

Neurokinin-1 receptor activation in Böttinger complex evokes bradypnoea

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In the present study, we examined the role of the neurokinin-1 receptor (NK1R) in the modulation of respiratory rhythm in a functionally identified bradypnoeic region of the ventral respiratory group (VRG) in the *in situ* arterially perfused juvenile rat preparation. In electrophysiologically and functionally identified bradypnoeic sites corresponding to the Böttinger complex (BötC), microinjection of the selective NK1R agonist [Sar⁹-Met(O₂)¹¹]-substance P (SSP) produced a significant reduction in phrenic frequency mediated exclusively by an increase in expiratory duration (T_E). The reduction was characterized by a significant increase in postinspiratory (post-I) duration with no effect on either late-expiratory duration (E2) or inspiratory duration (T_I). In contrast, in a functionally identified tachypnoeic region, corresponding to the preBöttinger complex (Pre-BötC), control microinjection of SSP elicited tachypnoea. Pretreatment with the NK1R antagonist CP99994 in the BötC significantly attenuated the bradypnoeic response to SSP injection and blunted the increase in T_E duration. This effect of SSP mimicked the extension of T_E produced by activation of the Hering-Breuer reflex. Therefore, we hypothesized that activation of NK1Rs in the BötC is requisite for the expiratory-lengthening effect of the Hering-Breuer reflex. Unilateral electrical stimulation of the cervical vagus nerve produced bradypnoea by exclusively extending T_E . Ipsilateral blockade of NK1Rs by CP99994 following blockade of the contralateral BötC by the GABA_A receptor agonist muscimol significantly reduced the extension of T_E produced by vagal stimulation. Results from the present study demonstrate that selective activation of NK1Rs in a functionally identified bradypnoeic region of the VRG can depress respiratory frequency by selectively lengthening post-I duration and provide evidence that endogenous activation of NK1Rs in the BötC appears to be involved in the expiratory-lengthening effect of the Hering-Breuer reflex. In conclusion, our findings demonstrate that selective activation of NK1Rs in discrete regions of the VRG can exert functionally diverse effects on breathing.

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The ventral respiratory group (VRG), a longitudinal column of heterogeneous cells located within the ventrolateral medulla, plays an integral role in the generation of respiratory rhythm (Richter, 1982; Richter & Spyer, 2001; Feldman & McCrimmon, 2003). This column of cells is divided into four rostral to caudal compartments, Böttinger complex (BötC), preBöttinger complex (Pre-BötC), rostral VRG (rVRG) and caudal VRG (cVRG), respectively, based on electrophysiological characterization of the respiratory neuronal phenotype (Ezure, 1990; Schwarzacher *et al.* 1991; Connelly *et al.* 1992; Sun *et al.* 1998; Chitravanshi & Sapru, 1999) and anatomical studies (Ellenberger & Feldman, 1988, 1990; Dobbins & Feldman, 1994; Alheid *et al.* 2002). It has

been shown that chemical excitation of the Pre-BötC with the glutamate receptor agonist dl-homocysteic acid (DLH) elicits tachypnoea, while activation of BötC and rVRG produces bradypnoea (Chitravanshi & Sapru, 1999; McCrimmon *et al.* 2000; Wang *et al.* 2002; Monnier *et al.* 2003). Together, these studies demonstrate a functional means to characterize VRG compartments (Chitravanshi & Sapru, 1999; McCrimmon *et al.* 2000; Wang *et al.* 2002; Monnier *et al.* 2003).

The role of substance P (SP) and its receptor, neurokinin-1 receptor (NK1R), in respiratory rhythm and pattern generation has received considerable attention. Exogenous SP is known to excite respiratory activity both *in vivo* and *in vitro* (Hedner *et al.* 1984; Chen *et al.* 1990;

Monteau *et al.* 1996; Johnson *et al.* 1996; Ptak *et al.* 1999, 2000; Shvarev *et al.* 2002; Morgado-Valle & Feldman, 2004). In addition, NK1Rs have also been implicated in mediating hypoxic respiratory responses and central chemosensitivity (Chen *et al.* 1990; Ptak *et al.* 2002; Nattie & Li, 2002; Wickstrom *et al.* 2004). Respiratory neurones throughout regions of the VRG, in particular the Pre-BötC, express NK1Rs (Nakaya *et al.* 1994; Gray *et al.* 1999; Wang *et al.* 2001; Guyenet *et al.* 2002). Furthermore, presumptive SPergic terminals have been shown to synapse onto NK1R expressing neurones in the VRG (Liu *et al.* 2004). Although genetic deletion of NK1R failed to alter basal respiratory rhythm in NK1R knockout mice (Ptak *et al.* 2000, 2002), basal respiratory rhythm was severely disrupted when NK1R expressing neurones were regionally ablated in the Pre-BötC (Gray *et al.* 2001; Wang *et al.* 2002; Wenninger *et al.* 2004; McKay *et al.* 2005). Moreover, a recent study has demonstrated that ablation of NK1R expressing neurones in the Pre-BötC resulted in a severe disruption in breathing during both sleep and wakefulness (McKay *et al.* 2005). Collectively, these data suggest that NK1R expressing neurones in the Pre-BötC, and perhaps in other VRG compartments, are required for normal breathing.

While previous studies have focused on the effect of NK1R activation in the Pre-BötC, the effect of SP in the adjacent BötC is not well understood. The BötC contains predominantly expiratory neurones (Schwarzacher *et al.* 1995; Sun *et al.* 1998; Chitravanshi & Sapru, 1999) with widespread projections throughout the VRG (Ezure & Manabe, 1988; Ezure *et al.* 2003). Chemical activation of the BötC by glutamate receptor agonists decreases respiratory frequency by selectively lengthening expiratory duration (Chitravanshi & Sapru, 1999; Wang *et al.* 2002; Monnier *et al.* 2003). The BötC has also been implicated in expiratory-lengthening reflexes such as the Hering-Breuer reflex (Manabe & Ezure, 1988; Ezure & Manabe, 1988; Hayashi *et al.* 1996). Since this region contains NK1R expressing neurones (Wang *et al.* 2001; Guyenet *et al.* 2002), the present study was designed to: (1) examine the effect of activating NK1R-expressing neurones in the BötC on respiratory frequency and (2) determine the role of NK1Rs in the BötC in expiratory-lengthening effect of the Hering-Breuer reflex using the *in situ* arterial-perfused juvenile rat preparation. We hypothesized that discrete microinjection of the selective NK1R agonist [Sar⁹-Met(O₂)¹¹]-substance P (SSP) into a functionally identified bradypnoeic region of the VRG would reduce respiratory frequency by increasing expiratory duration. Furthermore, we hypothesized that activation of NK1Rs in the BötC is necessary for the Hering-Breuer reflex. A portion of this work has been previously presented (Fong & Potts, 2005).

Methods

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

In situ working heart-brainstem preparation

Experiments were performed on juvenile male Wistar rats (55–120 g, $n = 38$, 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 (Paton, 1996; Potts *et al.* 2005). In brief, rats were deeply anaesthetized with halothane via spontaneous inhalation. 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 body immediately submerged in ice-cold Ringer solution bubbled with carbogen gas (95% O₂–5% CO₂). It was decerebrated precollicularly, and a portion of the posterior thoracic wall was removed to expose the heart and lungs, and it was then skinned. The descending aorta, phrenic nerves (PN) and in a subset of animals ($n = 7$) the right central vagus nerve (CVN) were blunt dissected, isolated and cut. The cerebellum was removed to exposure the dorsal surface of the brainstem. After the surgery, the preparation was transferred to an acrylic chamber and the thoracic aorta retrogradely cannulated with a double lumen catheter (16 and 18 ga; Braintree Scientific, Inc., Braintree, MA, USA) until the catheter tip was just caudal to the aortic arch and secured. Perfusion of Ringer solution bubbled with 95% O₂–5% CO₂ began immediately using a pump equipped with a multiroller pump head (model 505s pump drive; model 314DW2 pump head; Watson-Marlow Bredel, Inc., Wilmington, MA, USA). The perfusate was warmed to 31–33°C using an in-line heat exchanger, pumped through two in-line bubble traps and a filter (polypropylene mesh; pore size 40 µm, Millipore). Perfusion pressure was measured via one of the lumens of the double-lumen catheter using a pressure transducer (model PT300, Grass-Telefactor (Astro-Med), West Warwick, RI, USA), 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 and 36 ml min⁻¹ were used in the current study. Average elapsed time from induction of anaesthesia to start of systemic perfusion was 15 min.

Whole nerve activity was recorded from the PN and the CVN via suction electrodes (tip diameter, 0.2–0.3 mm). Raw neurograms were amplified (20 000–50 000×) and filtered (100 Hz to 3 kHz, model P511, Grass-Telefactor (Astro-Med)). The electrocardiogram (ECG) was measured via silver wires placed directly on the heart.

The ECG waveform was amplified (10 000–20 000 $\times$), filtered (100 Hz to 3 kHz, model P511; Grass-Telefactor (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). Neuromuscular paralysis was produced by addition of vecuronium bromide (50 μ g) directly to the perfusate.

Experimental protocol

Multibarrelled glass micropipettes containing four to five barrels (1.2 mm o.d., 0.68 mm i.d., World Precision Instruments, Sarasota, FL, USA) were pulled and broken back to yield a total tip diameter of <30 μ m. One barrel was filled with 3 M NaCl and a silver wire introduced into the NaCl solution for recording neuronal activity (electrode tip impedance = 8–14 M Ω). The neuronal activity was recorded, amplified using an AC amplifier (5 000–10 000 $\times$; NeuroLog NL104; Digitimer Ltd, Welwyn Garden City, UK) and filtered (500 Hz to 3 kHz; NeuroLog NL126; Digitimer). The remaining barrels were filled with different combinations of drugs as described in the following section. The ends of the microinjection barrels were sealed with polyethylene tubing connected to a pneumatic pressure injection system (Picospritzer II, NeuroLog). The micropipette was secured to a pipette holder attached to a piezoelectric stepper motor (Inch Worm, model IW-711-01; Burleigh Instruments, Toronto, ON, Canada) driven by a low-noise controller (model 6200ULN-1-1; Burleigh Instruments) mounted on a stereotaxic arm. The tip of the micropipette was placed at calamus scriptorius (CS) using a stereomicroscope (Carl Zeiss) and this point was used as relative zero for the rostro-caudal and lateral displacement of the pipette.

The pipette was implanted into the VRG between the following co-ordinates from CS: AP +0.6–1.8 mm, ML $\pm$ 1.4–2.0 mm and at a depth of 1800–2700 μ m from the dorsal surface of the medulla. Extracellular unit activity was recorded as the micropipette was advanced ventrally in the medulla and the locations of respiratory neurones noted. Once a respiratory region was identified electrophysiologically, the micropipette was allowed to rest for 10 min before microinjection of the glutamate receptor agonist, dl-homocysteic acid (DLH) to functionally identify the site, as described in the following section. Following the functional identification of the injection site, the experiment for the microinjection of SSP continued without altering the position of the micropipette. All injection volumes (6 nl) were monitored by direct observation of the movement of the fluid meniscus using a compound microscope (Wild Heerbrugg, Easley, SC, USA) equipped with a fine calibrated reticule (total

magnification $\times$ 62), allowing a resolution of 6 nl per reticule division.

Electrophysiological and functional identification of VRG injection site

In preliminary experiments, an effective dose of DLH was determined for the functional identification of an injection site. Both 6 and 30 pmol in 6 nl significantly altered phrenic frequency. However, since there was no difference between the peak responses produced by the two doses (data not shown) the lower dose of DLH (6 pmol in 6 nl) was used in subsequent experiments.

An example of the method used to electrophysiologically and functionally identify a microinjection site is illustrated in Fig. 1. Extracellular recording of mass respiratory neuronal activity was performed to locate an expiratory region in the VRG complex, containing post-I, E2 and/or tonic expiratory neurones (Fig. 1A), using stereotaxic co-ordinates that targeted the BötC (Fig. 1B). Once the region was identified, the broad spectrum glutamate receptor agonist DLH was microinjected to confirm that this was a functionally bradypnoeic region (Fig. 1C), as seen by the lengthening of the phrenic interburst interval and a transient reduction in phrenic amplitude, similar to responses previously described (Monnier *et al.* 2003). Recordings obtained from regions that demonstrated primarily inspiratory neuronal activity (Fig. 1D), which corresponding histologically to the Pre-BötC (Fig. 1E), produced tachypnoea (Fig. 1F).

Microinjection of SSP into functionally identified VRG injection sites

Once the injection site was both electrophysiologically and functionally identified as described above (Fig. 1), [Sar⁹, Met(O₂)¹¹]-substance P (SSP, 3–12 pmol in 6 nl) was microinjected into the same site to examine the effect of NK1R activation in predetermined regions of the VRG. Due to desensitization of NK1Rs, each injection site received only one injection of SSP in this current set of experiments. A minimum of 15 min was permitted between microinjection of DLH and SSP.

To further examine the effect of NK1R activation on the duration of different phases of respiratory cycle, the CVN was recorded in a subset of experiments ($n = 7$, Fig. 3). The CVN was chosen since it contains motor activity during the inspiratory (T_1) and postinspiratory (post-I) periods (Paton, 1996), thus permitting accurate identification of post-I and late-expiratory (E2) phases.

Verification of NK1R activation by SSP

To further confirm that the effect of SSP was via activation of NK1R, a selective NK1R antagonist, CP99994, was microinjected prior to a subsequent SSP microinjection. In

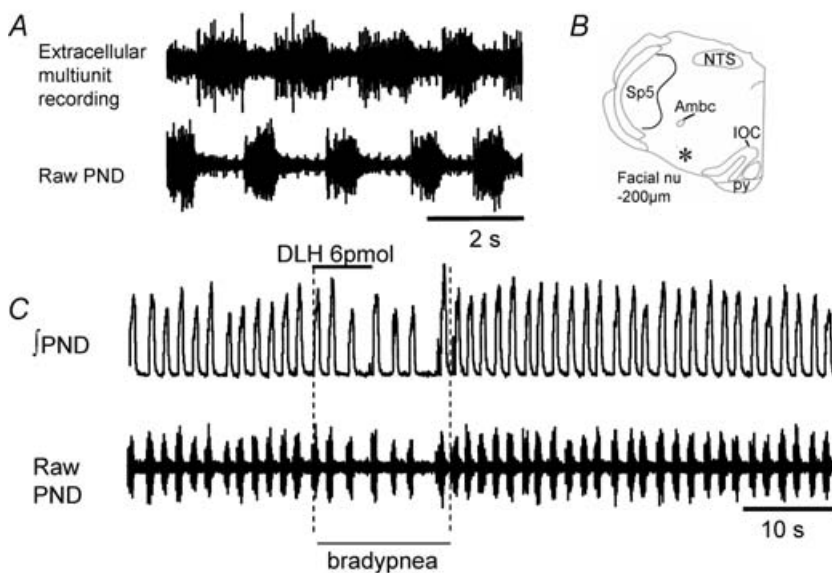
preliminary experiments, we determined that the response to SSP could be reproduced following a 60 min recovery period, while repeat microinjections of SSP at earlier time points (e.g. 30–45 min between microinjections) failed to elicit a response (unpublished personal observation). For this part of the study, the effect of SSP (6 pmol in 6 nl) was examined, the micropipette then remained in position for a further 60 min before either vehicle (Ringer solution, 6 nl, pH 7.4) or CP99994 (6 pmol in 6 nl, pH 7.4) was microinjected. Repeat microinjection of SSP was then performed within 2 min following vehicle or CP99994 microinjection.

Role of NK1Rs in BötC on vagal afferent stimulation

In a subset of preparations ($n = 5$), the cervical vagus nerve was isolated and electrically stimulated to examine the role of NK1Rs in the BötC on vagally mediated bradypnoea. The cervical vagus nerve was bluntly dissected, as described above, and two Teflon coated silver wires, with the last 5 mm exposed, were wrapped around the nerve 5 mm apart and embedded in low-melting-point wax.

The threshold of electrical stimulation (25–200 μ A, 50 Hz, pulse duration 1–3 ms for 12 s) required to

Bradypneic site



Tachypneic site

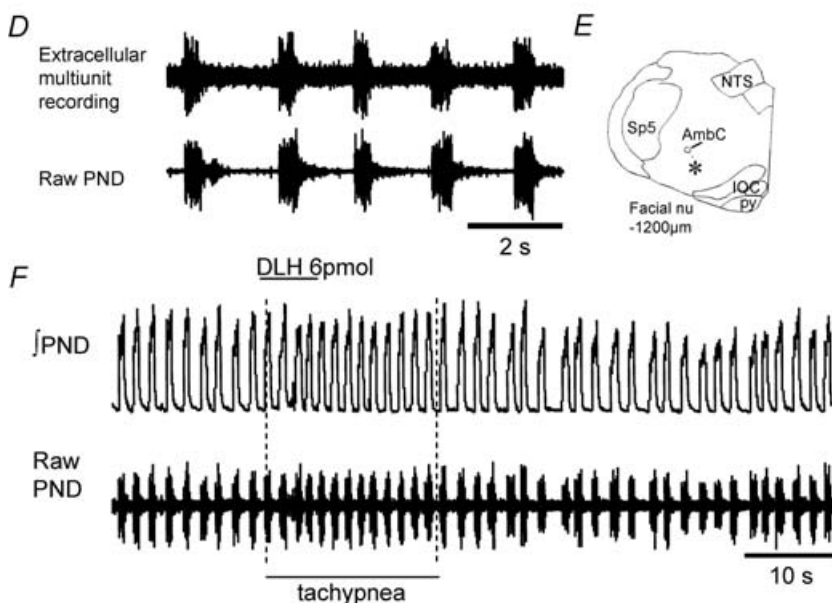


Figure 1. Examples of electrophysiological and functional identification of microinjection sites in the VRG

A, extracellular recording of respiratory neuronal activity correlated with raw phrenic nerve discharge (PND) used to electrophysiologically localize a microinjection site, illustrating predominantly neuronal activity during expiration. *B*, section reconstruction of the recording and injection site. The site, indicated by the asterisk (*), was located ventral to the compact formation of the nucleus ambiguus and is 200 μ m caudal to the caudal pole of the facial nucleus. *C*, rectified and integrated phrenic nerve discharge ($\int$ PND) and raw PND demonstrating the bradypnoea following microinjection of 6 pmol DLH in the site in *A*, demonstrating the functional identification of a bradypneic site. *D*, extracellular recording of respiratory neuronal activity demonstrating activity correlated to inspiration. *E*, section reconstruction of the recording and injection site. The site, indicated by the asterisk (*), was located ventral to the compact formation of the nucleus ambiguus and is 1200 μ m caudal to the caudal pole of the facial nucleus. *F*, raw PND and $\int$ PND demonstrating the tachypnoea following microinjection of 6 pmol DLH in the site in *D*, demonstrating the functional identification of a tachypneic site. The time of microinjections are indicated by horizontal bars and the duration of notable effect shown by the vertical dashed lines.

produce a detectable increase in T_E was determined in each preparation prior to the microinjection protocol. The stimulation current chosen during for each preparation was 1.5 times threshold. These parameters were similar to those previously described for activation of the Hering-Breuer reflex by vagal stimulation (Hayashi *et al.* 1996; Wang *et al.* 2005; Li *et al.* 2006).

To allow for the examination of the role of NK1Rs in the BötC ipsilateral to the stimulation, the contralateral BötC was first chemically inactivated by microinjection of the GABA_A agonist muscimol (30 pmol in 30 nl), following functional identification of the injection site. The micro-pipette was then retracted and moved to the brainstem ipsilateral to the vagus nerve and a DLH bradypnoeic region was again identified. Once a bradypnoeic region was located, the NK1R antagonist, CP99994 (500 pmol in 50 nl) was microinjected into the same site. The average time between microinjection of muscimol and CP99994 in both BötC was 20 min. The vagus nerve was electrically stimulated before microinjection of any drugs (control) and repeated after microinjection of muscimol and CP99994. The recovery response was tested 30 min after CP99994 microinjection.

Histological verification

At the end of each experiment, fluorescent microspheres labelled with Alexa-Fluor 488[®] (0.4% solids in Ringer solution, 0.4 μ m, Molecular Probes, Eugene, OR, USA) were microinjected to mark the injection site. The brainstem was then removed, fixed in 10% formalin for 4–7 days and cryoprotected in 30% sucrose. Coronal cryosections (50 μ m) were mounted onto glass slides in 0.5% gelatin, coverslipped with Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA) and sealed with clear nail polish.

Microscopy and image analysis

The microinjection site was determined in sections using an Olympus microscope (BX51), equipped with the appropriate filter set for Alexa Fluor 488. Once the section containing the microinjection site was identified, the section was reconstructed and major landmarks drawn and the injection site plotted using NeuroLucida imaging software (ver. 6.50, MicroBrightField, Inc., Williston, VT, USA) controlling a 3-axis motorized stage (Ludl Instruments, Hawthorne, NY, USA) and a cooled monochrome digital camera (ORCA-AG, Hamamatsu). Each injection site was further characterized by identification of the caudal pole of the facial nucleus and the distance between this landmark and the injection site determined based on the number of sections separating the two.

NK1R immunohistochemistry

Once sections containing the injection sites were identified and photographed and the outlines drawn, they were carefully removed from the slides and processed for NK1R immunohistochemistry to examine the relationship between the injection site and NK1R immunopositive cells. All incubations were performed in 48-well culture plates at room temperature on a shaker table and all washes were performed in 0.1 M phosphate buffered saline (PBS, pH 7.4) at room temperature. The sections were incubated for 30 min in a preblocking solution of 10% normal goat serum (NGS) with 0.3% Triton X-100 in 0.1 M PBS prior to three brief washes (5 min each). The free-floating sections were then incubated overnight in the primary antibody, rabbit polyclonal raised against NK1R (1:1000, Novus Biologicals, Inc., Littleton, CO, USA), with 1% NGS and 0.3% Triton X-100 in 0.1 M PBS (pH 7.4). The following day, the sections were washed (3 $\times$ 10 min) prior to incubation in the secondary antibody, goat antirabbit-IgG conjugated to Cy3 (1:300, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) with 1% NGS and 0.3% Triton X-100 in 0.1 M PBS. Sections were again washed prior to mounting onto glass slides in 0.5% gelatin, coverslipped with Vectashield (Vector Laboratories) and sealed with clear nail polish.

Representative photomicrographs of NK1R immunoreactivity and the fluorospheres labelling the injection sites were captured using an Olympus microscope (BX51), equipped with the appropriate filter sets for Alexa Fluor 488 and Cy3, together with a cooled monochrome digital camera (ORCA-AG, Hamamatsu) and NeuroLucida imaging software (v. 6.50, MicroBrightfield). The digital images were imported and colourized using Adobe Photoshop 6.0 (Adobe Systems, Mountain View, CA, USA).

Data and statistical analyses

All data were collected on-line using commercially available data acquisition hardware and software (micro1401 analog-to-digital converter, Spike2 software, v. 5.12; Cambridge Electronic Design, Cambridge, UK). Raw neurograms were sampled at 2.5 kHz, while extracellular recording was sampled at 10 kHz. Analyses were performed off-line using custom written scripts. Changes in respiratory measurements in response to SSP microinjection were expressed as the percentage change from baseline. The baseline was determined by averaging the variables measured for 10 respiratory cycles prior to microinjection. All variables, as described below, were continuously measured for 2 min following each injection. This time frame was chosen as this is sufficient for respiratory function to recover to control values.

The following variables were measured for phrenic nerve discharge (PND): inspiratory duration (T_I), determined as duration of phrenic burst; expiratory duration (T_E) determined as the duration from the end of one phrenic burst to the onset of the following burst; and frequency, calculated from the sum of T_E and T_I and expressed in hertz (Hz). In the subset of experiments where the CVN was recorded, the duration of the post-I and E2 phases was determined, as well as the peak amplitude of post-I activity and the area under the curve during post-I period was calculated using the rectified and integrated CVN neurogram. The duration of the post-I phase was determined as the period between the end of inspiration, as determined from the PND and the abrupt decrease in CVN activity, as illustrated in Fig. 3 (Potts *et al.* 2005). E2 phase duration was calculated as the difference between T_E and post-I duration. For the vagal nerve stimulation studies, baseline respiratory measurements (phrenic frequency, T_E and T_I) were obtained prior to the start of the stimulation and throughout the duration of stimulation and for at least 10 phrenic burst cycles following cessation of the stimulation. The time of 10 phrenic bursts was chosen as the phrenic frequency was found to return to baseline measurements in that time frame.

All graphs were plotted using the software package GraphPad Prism v. 4.03 (San Diego, CA, USA). All data are presented as the mean $\pm$ s.e.m. All statistical analysis was performed using the software program SAS (v. 9.1, SAS Institute, Cary, NC, USA). Statistical significance for the effect of drug microinjection on respiratory variables was determined using one-way repeated measures ANOVA. The peak response to vagal stimulation and the respiratory frequency before and after chemical inactivation by muscimol, NK1R blockade by CP99994 and following CP99994 recovery was compared using a one-way repeated measures ANOVA. The peak effect of each dose of SSP microinjection was compared to baseline using Student's *t* test for one-sample. The peak response between the two doses of SSP was compared using an unpaired *t* test since each preparation received only a single dose of SSP due to desensitization of NK1Rs, as described earlier. A paired *t* test was used to compare the respiratory parameters before and after CP99994 microinjection, as well as the effect of vagal stimulation on T_E compared to baseline. $P < 0.05$ was considered significant in all cases.

Solutions and drugs

The Ringer's solution, containing 125 mM NaCl, 2.4 mM NaHCO_3 , 5 mM KCl, 2.5 mM CaCl_2 , 1.25 mM MgSO_4 , 1.25 mM KH_2PO_4 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 solution as an oncotic agent, resulting in an oncotic

pressure of the perfusate of approximately 310 mosmol ($\text{kg H}_2\text{O}$) $^{-1}$. The neuromuscular blocking agent, vecuronium bromide ($0.04 \mu\text{g ml}^{-1}$, Sico Pharmaceuticals), was also applied to the perfusate. All drugs (DLH, SSP, CP, muscimol) were dissolved in the Ringer solution and pH adjusted to 7.4. The concentrations of drugs were DLH (1–5 mM), SSP (500 μM to 2 mM), CP99994 (1 mM), muscimol (1 mM). The dose of each drug calculated per 6 nL microinjection volume was DLH (6–30 pmol), SSP (3–12 pmol) and CP99994 (6 pmol). For the vagal stimulation experiments, the dose of muscimol calculated for 30 nL was 30 pmol, and CP99994 was 500 pmol in 50 nL. Fluorescent microspheres (0.4% final concentration, Molecular Probes) were diluted from a 2% stock solution in Ringer solution immediately prior to use.

Results

In 38 *in situ* working heart–brainstem preparations, baseline perfusion pressure, heart rate and phrenic burst frequency were 64 ± 2.2 mmHg, 383 ± 8 bpm and 0.58 ± 0.04 Hz, respectively.

NK1R activation in the BötC

Microinjection of SSP (6 pmol) into a predetermined DLH sensitive bradypnoeic region resulted in bradypnoea (Fig. 2A). In initial experiments, a dose of 3 pmol SSP failed to elicit consistent changes in phrenic frequency (data not shown). However, microinjection of both 6 pmol ($n = 16$) and 12 pmol ($n = 7$) SSP consistently reduced phrenic frequency (Fig. 2B), although a significant decrease in frequency was only observed for 6 pmol SSP ($P < 0.05$, One way repeated measures ANOVA). This was likely to be due to the considerable variability in phrenic frequency produced by 12 pmol SSP which persisted over 30–50 cycles. A similar observation of variable respiratory responses was reported by Gray *et al.* (2001) in the Pre-BötC. Although the peak reduction in phrenic frequency following each dose of SSP was significantly different from baseline, there was no statistical difference between these doses (6 pmol: $-16.2 \pm 4.3\%$; 12 pmol: $-28.3 \pm 8.9\%$, $P > 0.05$, unpaired *t* test). Both doses produced an immediate and significant lengthening of T_E (Fig. 2C, 6 pmol: $+53.4 \pm 20.5\%$; 12 pmol: $+95.7 \pm 21.5\%$, $P < 0.05$, one way repeated measure ANOVA). In contrast, SSP failed to alter T_I (Fig. 2D, 6 pmol: $+9.1 \pm 7.7\%$; 12 pmol: $+7.6 \pm 6.7\%$, $P > 0.05$, one way repeated measure ANOVA). An example a bradypnoeic injection site, corresponding to the BötC, from data obtain in Fig. 2A is shown in Fig. 2E. Since the lower dose of SSP produced consistent effects, without producing the variability of the higher dose, this dose, 6 pmol SSP, was chosen for the remainder of

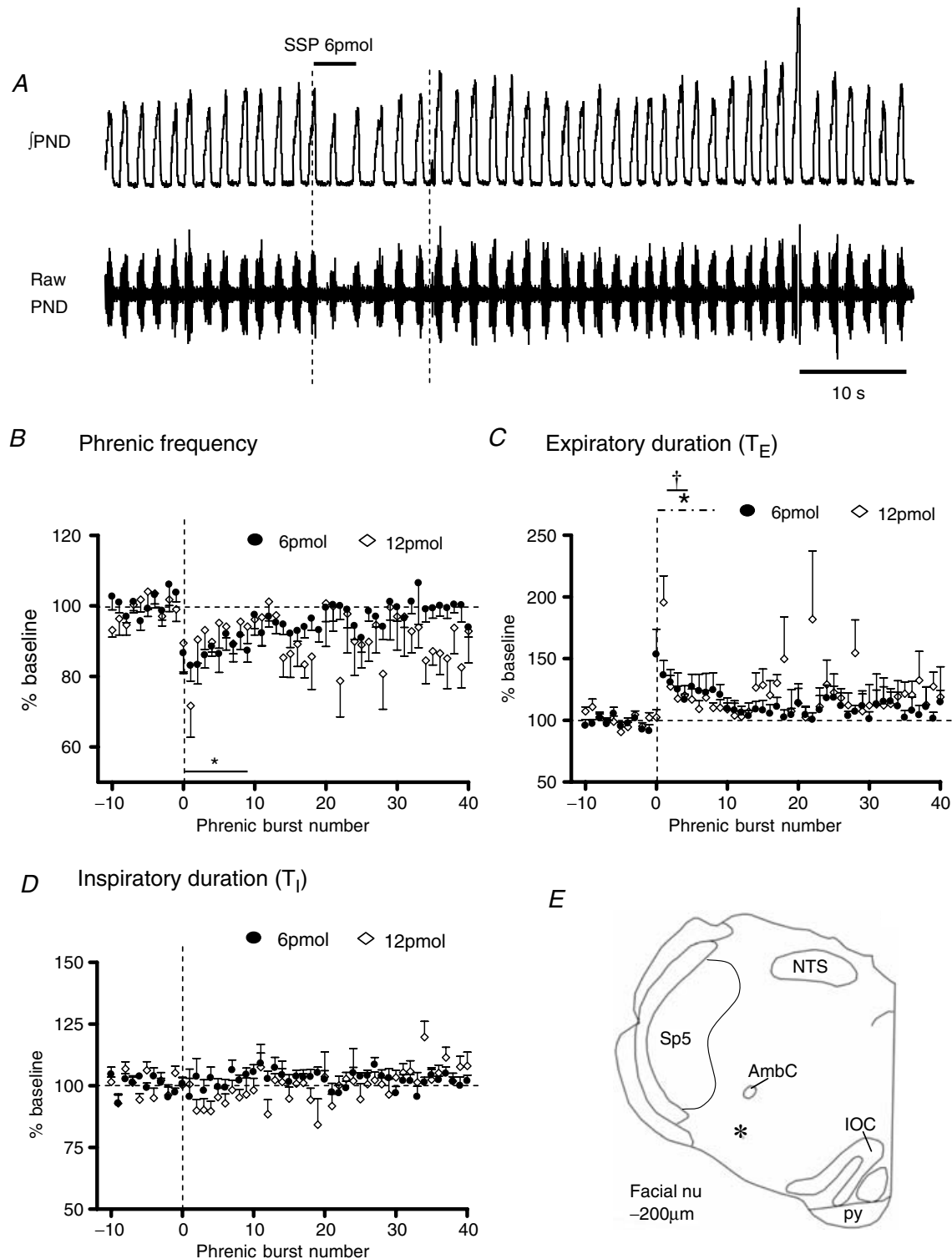


Figure 2. Effect of microinjection of $[\text{Sar}^9\text{-Met}(\text{O}_2)^{11}\text{-substance P (SSP)}$ into BötC

A, microinjection of 6 pmol SSP into a functionally identified bradypnoeic region also produces bradypnoea. B, microinjection of 6 pmol (●) of SSP significantly decreased in phrenic burst frequency, but microinjection of 12 pmol failed to reach statistical significance (one-way repeated measures ANOVA, $P > 0.05$). C, both doses of SSP significantly increased expiratory duration (T_E). D, neither dose of SSP had any effect on inspiratory duration (T_I). B–D, microinjection of SSP occurred at phrenic burst number 0 (indicated by the vertical dashed line). The period when a significant change was observed is indicated by the horizontal line. * $P < 0.05$ (6 pmol, $n = 16$); † $P < 0.05$ (12 pmol, $n = 6$). E, histological verification of the injection shown in A.

the experiments. In addition, the lower dose minimized desensitization of NK1Rs.

Microinjection of SSP on expiratory phase duration

To determine which phase of expiration was altered, we recorded PND and CVN neurograms simultaneously in a subset of preparations ($n=7$). A representative tracing of one of these recordings is shown in Fig. 3. Microinjection of SSP into a DLH-sensitive bradypnoeic region produced an approximate doubling of post-I duration (601–1180 ms) with very little effect on E2 duration (341–370 ms). Group data showed that microinjection of SSP significantly increased post-I duration (Fig. 4A, $+93.0 \pm 63\%$, $P < 0.05$, one way repeated measure ANOVA) while having no significant effect on E2 duration (Fig. 4B, $+5.5 \pm 10\%$, $P > 0.05$, one way repeated measures ANOVA). The increase in post-I duration was confirmed by a significant increase of area under the

curve (AUC) of the integrated CVN recording during the post-I period (Fig. 4C, $+135.3 \pm 92\%$, $P < 0.05$, one way repeated measures ANOVA). However, there was no significant difference in the amplitude of CVN discharge during the post-I period (Fig. 4D, $+13.1 \pm 7\%$, $P > 0.05$, one way repeated measures ANOVA).

NK1R antagonism on repeat SSP microinjection

In a subset of preparations ($n=4$), a repeated SSP microinjection was performed 60 min following the initial microinjection. Both microinjections resulted in a similar bradypnoeic response (Fig. 5A) and increase in T_E (Fig. 5B). The initial SSP microinjection resulted in a significant decrease in phrenic burst frequency (Fig. 5A, $-31.4 \pm 5\%$, $P < 0.05$, one sample t test) and a significant increase in T_E (Fig. 5B, $+68.8 \pm 12\%$, $P < 0.05$, one sample t test) compared to baseline. Sixty minutes later, the second SSP microinjection also produced a

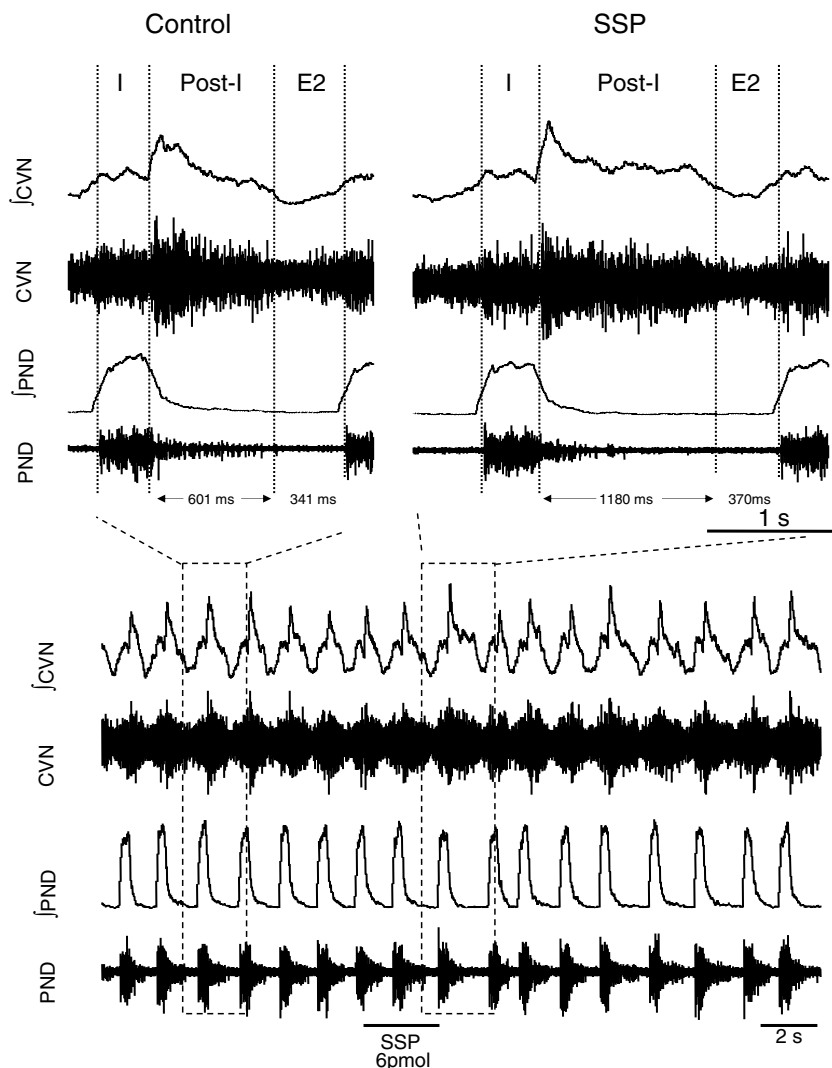


Figure 3. Microinjection of [Sar⁹-Met(O₂)¹¹]-substance P (SSP) in the BötC on expiratory phases

Representative tracings of simultaneous recording of phrenic nerve discharge (PND) and central vagus nerve discharge (CVN) illustrating the prolongation of postinspiratory (post-I) period following microinjection of 6 pmol SSP in the BötC. The two inserts illustrate a typical respiratory cycle during the baseline period and a respiratory cycle immediately following SSP microinjection, demonstrating an increase in post-I duration with not effect on late-expiration (E2). Time of injection is indicated by the horizontal line.

significant decrease in phrenic burst frequency (Fig. 5A, $-26.8 \pm 7.5\%$, $P < 0.05$, one sample t test) and extended T_E (Fig. 5B, $+54 \pm 18.5\%$, $P > 0.05$, one sample t test). No differences were found between these responses when compared using a paired t test.

To verify that the effects of SSP were mediated via activation of NK1R, SSP microinjection was repeated following blockade of NK1Rs with the selective antagonist CP99994. In a separate subset of preparations ($n = 5$), NK1R blockade was performed followed by repeat SSP microinjection. The initial SSP microinjection resulted in a significant reduction in both phrenic burst frequency (Fig. 5C, $-23.3 \pm 2.5\%$, $P < 0.05$, one sample t test) and extension of T_E (Fig. 5D, $+45.6 \pm 8.7\%$, $P < 0.05$, one sample t test). Following a 60 min recovery period and pretreatment with CP99994, SSP failed to elicit a change in phrenic frequency (Fig. 5C, $-7.5 \pm 3.7\%$)

and T_E (Fig. 5D, $+11.7 \pm 5.1\%$) compared to baseline ($P > 0.05$, one sample t test). The responses to the repeat microinjections of SSP in the presence of CP99994 antagonism was significantly attenuated from the initial SSP microinjection when compared using a paired t test. Unilateral microinjection of CP99994 into a predetermined bradypnoeic region ($n = 4$) had no effect on basal phrenic burst frequency, T_E , post-I or E2 durations (Table 1).

Microinjection of SSP induced tachypnoea

To verify that our microinjection volumes and injection sites were discrete and could elicit functionally distinct responses when injected into different VRG compartments, as described by Monnier *et al.* (2003), we microinjected SSP into a functionally identified

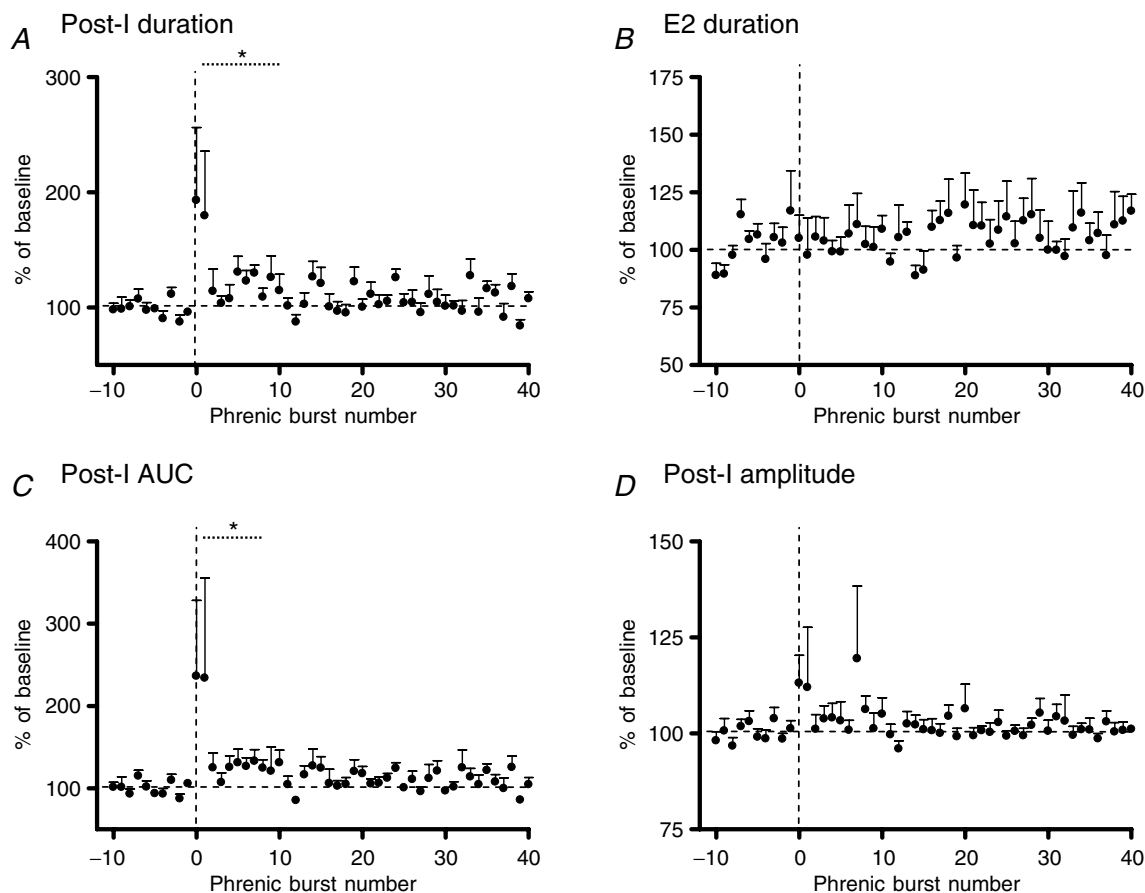


Figure 4. Quantification of the effect of microinjection of [Sar⁹-Met(O₂)¹¹]-substance P (SSP) in the BötC on expiratory phases

Microinjection of SSP (6 pmol) significantly increased postinspiratory duration (A) but did not affect E2 duration (B). The increase in post-I duration resulted in a corresponding increase in the area under the curve (AUC) of the cervical vagus nerve during the post-I phase (C); however, the peak amplitude during post-I duration (D) was not affected. Data presented as percentage of baseline. Injection occurred at phrenic burst number 0 (indicated by the vertical dashed line). Period when significant change was noted is indicated by the horizontal lines. * $P < 0.05$ 6 pmol SSP; one way repeated measures ANOVA, $n = 7$.

Table 1. Effect of unilateral NK1R antagonism on respiratory rhythm in a functionally identified bradypnoeic region of the VRG complex

	Before CP99994	After CP99994
Phrenic frequency (Hz)	0.77 ± 0.04	0.75 ± 0.04
Expiratory duration (T_E) (ms)	917 ± 100	950 ± 111
Post-inspiratory (post-I) duration (ms)	469 ± 74	450 ± 80
Late-expiratory (E2) duration (ms)	450 ± 81	460 ± 32

Paired *t* test, $P > 0.05$, $n = 4$.

tachypnoeic region which corresponded to the Pre-BötC ($n = 4$). Microinjections of SSP elicited tachypnoeic responses (Fig. 6A) that resulted in a significant increase in phrenic frequency (Fig. 6B, $P < 0.05$, one way repeated measures ANOVA). This was also accompanied by a significant reduction in T_E duration ($-35.5 \pm 6.7\%$,

$P < 0.05$, one way repeated measures ANOVA) and no change in T_I duration ($-26.2 \pm 15.1\%$, $P > 0.05$, one way repeated measures ANOVA).

Localization of microinjection sites

Microinjection sites for SSP ($n = 30$) are illustrated in Fig. 7A. Injections that elicited bradypnoea were localized within a region ventral to the compact formation of nucleus ambiguus (AmbC), extending $\sim 600 \mu\text{m}$ caudal of the facial nucleus. On the other hand, microinjections of SSP that elicited tachypnoeic responses were localized approximately $900 \mu\text{m}$ caudal to facial nucleus, corresponding to the Pre-BötC (Fig. 7A and B). An example of a microinjection site marked by fluorescent microspheres (in green), located amongst dense NK1R immunoreactivity (red) is illustrated in Fig. 7C.

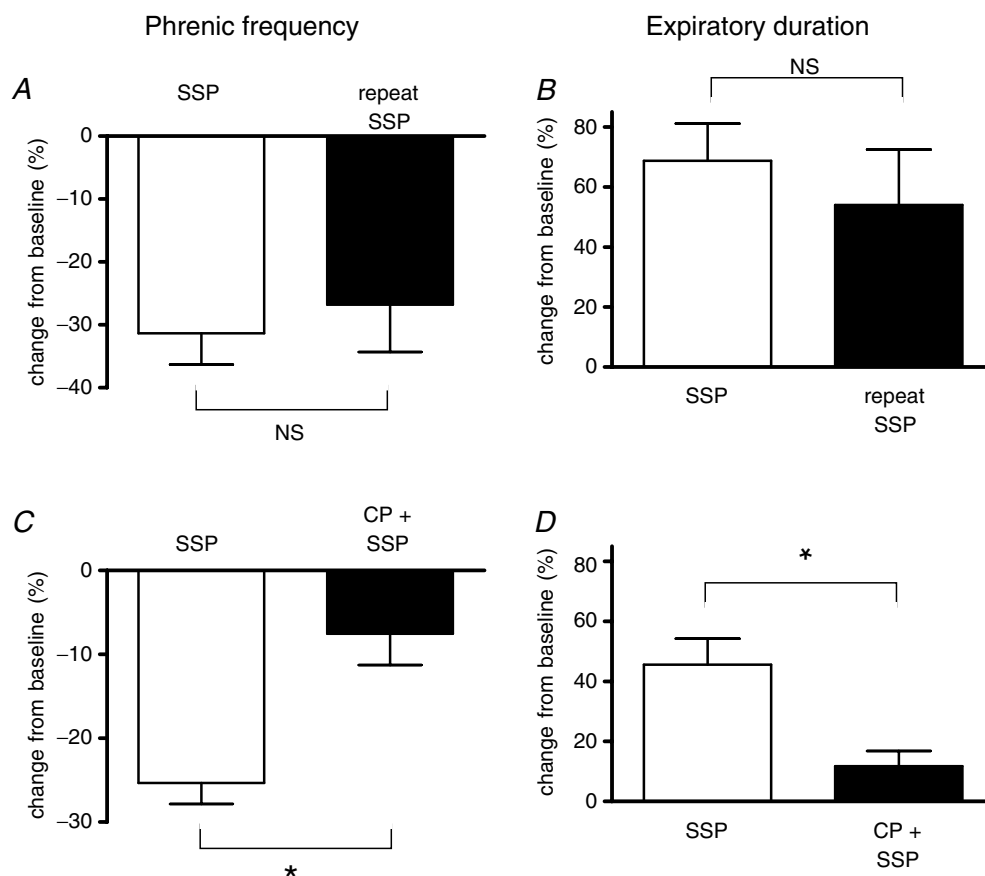


Figure 5. Effect of the selective neurokinin-1 receptor (NK1R) antagonist CP99994 on $[\text{Sar}^9\text{-Met}(\text{O}_2)^{11}]$ -substance P (SSP) induced bradypnoea

In time control experiments, repeated SSP (6 pmol, $n = 4$) microinjection following a 60 min recovery period resulted in reproducible bradypnoea (A), characterized by an increase in expiratory duration (B). In a separate group of experiments, pretreatment with the selective NK1R antagonist, CP99994, prior to repeated microinjection of SSP ($n = 5$), blocked both the SSP induced bradypnoea (C) and the increase expiratory duration (D). A–D: $*P < 0.05$ compared to first SSP microinjection; NS: not significant, paired *t* test.

Vehicle control

Microinjection of vehicle ($n = 7$, Ringer solution, pH 7.4) into predetermined DLH bradypnoeic regions resulted in no change to phrenic frequency (0.68 ± 0.03 Hz *versus* 0.70 ± 0.036 Hz, before *versus* after, $P > 0.05$, paired t test), T_E duration (1.12 ± 0.06 s *versus* 1.13 ± 0.06 s, before *versus* after, $P > 0.05$, paired t test) or T_I duration (0.54 ± 0.02 s *versus* 0.56 ± 0.025 s, before *versus* after, $P > 0.05$, paired t test).

Role of NK1Rs in the BötC on vagally mediated bradypnoea

No difference was observed in the basal phrenic burst frequency, T_E duration or T_I duration following either unilateral chemical inactivation of the contralateral BötC by the GABA_A receptor agonist muscimol or following ipsilateral NK1R blockade by CP99994 in conjunction with the contralateral BötC inactivation (Table 2).

Unilateral electrical stimulation of the vagus nerve (ipsilateral to CP99994 microinjection) resulted in prolongation of T_E (Fig. 8A, 1.2–3.4 s), with the effect gradually decreasing during stimulation. The peak extension of T_E produced by unilateral stimulation of the vagus nerve following inactivation of the contralateral BötC was not different from control stimulation (4.3 ± 0.5 s *versus* 3.3 ± 0.4 s, control *versus* muscimol, $P > 0.05$, one way repeated measures ANOVA, Fig. 8D). In contrast, following ipsilateral blockade of NK1R by CP99994, the peak extension of T_E was significantly attenuated compared to the control vagal stimulation (Fig. 8B and D). The peak extension of T_E evoked by vagal stimulation returned to control level following recovery from NK1R blockade (Fig. 8C and D). Group data for the peak response to vagal stimulation under each condition is shown in Fig. 8D. The locations of bilateral microinjections for muscimol and CP99994 are illustrated on a representative section reconstruction in Fig. 8E. All injection sites were located within 200 μ m from the caudal pole of the facial nucleus.

Discussion

The major findings in the present study are that: (1) selective activation of NK1Rs in an electrophysiologically and functionally identified bradypnoeic region of the VRG reduces phrenic frequency; (2) the decrease in phrenic frequency is mediated exclusively by an increase in expiratory duration, in particular, a lengthening of post-inspiratory duration; and (3) blockade of NK1Rs in the BötC significantly attenuates the bradypnoeic response evoked by vagal stimulation (simulating activation of the Hering-Breuer reflex), while in contrast, microinjection of SSP into the Pre-BötC increased phrenic frequency. These

results demonstrate that selective activation of NK1Rs within discrete regions of the VRG can either stimulate or depress respiratory frequency suggesting that NK1R expressing neurones have functionally diverse roles within these regions of the VRG complex. Furthermore, these findings suggest that substance P may be endogenously released in the BötC and that activation of NK1Rs in this region may be required to evoke reflex bradypnoea during vagal afferent stimulation.

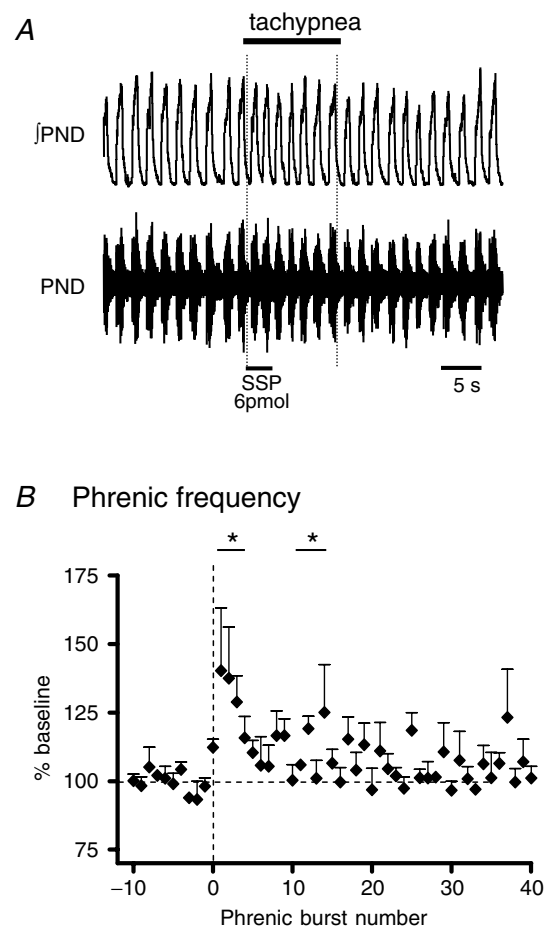


Figure 6. Microinjection of [Sar⁹-Met(O₂)¹¹]-substance P (SSP) into the Pre-Bötzinger complex (Pre-BötC) elicits tachypnoea A, representative integrated phrenic nerve discharge (/PND) and the raw PND demonstrating the tachypnoeic effect of microinjection of SSP (6 pmol) into the Pre-BötC evokes tachypnoea. The time of injection is indicated by the horizontal bars and the duration of notable effect is shown by the vertical dashed lines. B, average data, represented as percent of baseline, showing a significant increase in phrenic frequency following microinjection of SSP. Injection occurred at phrenic burst number 0 (indicated by the vertical dashed line). The period that a significant effect was observed is indicated by the horizontal bars. †, $*P < 0.05$, one-way repeated measures ANOVA, $n = 4$).

Table 2. Effect of unilateral muscimol and NK1R antagonism on respiratory rhythm in a functionally identified bradypnoeic region of the VRG complex

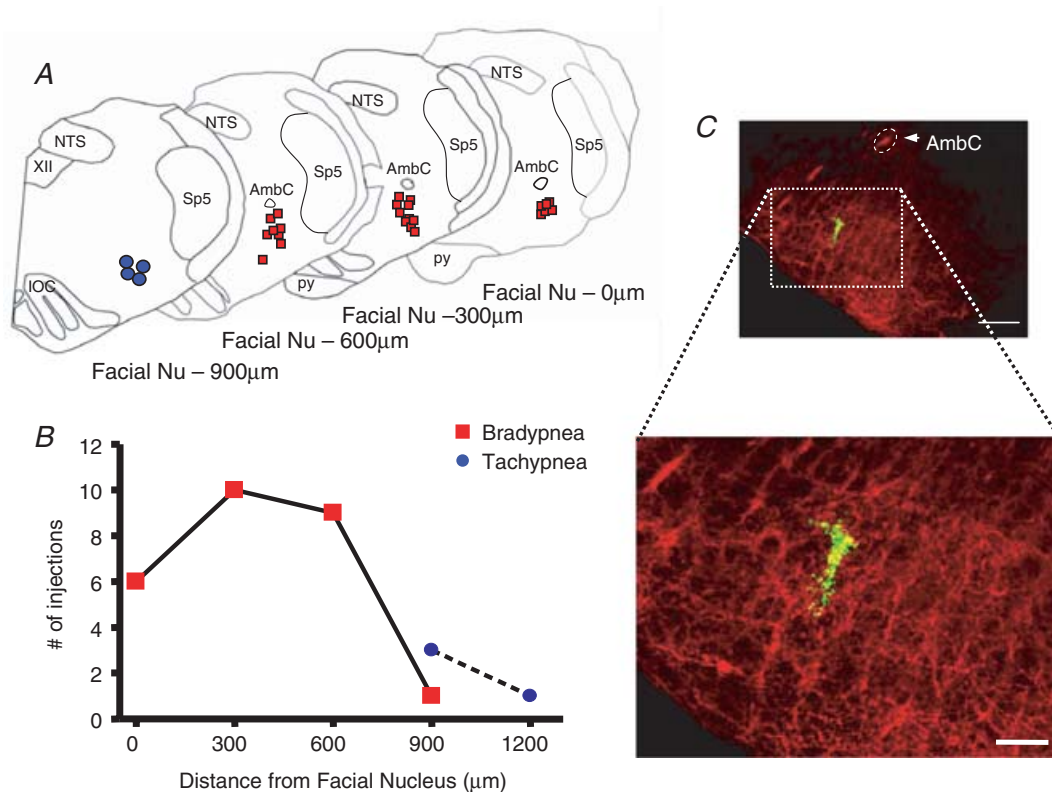
	Control	Muscimol	CP99994
Phrenic frequency (Hz)	0.54 ± 0.07	0.63 ± 0.16	0.68 ± 0.15
Expiratory duration (T_E) (ms)	1240 ± 160	1020 ± 170	980 ± 200
Inspiratory duration (T_I) (ms)	770 ± 90	720 ± 140	680 ± 130

One way repeated measures ANOVA, $P > 0.05$, $n = 5$.

Physiological significance of NK1Rs in BötC

The role of SP in modulating respiratory rhythm has received considerable attention, in particular, the stimulatory effect of SP in the Pre-BötC (Johnson *et al.* 1996; Gray *et al.* 1999, 2001; Morgado-Valle & Feldman, 2004; Pena & Ramirez, 2004). The results from our study contrast data from these previous studies by showing that NK1R activation in the BötC decreases respiratory frequency by extension of T_E . We found

that the bradypnoeic response produced by SSP was quite surprising since there is a relatively low expression of NK1Rs in the BötC compared to the adjacent Pre-BötC region (Wang *et al.* 2001; Guyenet *et al.* 2002). Nevertheless, our study demonstrates that SSP microinjection can induce different functional respiratory responses dependent on the VRG location and suggests that neurones expressing NK1Rs in the BötC may also be involved in modulating respiratory rhythm.

**Figure 7. Localization of microinjection sites of [Sar⁹-Met(O₂)¹¹]-substance P (SSP)**

A, microinjection sites for SSP resulting in bradypnoea (red squares) or tachypnoea (blue circles) were plotted on representative section reconstructions, labelled with their relative distance from the caudal pole of the facial nucleus. B, injections that elicited bradypnoea were localized within a region spanning $<900 \mu\text{m}$ caudal to the caudal pole of the facial nucleus, whereas the tachypnoeic responses were localized between 900 and $1200 \mu\text{m}$ from the caudal pole of the facial nucleus. C, a representative injection site, marked by fluorescent microspheres (green) was localized amongst dense neurokinin-1 receptor immunoreactivity (red) ventrolateral to the compact formation of nucleus ambiguus (AmbC), in a region corresponding to the ventral respiratory group complex. Abbreviations: AmbC, compact formation of nucleus ambiguus; IOC, inferior olivary complex; NTS, nucleus tractus solitarius; py, pyramidal tract; Sp5, spinal trigeminal nucleus; XII, hypoglossal nucleus.

In the present study, the depression of respiratory frequency by exogenous application of SSP to the BötC was mediated exclusively by lengthening of post-I duration, similar to that reported during activation of the Hering-Breuer reflex, either by lung inflation or by electrical stimulation of the vagus nerve (Hayashi *et al.* 1996; Wang *et al.* 2005; Li *et al.* 2006). Due to the similarity in responses evoked by Hering-Breuer reflex and exogenous SSP in the present study, we hypothesized that the extension of post-I duration by vagal stimulation may be dependent upon the endogenous activation of NK1Rs

in the BötC. Indeed, we found that the bradypnoea evoked by unilateral electrical stimulation of the vagus nerve (simulating the Hering-Breuer reflex) was significantly attenuated following NK1R antagonism in the BötC. This result suggests that NK1Rs may be critical for the respiratory lengthening effect of the Hering-Breuer reflex. On the other hand, unilateral antagonism of NK1R in the present study had no effect on basal respiratory frequency, suggesting that SP may not be constitutively released in the BötC. However, the absence of lung inflation and pulmonary afferent vagal feedback under the current

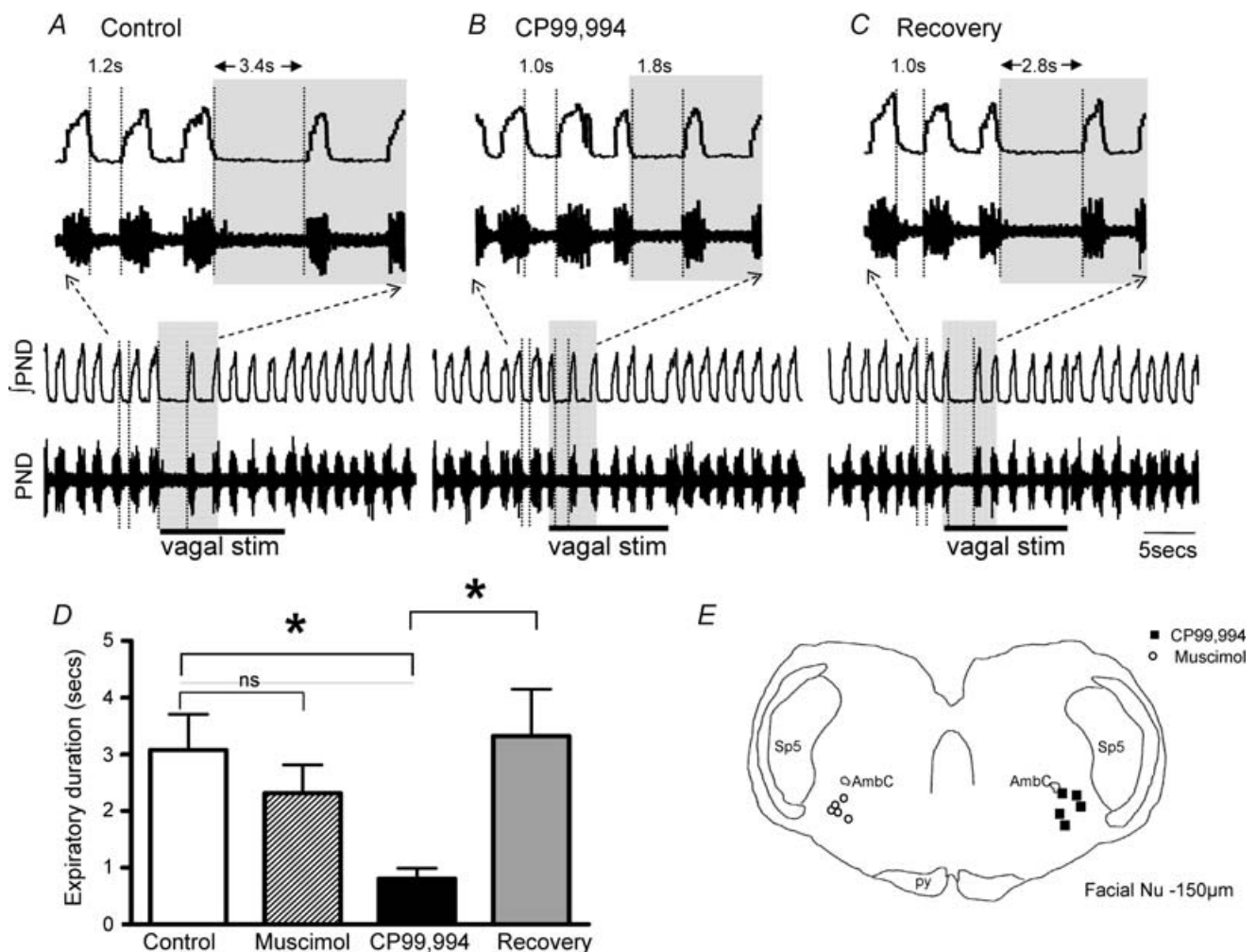


Figure 8. Effect of NK1R antagonism on vagally mediated bradypnoea

Representative integrated phrenic nerve discharge ($jPND$) and the raw PND demonstrating the effect of electrical stimulation of the vagus nerve ($125 \mu A$, 50 Hz, 1 ms pulse duration, 12 s stimulus duration) prior to, during and following NK1R blockade. The duration of stimulation is indicated by the bar. *A*, electrical stimulation of the vagus nerve produced a reduction in phrenic frequency by extension of expiration (T_E). *B*, in the presence of NK1R antagonism by CP99994, the extension of T_E was attenuated compared to control stimulation in *A*. *C*, the extension of T_E recovered towards control levels 30 min after CP99994. *D*, group data demonstrating a significant attenuation of the prolongation of T_E by CP99994, which recovered following washout (one-way repeated measures ANOVA, $P < 0.05$, $n = 5$). *E*, histological verification of the injection sites for muscimol (○) and CP99994 (■) plotted on a representative section reconstruction. All injection sites are within $150 \mu m$ of the caudal pole of the facial nucleus. Abbreviations: AmbC, compact formation of nucleus ambiguus; py, pyramidal tract; Sp5, spinal trigeminal nucleus.

experimental conditions may have contributed to this apparent lack of effect on basal frequency. Nevertheless, these results suggest that NK1R activation in the BötC decreases respiratory frequency by lengthening post-I duration and that endogenous activation of NK1Rs appears to be required to evoke the expiratory-lengthening effect of the Hering-Breuer reflex.

This is the first study to demonstrate the involvement of NK1Rs in the Hering-Breuer reflex at the level of the VRG. However, the precise source(s) of SP in the VRG in the modulation of the Hering-Breuer reflex remains to be determined. One possible source may be the second order neurons in the Hering-Breuer reflex pathway that receive projections from the slowly adapting pulmonary stretch receptors (SARs). SARs project their afferents via the vagus nerve and terminate on Pump cells (P-cells) in a discrete region of the NTS (Bonham & McCrimmon, 1990), a region that also contains substance P neurones (Maley, 1996; Li *et al.* 2005). The P-cells within the NTS project widely throughout the medulla, including the BötC (Ezure & Tanaka, 1996; Ezure *et al.* 2002). Thus, it is plausible that some P-cells may be SPergic and may project to the BötC where they could release SP during Hering-Breuer reflex activation, although this remains to be confirmed. Another source of SP may be from the dorsolateral pons, including the parabrachial nucleus (Douglas *et al.* 1982; Leger *et al.* 1983; Block & Hoffman, 1987), which receives P-cell projections from the NTS (Ezure & Tanaka, 1996; Ezure *et al.* 1998, 2002) and has been implicated in the Hering-Breuer reflex (Cohen & Shaw, 2004; Ezure, 2004). The parabrachial nucleus can also modulate respiratory rhythm (Lumsden, 1923) and has recently been implicated in coupling respiratory rhythms to locomotor activity (Potts *et al.* 2005), vocalization (Smotherman *et al.* 2006) and the respiratory response to nociception (Jiang *et al.* 2004). In addition, a further source of SP to the VRG may be from the midline raphe (Holtman & Speck, 1994) since a recent study has demonstrated that the midline raphe can modulate the Hering-Breuer reflex (Li *et al.* 2006). The precise source of SP released in the VRG, however, awaits future work.

Proposed mechanisms

Neuronal mechanisms involved in the generation of respiratory rhythm have been the subject of rigorous research and debate. Based on current knowledge, several neuronal models have been proposed: (1) the pacemaker model (Feldman *et al.* 1990; Smith *et al.* 1991), (2) the hybrid-pacemaker model (Smith *et al.* 2000), and (3) the network model (Richter *et al.* 1986; Richter & Spyer, 2001; Rybak *et al.* 2004). It is believed that there is a shift in the neural mechanisms responsible for generating respiratory rhythm during development

and maturation, with an increasing role for network inhibition in the mature, adult system (Smith *et al.* 2000). The BötC contains both postinspiratory (post-I) and expiratory-augmenting neurones (E_{aug}) (Ezure, 1990; Schwarzacher *et al.* 1991; Connelly *et al.* 1992; Sun *et al.* 1998; Chitravanshi & Sapru, 1999) that establish expiratory duration, and thus respiratory rhythm, through their reciprocal interactions with inspiratory neurones (Richter *et al.* 1986; Smith *et al.* 2000; Richter & Spyer, 2001; Rybak *et al.* 2004). Previously, we have shown that excitation of expiratory neurones, in particular E_{aug} , played a key role in coupling locomotor activity and respiratory rhythm by reducing expiratory duration and increasing respiratory frequency (Potts *et al.* 2005). In the present study, however, we found that excitation of expiratory neurones by SSP increased expiratory duration and decreased respiratory frequency. Based on current network models that incorporate inspiratory, postinspiratory and expiratory neurones (Richter *et al.* 1986; Smith *et al.* 2000; Richter & Spyer, 2001; Rybak *et al.* 2004), we propose that the overall effect of SSP-induced reduction of respiratory frequency may be via excitation of post-I neurones, which would result in an extension of post-I duration and a lengthening of expiration. It has been reported that post-I neurones in the BötC are activated by vagal stimulation and they have been implicated in the Hering-Breuer reflex (Remmers *et al.* 1986; Manabe & Ezure, 1988; Ezure & Manabe, 1988; Hayashi *et al.* 1996; Rybak *et al.* 2004). Therefore, based on the findings from the present study, we propose that NK1Rs may be expressed on post-I neurones and this expiratory population may be involved in mediating expiratory lengthening reflexes, such as the Hering-Breuer reflex. Although direct evidence to demonstrate the expression of NK1Rs on post-I neurones is currently not available, results from the present study support this idea.

Technical considerations and limitations

The vagus nerve contains a heterogeneous population of afferent fibres, including rapidly adapting stretch receptors, bronchiopulmonary C-fibres, cardiopulmonary C-fibres and baroreceptor afferents. While activation of bronchiopulmonary C-fibres has been reported to increase respiratory frequency (Lee & Pisarri, 2001; Kubin *et al.* 2006), activation of both cardiopulmonary C-fibres (Paton, 1998) and baroreceptors (Brunner *et al.* 1982; Paton *et al.* 2001; Potts *et al.* 2003) result in bradypnoea by extension of expiration. Thus, electrical stimulation of the vagus nerve in the present study may have activated other afferent fibres together with SARs involved in the Hering-Breuer reflex. However, the stimulus parameters used in the current study were similar to those used in a number of previous studies that incorporated

vagal stimulation to examine the Hering-Breuer reflex (Hayashi *et al.* 1996; Siniatia *et al.* 2000; Wang *et al.* 2005; Li *et al.* 2006). In addition, the lowest stimulation current was used to minimize non-specific activation of higher threshold C-fibres (Kubin *et al.* 2006). Despite these precautions, however, activation of other afferent populations should be considered when interpreting these results.

A potential limitation of central microinjection is selectivity of the site of activation. Previous studies have demonstrated that injection of nanolitre volumes and picomole doses of selective receptor agonists is capable of activating discrete regions of the VRG (McCrimmon *et al.* 1986; Wang *et al.* 2002; Monnier *et al.* 2003). While it is difficult to directly gauge the spread of micro-injected drugs, similarities in the data between the present study and previous reports suggest that the volume and dosage of drugs used in the present study was capable of activating discrete VRG compartments. Furthermore, the functionally distinct responses following microinjection of SSP into the BötC and Pre-BötC further support our ability to selectively activate each VRG compartment.

While a number of previous studies have examined the effect of SP in the VRG by microinjection (Chen *et al.* 1990; 1991; Makeham *et al.* 2005), our study is unique for several reasons. Firstly, our microinjection sites were identified electrophysiologically, followed by further functional characterization with DLH prior to NK1R activation, to ensure that the pipette was indeed located in a bradypnoeic or tachypnoeic region. Secondly, the dose of SP used in earlier studies (Chen *et al.* 1990, 1991; Makeham *et al.* 2005) was at least 100-fold greater than that used in the present study and the injected volumes used were substantially larger (50–500 nL *versus* 6 nl). These larger injection volumes would certainly have spread over a much greater area of the rostral and ventral surface of the medulla and may have included the ventral surface at the level of the facial nucleus, which has recently been proposed as an additional site for respiratory rhythm generation (Onimaru & Homma, 2003; Janczewski & Feldman, 2006). Together, the lower dose and smaller injection volume used in the present study have allowed for a greater resolution in identifying the site of NK1R activation.

Concluding remarks

The current study has demonstrated that NK1R activation in the Pre-BötC and BötC produce opposing effects on respiration. In light of current network models for respiratory rhythm generation, our data suggest that SP may selectively activate post-I neurones in the BötC. Furthermore, NK1R expressing neurones in the BötC have a powerful effect on respiratory rhythm generation and these data provide additional information on the role of

NK1Rs in the VRG. Our data provide the first evidence that SP may be endogenously released in the BötC during vagal afferent stimulation, and thus activation of NK1Rs may be required for expiratory lengthening reflexes such as the Hering-Breuer reflex. In conclusion, the present study has demonstrated that selective activation of NK1Rs within discrete regions of the VRG can either stimulate or depress respiratory frequency suggesting that NK1R expressing neurones can exert functionally diverse roles on breathing.

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