratory duration; Tₜtot, total respiratory cycle time; PN, phrenic nerve. Values presented as means ± SEM. One way repeated measures ANOVA on ranks, p > 0.05, n = 20.
TABLE 4: Response of tonic-expiratory neurons to picoejection of neurokinin-1 receptor blockade by CP99,994 (CP)

<table>
<thead>
<tr>
<th></th>
<th>Before CP</th>
<th>After CP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Burst dur’n (sec)</strong></td>
<td>0.56 ± 0.15</td>
<td>0.49 ± 0.13</td>
</tr>
<tr>
<td><strong>Number of APs per burst</strong></td>
<td>10.27 ± 3.19</td>
<td>9.49 ± 3.43</td>
</tr>
<tr>
<td><strong>Mean freq (Hz)</strong></td>
<td>19.80 ± 6.36</td>
<td>19.38 ± 70.81</td>
</tr>
<tr>
<td><strong>Peak freq (Hz)</strong></td>
<td>55.04 ± 19.91</td>
<td>54.80 ± 23.77</td>
</tr>
</tbody>
</table>

Abbreviations: AP, action potential. Paired student’s t-test, n = 5 neurons. No difference between before and after CP picoejection. Mean ± SEM.
Table 5: Effect of somatic afferent stimulation on decrementing-expiratory neurons before and after NK1-R blockade by CP99,994

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CP99,994</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Burst duration (sec)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.83 ± 0.16</td>
<td>0.74 ± 0.11</td>
</tr>
<tr>
<td>Stimulation</td>
<td>0.40 ± 0.07*</td>
<td>0.47 ± 0.06*</td>
</tr>
<tr>
<td><strong>Number of action potentials per burst</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>26.31 ± 4.05</td>
<td>22.08 ± 3.02</td>
</tr>
<tr>
<td>Stimulation</td>
<td>15.49 ± 3.30*</td>
<td>14.22 ± 2.26*</td>
</tr>
<tr>
<td><strong>T&lt;sub&gt;E&lt;/sub&gt; (sec)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.30 ± 0.18</td>
<td>1.22 ± 0.14</td>
</tr>
<tr>
<td>Stimulation</td>
<td>0.86 ± 0.08*</td>
<td>0.89 ± 0.12*</td>
</tr>
</tbody>
</table>

Abbreviations: T<sub>E</sub>, expiratory duration. Values presented as means ± SEM. * = p < 0.05, Paired t-test, baseline vs. stimulation during control or following CP99,994 (n = 7). No difference between any measurements during control and following CP99,994.
FIG 2

A

SSP

Inst Freq (Hz)

VRG Unit

nPND

2sec

B

VRC Unit

PNC

398 spikes

391 spikes

C

D

Av # of action potentials

E

% of baseline

Burst dur' AP / burst Mean freq Peak freq

n = 9

Baseline SSP Recovery

* * *
FIG 3

A

Inst Freq (Hz)

VRG Unit

∫ PND

2 sec

B

VRG Unit

PND

111 spikes

112 spikes

C

D

E

Av. # action potentials

Time (sec)

% of baseline

Burst dur't AP / burst Mean freq Peak freq

Baseline SSP Recovery

n = 4

*
FIG 4

A

Inst Freq (Hz)

VRG Unit

∫ PND

2sec

B

VRG Unit

PND

C

231 spikes

328 spikes

D

Av # action potentials

PND

SSP

Time (sec)

E

% of baseline

Burst dur' AP / burst Mean freq Peak freq

Baseline SSP Recovery
**FIG 7**

**A  Control**

Inst. Freq (Hz)
VRG Unit
PND
Tension (g)

![Control Waveform]

2.1sec 1.3sec
151 spikes

**B  CP99,994**

Inst. Freq (Hz)
VRG Unit
PND
Tension (g)

![CP99,994 Waveform]

1.8sec 1.4sec
196 spikes

**C**

![Bar Chart]

Mean freq  peak freq

* = stimulus artifact

Baseline
Contraction

** = stim. art.
HB reflex: Inspiratory off-switch

Locomotor-respiratory coupling

FIG 10