Neurokinin Receptor-Expressing Pre-Bötzinger Complex Neurons in Neonatal Mice Studied In Vitro

John A. Hayes and Christopher A. Del Negro

Department of Applied Science, The College of William and Mary, Williamsburg, Virginia

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INTRODUCTION

Breathing behavior in mammals is generated by respiratory neurons in the medullary brain stem (Ballanyi et al. 1999; Bianchi et al. 1995; Blessing 1997). A critical issue is the role of neurons that both express neurokinin-1 receptors (NK1Rs) and reside in the critical site for inspiratory breathing behavior, the pre-Bötzinger complex (preBo¨tC) (Gray et al. 1999, 2001; Guyenet and Wang 2001; Janczewski and Feldman 2006; Smith et al. 1991; Stornetta et al. 2003a; Wang et al. 2001).

Substance P (SP) is an endogenous agonist for NKRs, which are 36% TMR-SP+ and 64% TMR-SP-. Also, late inspiratory neurons with large Cm are 67% TMR-SP+ and 33% are TMR-SP-. Thus NKR+ and NKR- neurons exhibit the same phenotypic properties, which suggests that they may share functional roles also. Substance P (SP) alone evoked a voltage-insensitive inward current (I(SP)) that reversed at −19 mV and was associated with an increase in membrane conductance in both NKR+ and NKR- neurons. Gap junctions may be needed to confer SP sensitivity to neurons that appear to lack NKR expression. We propose that cell death in NKR+ preBo¨tC neurons, by targeted lesion or neurodegeneration, may impair breathing behavior by killing less than one half of the rhythmogenic preBo¨tC neurons and a large number of respiratory premotor neurons.

METHODS

The Institutional Animal Care and Use Committee at the College of William and Mary approved all protocols. Transverse slices (550 μm thick) from neonatal (P0–P7) C57BL/6 mice were dissected as described previously (Del Negro et al. 2005; Pace et al. 2007). With the neuraxis pinned to a paraffin-coated block, oriented rostral side up and imaged inspiratory neurons in neonatal mouse slices that isolate the preBo¨tC and generate respiratory motor output in vitro. Using tetramethylrhodamine conjugated to the endogenous NKR agonist Substance P (TMR-SP) to tag neurons that express NKRs, we show that early inspiratory neurons with small whole cell capacitance (Cm) are 36% TMR-SP+ and 64% TMR-SP-. Also, late inspiratory neurons with large Cm are 67% TMR-SP+ and 33% are TMR-SP-. Thus NKR+ and NKR- neurons are 36% TMR-SP+ and 64% TMR-SP-. Substance P (SP) alone evoked a voltage-insensitive inward current (I(SP)) that reversed at −19 mV and was associated with an increase in membrane conductance in both NKR+ and NKR- neurons. Gap junctions may be needed to confer SP sensitivity to neurons that appear to lack NKR expression. We propose that cell death in NKR+ preBo¨tC neurons, by targeted lesion or neurodegeneration, may impair breathing behavior by killing less than one half of the rhythmogenic preBo¨tC neurons and a large number of respiratory premotor neurons.

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Address for reprint requests and other correspondence: C. A. Del Negro, Dept. of Applied Science, McGlotten-Street Hall, Rm. 303, The College of William and Mary, Williamsburg, VA 23187-8795 (E-mail: cadeln@wm.edu).

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“on-line histology” atlas published by the Ballanyi group (Ruangkitisakul et al. 2006), we made the first cut above the rostral-most XII nerve roots at the level of the dorsomedial cell column and principal lateral loop of the inferior olivary nucleus; thus the preBoC is located at or near the rostral surface (Ruangkitisakul et al. 2006). The caudal cut always captured the obex. Therefore we recorded from the rostral side of the slice where the preBoC neurons were exposed and never from the caudal side.

Slices were perfused at 26–28°C with artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 9 KCl, 0.5 NaH2PO4, 25 NaHCO3, 30 d-glucose, 1.5 CaCl2·H2O, and 1 MgSO4. We used 21 slices for the electrophysiology electrophysiology/imaging experiments (Figs. 1, 2, and 7 and Supplementary Figs. S1–S3), and report data from 29 slices for the two-photon/confocal imaging experiments (Figs. 6 and 7), and 33 slices for the voltage-clamp experiments (Figs. 3–5 and 7).

To avoid tachyphylaxis and other consequences of multiple drug applications, each slice was used for one type of experiment: electrophysiological recordings of preBoC neurons after TMR-SP labeling, voltage-clamp experiments to characterize SP-evoked membrane current, or acquisition from a two-photon/confocal imaging experiment.

**Electrophysiology**

Voltage-clamp and current-clamp experiments were performed with a HEKA EPC-10 patch-clamp amplifier (Lambrecht, Germany). Network activity was monitored from XII nerves with extracellular suction electrodes and a high-gain differential amplifier with bandpass filtering (0.3–1 kHz). The root-mean-square (RMS) of voltage input to the differential amplifier (Dagan Instruments, Minneapolis, MN) was conditioned using a true RMS-to-DC converter (Analog Devices, Norwood, MA) to provide a full-wave rectified and smoothed XII waveform. Data were acquired digitally and analyzed using Igor Pro 5 (WaveMetrics), Chart 5 (AD Instruments, Colorado Springs, CO), Excel (Microsoft, Redmond, WA), and custom software. An 8-mV liquid junction potential was corrected off-line in current-clamp recordings and on-line in voltage-clamp recordings.

Whole cell capacitance (C_M) was measured using 50-ms voltage steps from −60 mV to command potentials from −75 to −65 mV in a 10-step sequence. Charge (Q) was computed by integrating leak-subtracted capacitative current (ΔQ = ∫i_C dt) and C_M was calculated from C_M = ΔQ/ΔV. Series (access) resistance (R_S) was monitored throughout voltage-clamp recordings according to the Thevenin equivalent circuit, which allows R_S to be calculated from the decay time constant (τ_m) in response to small voltage steps with R_S = τ_m/C_M as long as R_S ≪ R_NC. We monitored input resistance (R_I) through P/N on-line leak protocols. To avoid voltage-clamp errors, we discarded experiments in which R_S > 0.1 × R_NC. We compensated for R_S as much as possible without loss of stability. We also rechecked R_S and R_NC before each I-V protocol to assess voltage-clamp viability. The average uncompensated R_S was 20.2 ± 2.0 MΩ with an average of 37 ± 3% R_C compensation, and the average R_NC was 355.3 ± 84.2 MΩ (n = 24). Firing patterns of recorded neurons were consistent in on-cell and whole cell and remained constant for the duration of the experiments, which in current clump could last 40–60 min.

Voltage-clamp experiments (Figs. 1 and 2) and some voltage-clamp recordings (Fig. 3) used the following patch solution containing (in mM) 140 K-glucuronate, 5 NaCl, 0.1 EGTA, 10 HEPES, 2 Mg-ATP, and 0.3 Na(3)-GTP. KOH was used to equilibrate pH at 7.2.

Voltage-clamping experiments in Figs. 4 and 5 used ACSF containing (in mM) 84 NaCl, 3 KCl, 20–40 TEA-Cl, 25 NaHCO3, 5 NaH2PO4, 5 d-glucose, 0.5 CaCl2, 2 MgSO4, 2 CsCl, 0.2 CdCl2, 20–40 sucrose (for equimolar balancing with TEA-Cl), and 0.001 TTX. Patch electrodes (4–6 MΩ) contained the following solution (in mM): 140 d-gluceric acid, 140 CsOH monohydrate, 10 TEA-Cl, 10 NaCl, 10 HEPES, 2.5 EGTA, 1.2 CaCl2, dihydrate, 2 Mg-ATP, and 0.3 Na(3)-GTP, with pH adjusted to 7.2 using HCl.

**Epifluorescence microscopy**

Slices were incubated in 1 μM TMR-SP (Invitrogen, Carlsbad, CA) for 8–12 min at 32°C and moved to the perfusion chamber for intracellular recording. We visualized preBoC neurons with Koehler illumination and differential interference contrast (DIC) videomicroscopy, which facilitated patch-clamp recordings, and switched to epifluorescence (X-cite-120, EXFO, Mississauga, Ontario, Canada) and a rhodamine filter to capture TMR-SP. Positively labeled neurons (TMR-SP+*) were distinguished by labeling around the somatic border, in a perinuclear area, and occasionally along dendrites (e.g., Figs. 2 and S1). TMR-SP labeling became more diffuse over time (>1–2 h) (Grady et al. 1995).

Control experiments were performed (Fig. S2) by preincubating a slice in 10 μM unconjugated SP for 5 min at 32°C and applying 1 μM TMR-SP following the protocol described above. After exposure to h-SP labeling, only sparse TMR-SP labeling could be detected in regions that included the preBoC; the extent of the labeling was dramatically less than when TMR-SP was applied without prior exposure to unconjugated SP. This is most apparent in the nucleus ambiguous (NA; Bieger and Hopkins 1987), which is heavily populated by NK1R+ neurons and is adjacent to the preBoC dorsally (Gray et al. 1999; Pagliardini et al. 2005). In control experiments, the NA showed substantially less TMR-SP labeling (Fig. S2A) than using the standard TMR-SP loading protocol (Fig. S2B).

Epifluorescence images were acquired with a 12-bit charge-coupled device (CCD) monochromatic camera, the QImaging Retiga 1300i (Surrey, British Columbia, Canada) using a long-working distance water immersion ×40 objective with a 0.80 numerical aperture. Before image acquisition, the pipette tip or somatic border was focused with IR-DIC videomicroscopy; IR-DIC images were typically exposed for 150–250 ms with 1 × 1 binning. Epifluorescence images were exposed for 10 s with 1 × 1 binning at maximum fluorescence intensity. The images were pseudocolored with a black-to-red look-up table in iVision software (Biovision Technologies, Exton, PA). Background subtraction was performed by plotting a histogram of pixel intensities and truncating all values less than the lowest peak. For publication figures, we copied images to Photoshop (Adobe Systems, San Jose, CA) and enhanced contrast and applied a 1-pixel radius Gaussian blur.

**Confocal and two-photon microscopy**

Fifty micrograms of fluo-4 acetoxyethyl ester (fluo-4 AM; Invitrogen) was dissolved in 50 μl of pluronic (20%) + DMSO (Invitrogen) and vortexed for 10 min. After that, 750 μl of 30°C 9 mM [K+] ACSF was added to the dye solution and vortexed until the dye was evenly distributed. The solution was divided into two tubes (~375 μl each), and an additional 375 μl of 30°C 9 mM [K+] ACSF was added to each tube for a final concentration of 29.4 μM fluo-4-AM. Slices were incubated 40–50 min in 29.4 μM fluo-4-AM at 32°C and incubated in a separate chamber of 1 μM TMR-SP at 32°C for 10–14 min. We imaged cellular Ca2+ fluctuations using an inverted Nikon Radiance microscope (Nikon, Melville, NY) and a Mai Tai Sapphire femtosecond laser (Spectraphysics, Mountain View, CA) tuned to 800-nm excitation wavelength. Data were acquired digitally and saved to disk on a PC running Windows NT and LaserSharp software by Zeiss Microimaging (Thornwood, NY).

TMR-SP labeling was measured at the same workstation (without moving the slice) using a 543-nm Green HeNe laser at full intensity (1.5 mW), a pinhole size of 2.2 Airy units, and 512 × 512 pixels scanned with 25 lines per second (lps) using an accumulate feature to optimize the signal-to-noise ratio. This involved 30 scans per image where the pixel intensities of each scan were divided by two and

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1 The online version of this article contains supplemental data.
added to the previous scans (~10-min acquisition time). We used a Nikon \( \times 40 \) Plan Fluor objective with a numerical aperture of 0.75, which resulted in a 5-\( \mu \)m depth of field. We acquired one green (500–530 nm) and one red (600–650 nm) channel for TMR-SP images. For each image, background correction was accomplished by subtracting the RMS of the pixel intensity across the image from every pixel value. We renormalized each image and subtracted 8% of the green signal (the fluo-4 channel) from the red channel to correct for the overlap of the emission spectra, i.e., the portion of fluo-4 emission expected in the 600- to 650-nm band. Images were analyzed using iVision and ImageJ (National Institutes of Health, Bethesda, MD). Like the epifluorescence data, for publication figures, we copied images to Photoshop and enhanced contrast and applied a 1-pixel radius Gaussian blur.

Peak acquisitions (i.e., Fig. 6A, fluo-4) were achieved by scanning the focal plane repeatedly at 25 lps (1,024 \( \times \) 1,024 pixels) until at least one pixel saturated. Time-series recordings of Ca\( \text{2}^{+} \) activity (Fig. 6B) were scanned at 256 \( \times \) 256 pixels and 500 lps (\( \sim \)2 Hz) for \( \sim \)125 frames. Summed Ca\( \text{2}^{+} \) activity of time-series two-photon experiments (Fig. 6C, small inset panels) was plotted by taking the minimum pixel value for the whole time-course of the acquisition bout and subtracting this baseline from the entire time series. We summed all the fluorescence measurements for the whole time series collapsed onto one aggregate image. This produces an image that convolves expiratory and inspiratory neurons (and other transiently active cells), highlighted in warm colors.

We scanned one plane per slice preparation for experiments that contained both TMR-SP and fluo-4; the imaging plane was \( \leq 5 \mu \)m in thickness. Using the two-photon (Ti:Sapphire) laser to detect Ca\( \text{2}^{+} \) transients through fluo-4 fluorescence changes, we first probed for inspiratory neurons. If we detected inspiratory neurons in a given plane, we applied a long-duration confocal scan to detect TMR-SP labeling using the 543-nm laser (HeNe). This long-lasting exposure at high intensity bleached TMR-SP; thus we terminated the experiment after acquiring these data in the selected plane. If in a given plane we failed to detect inspiratory neurons, we did not apply the long-duration confocal scan but instead we incremented our adjacent tissue layer.

Some pilot experiments with just TMR-SP were performed using the Ti-Sapphire laser to study the viability of the labeling technique (Fig. S1C).

Statistics

We compared neurons on the basis of drive potential latency (Fig. 1B), \( C_M \), and TMR-SP labeling. We tested for normality and applied Student’s \( t \)-test or Wilcoxon signed ranks tests as appropriate to detect statistically significant differences. Mean values are reported as mean \( \pm \) SE.

We compared NKR expression in six different experimental approaches using a resampling method (Manly 2007). We used the fraction of NKR\( \text{+} \) neurons detected in a given experiment as our benchmark and counted the number of times a uniformly distributed randomly generated number in the interval [0,1] fell below that fraction when drawing the same number of samples. We repeated this algorithm in 10,000 simulated experiments and tallied the outcomes to generate a histogram that reports the likelihood of each experimental sample drawn by chance. Removing the highest and lowest 250 counts yields 95% credible intervals for the experiment.

We tested whether the fraction of SP-sensitive neurons detected in voltage clamp (Fig. 4; 86.7%) was significantly higher than the TMR-SP\( \text{+} \) fraction detected with other techniques. This was performed by comparing the SP-sensitive fraction detected in voltage clamp (control) to the fraction of TMR-SP\( \text{+} \) neurons found in other conditions. These include 1) epifluorescence imaging (Fig. 2), 2) confocal imaging (Fig. 6), 3) the SP-sensitive fraction in voltage clamp with carbenoxolone (CBX, Sigma-Aldrich, St. Louis, MO; Fig. 5), 4) the meta-analyzed NK1R immunoreactive (NK1R-ir) data set from adult rats (Guyenet and Wang 2001), and finally 5) single-cell RT-PCR evidence for NK1Rs in inspiratory neurons of neonatal rats (Manzke et al. 2003). Altogether, the pooled fraction of neurons that show evidence of NKR/NK1R expression was 40% (Fig. 7). To calculate the likelihood of the voltage-clamp experiment being different from the other experiments by chance, we used the above resampling technique and calculated a \( P \) value by dividing the number of samples drawn that equaled or exceeded 86.7% (the SP-sensitive fraction in this experiment) by 10,000 simulated experiments.

RESULTS

Electrical properties and TMR-SP labeling in inspiratory neurons

Inspiratory neurons were separable on the basis of multiple phenotypic properties (Figs. 1 and 2). We measured \( C_M \) in preBötC neurons satisfying reliable voltage-clamp conditions (see Methods). We also characterized inspiratory drive latency in all neurons with reliable current-clamp recordings, defined as the difference between the onset of inspiratory excitatory postsynaptic potentials (EPSPs) and the beginning of the XII motor output (Rekling et al. 1996). Figure 1A plots inspiratory drive latency versus \( C_M \) in neurons that had both reliable voltage- and current-clamp recordings. We found a subset of early inspiratory neurons with significantly longer latency (241.2 \( \pm \) 8.4 ms, \( n = 8 \)) and lower \( C_M \) (45.6 \( \pm \) 1.5 pF, \( n = 8 \)) compared with a subset of late inspiratory neurons that had significantly larger \( C_M \) (85.9 \( \pm \) 6.5 pF, \( n = 5 \); \( t \)-test: \( P < 0.01 \)) and shorter latency (103.7 \( \pm \) 8.2 ms, \( n = 5 \); Wilcoxon: \( P < 0.05 \)).
Early inspiratory neurons showed an incremental discharge pattern (Fig. 1B, left, arrow), whereas late inspiratory neurons exhibited a rapid onset with decrementing discharge pattern (Fig. 1B, right, arrow).

Once we recognized the correlation between $C_M$ and inspiratory drive latency, we divided preBo C neurons into two classes: 1) early inspiratory neurons had an average drive latency of 201.5 ± 8.1 ms ($n = 22$, with a total of 256 latency measurements) and 2) late inspiratory neurons had an average drive latency of 88.9 ± 6.5 ms ($n = 6$, with a total of 66 latency measurements). Inspiratory drive latency was significantly different between these two classes ($t$-test: $P < 0.05$).

Nine early inspiratory neurons showed a low baseline membrane potential and thus a silent interburst phase (Fig. 1B, left, arrow), whereas late inspiratory neurons exhibited a rapid onset with decrementing discharge pattern (Fig. 1B, right, arrow).

After patch-recording, we tested for TMR-SP labeling. TMR-SP labeled 8 of 22 (36.4%) early inspiratory neurons. Figure 2 shows one TMR-SP $^-^{-}\text{inspiratory neuron (Fig. 2A)}$ and two TMR-SP $^+\text{inspiratory neurons (Fig. 2, B and C)}$ with discharge properties described above. Four of six late inspiratory neurons exhibited TMR-SP labeling (66.6%) as shown in Fig. 2D. For the combined sample of 28 early and late inspiratory neurons, 42.8% were TMR-SP $^+$. We also recorded TMR-SP $^+\text{expiratory neurons, which discharge at high rates throughout the interburst interval but are actively inhibited during the inspiratory phase (Fig. 2E, n = 18). These data were surprising because adult rat expiratory neurons showed no NK1R immunoreactivity (Guyenet and Wang 2001). Finally, nonrhythmic TMR-SP $^+\text{cells were detected within the preBoC region but were not counted because their identity could not be verified in vitro (Fig. S3).}$

**FIG. 2.** Tetramethylrhodamine conjugated to substance P (TMR-SP) labeling in preBoC neurons with different phenotypic properties. IR-DIC and epifluorescence images are shown in left columns, with corresponding intracellular traces to the right. A: TMR-SP $^-\text{early inspiratory neuron with silent interburst intervals. B: TMR-SP}^+\text{early inspiratory neuron with tonic low-frequency spiking properties. C: TMR-SP}^+\text{early inspiratory neuron with voltage-dependent pacemaker properties. D: TMR-SP}^+\text{late inspiratory neuron. E: TMR-SP}^+\text{expiratory neuron. Scale bar (25 μm) applies to all images in A–E.}$
Excitation of inspiratory neurons by substance P

SP has been hypothesized to excite preBöC neurons by evoking a low-threshold, voltage-dependent, and TTX-insensitive Na⁺ current (Pena and Ramirez 2004). Because we were interested in SP-evoked excitation, we did not prelabel neurons with TMR-SP to avoid NKR desensitization. We measured the steady-state current-voltage (I-V) relationship with a K-glucurate patch solution and standard ACSF while blocking TTX-sensitive Na⁺ currents and Cd²⁺-sensitive voltage-gated Ca²⁺ currents (Fig. 3). The slope of the I-V curve increased in 0.5–1 µM SP and crossed the control I-V curve at approximately –20 mV, which suggests the opening of a mixed cation channel (n = 4).

To isolate the SP-induced current (Iₚₛₚ), we used patch solution containing Cs⁺ and TEA, with Cs⁺, TEA, and 4-AP in the ACSF to block K⁺ currents and hyperpolarization-activated cation current (Iₚₖ). We identified inspiratory neurons in the on-cell configuration (Fig. 4A) by observing the onset latency of inspiratory discharge. It was impossible to determine early versus late inspiratory phenotypes because Cs⁺-patch solution elevates input resistance and depolarizes the reversal potential for chloride. Nevertheless, Cₛ ranged from 23.3 to 78.9 pF, (mean Cₛ = 49.2 ± 7.0 pF, n = 9), which suggests both early and late inspiratory phenotypes were sampled (Fig. 1). We measured the I-V relationship in control and 1 µM SP and obtained Iₛₚ by subtraction (Fig. 4, B and C). Iₛₚ was linear (n = 13) and reversed at Eₛₚ = –19.4 ± 0.02 mV (n = 9), similar to the Iₛₚ reversal potential measurement with the K-glucurate patch solution (Fig. 3).

Next, we examined whether Iₛₚ expressed any voltage-dependent properties. Because the I-V protocol could cause voltage-dependent inactivation of Iₛₚ during 500-ms-long voltage steps, we analyzed tail currents from –60 to +10 mV after a prepulse to +10 mV for 100 ms (Fig. 4D). Iₛₚ was computed by subtraction. We compared Iₛₚ tail currents to steady-state Iₛₚ in the range of –60 to 0 mV. In both cases, Iₛₚ was identical throughout the voltage range (Fig. 4C, inset), suggesting no voltage-dependent inactivation of Iₛₚ.

We detected Iₛₚ in 13/15 neurons (87%). These data are consistent with current-clamp studies showing that SP depolarizes every inspiratory neuron tested in vitro (Gray et al. 1999; Pena and Ramirez 2004). However, TMR-SP labeling was only present in 42.8% of inspiratory neurons in our previous experiments. This disparity could reflect a failure to detect TMR-SP labeling in some NKR⁺ neurons or that SP-sensitive glial cells play some role in exciting inspiratory preBöC neurons that are otherwise SP-insensitive and NKR⁻.

An alternative explanation for the disparity between the large number of preBöC neurons with measurable Iₛₚ and the smaller TMR-SP⁺ subset is that gap junctions (Rekling et al. 2000) might confer the effects of SP to NKR⁻ neurons. To test whether gap junctions were required to evoke Iₛₚ, we repeated the voltage-clamp protocols from Fig. 4 after >15-min exposure to 100 µM CBX to block gap junctions. Our intra- and extracellular solutions minimized undesired effects of CBX on intrinsic membrane properties such as leak currents (Rekling et
al. 2000; Rouach et al. 2003). We evoked $I_{SP}$ in 7 of 14 inspiratory neurons with CBX present, whereas 7 inspiratory neurons did not respond to SP either in I-V or tail-current protocols (Fig. 5).

**TMR-SP labels respiratory phasic and nonrhythmic neurons in the mouse preBo¨tC**

We measured the rhythmic Ca$^{2+}$ activity of inspiratory neurons using two-photon laser-scanning microscopy (TPLSM), which enabled us to scan 5-µm-thick focal planes in a defined 293 × 293-µm area within the preBo¨tC. In 49 planes from 29 slices, we observed 344 inspiratory neurons and detected a maximum of 11 and a minimum of 1 inspiratory neuron per plane (average 6), which is commensurate with inspiratory neuron counts recently reported using TPLSM in neonatal rats (Ruangsakkiatkul et al. 2006). Fluorescence changes could not differentiate early versus late inspiratory phenotypes; with drive latencies typically <500 ms (see Fig. 1), our maximum 4-Hz sampling rate was too low to make reliable distinctions.

Figure 6A (top) shows the peak acquisition of Ca$^{2+}$ emission over several respiratory cycles in a typical experiment. Figure 6A (bottom) shows TMR-SP emission in the same region detected with confocal laser-scanning microscopy (CLSM). Cycle-to-cycle activity from neurons in Fig. 6A are plotted with XII activity in Fig. 6B: neurons 1–7 were inspiratory, whereas neuron 8 was expiratory. Neurons 4, 7, and 8 are shown at higher magnification in Fig. 6C. We detected 13/31 (41.9%) TMR-SP$^+$ inspiratory neurons with TPLSM/CLSM and 3/3 (100%) TMR-SP$^+$ expiratory neurons in a total of four imaging planes acquired in four slices. Several TMR-SP$^+$ nonrhythmic cells were situated among the inspiratory neurons (such as 9–11, Fig. 6C).

**Comparing the relative fraction of preBo¨tC neurons with evidence for NKR expression in several experimental conditions**

Altogether we used three methods to quantify the fraction of NKR$^+$ inspiratory neurons in the preBo¨tC: epifluorescence yielded 12/28 (42.9%) TMR-SP$^+$, TPLSM/CLSM yielded 13/31 (41.9%) TMR-SP$^+$, and $I_{SP}$ was measured in voltage clamp in 7/14 (50%) inspiratory neurons in the presence of 100 µM CBX. These measurements in the neonatal mouse preBo¨tC are comparable with the fraction of early inspiratory neurons, dubbed preinspiratory (preI) by the authors, which were recorded in adult rats in vivo and subsequently found to be NK1R$^+$ by immunohistochemistry: 11/32 (34.4%) (Guyenet and Wang 2001). Additionally, our results are consistent with the fraction of inspiratory neurons (4/13, 30.7%) with NK1R expression measured using single-cell RT-PCR methods (Manzke et al. 2003) and qualitatively similar to the conclusion by Manzke et al. that there is a large presence of noninspiratory NK1R$^-$ir neurons in the preBo¨tC.

We tested the null hypothesis that these independent measurements reflect the same underlying fraction of NKR$^+$/ NK1R$^+$ neurons in the preBo¨tC. A virtual preBo¨tC in silico containing 40% NKR$^+$/NK1R$^+$ neurons (the pooled fraction of NKR$^+$/NK1R$^+$ neurons detected using all methods excluding the control $I_{SP}$ experiment) and 60% NKR$^-$/NK1R$^-$ neurons was used to randomly sample 14, 28, 31, 32, and 13 neurons (with replacement) corresponding to the experiments above. Each sample was repeated 10,000 times. We tallied the results in a frequency histogram and found that, for a population containing 40% NKR$^+$/NK1R$^+$ neurons, drawing empirical samples of 42.9, 41.9, 50, 34.4, and 30.7% were statistically indistinguishable ($P \gg 0.05$). Finally, we considered the possibility that the early inspiratory neurons we found in the neonatal mouse preBo¨tC are phenotypically the same as the preI neurons recorded in adult rats in vivo (Guyenet and Wang 2001); again, the fraction of NKR$^+$/NK1R$^+$ neurons was statistically indistinguishable (8/22, 36.4% vs. 11/32 34.4%, $P \gg 0.5$).

In contrast, we evoked $I_{SP}$ in 13/15 (86.7%) inspiratory neurons with gap junctions intact (Fig. 4). In resampling simulations, this outcome (i.e., drawing a sample fraction of 86.7% NKR$^+$ neurons) occurred by chance <1% of the time, so we rejected the null hypothesis at $P < 0.01$. Figure 7 plots the sample mean fraction of NKR$^+$ neurons with 95% credible intervals to show the consistency between the fraction of NKR$^+$ neurons detected with imaging experiments, immunohistochemistry, RT-PCR, and voltage-clamp experiments in the presence of CBX compared with the much larger number of NKR$^+$ neurons with measurable $I_{SP}$ with gap junctions unblocked.
confocal/H11001 using single-cell RT-PCR to determine NK1R. Guyenet and Wang (2001) showed 95% credible intervals. Overlapping regions of epifluor., confocal LSM, included. Bold horizontal lines in each category show mean, and thin lines P rectangle. **Statistical significance at P < 0.01.

DISCUSSION

Our data suggest that the preBoëtC comprises ~40% NK1R+ inspiratory neurons in rodents. Nonetheless, SP may exert widespread excitatory effects caused by gap junctions that activate $I_{SP}$ in both NK1R+ and NK1R- neurons. The functional roles of NK1R+ and NK1R- neurons may overlap because both subsets showed early and late inspiratory phenotypes, and both respond to SP-mediated modulation (Gray et al. 1999) in the absence of gap junction blockers. Therefore the NK1R expression per se may not be a reliable means to classify preBoëtC neurons functionally. Destruction of NK1R+ neurons disrupts normal respiratory physiology (Gray et al. 2001; McKay et al. 2005). Because NK1R- neurons exhibit both early inspiratory and late inspiratory phenotypic properties, as well as expiratory and nonrespiratory phenotypes, the loss of all of these neuron types must be considered when interpreting the functional consequences of lesion or natural NK1R+ cell death.

Biophysics of $I_{SP}$ in inspiratory neurons

$I_{SP}$ is measurable in early and late inspiratory neurons using doses of SP that have clear respiratory effects in previous studies (Gray et al. 1999; Pagliardini et al. 2005; Pena and Ramirez 2004). $I_{SP}$ does not depend on extracellular Ca2+ and is TTX-insensitive, and Na+ is the dominant inward charge carrier (Pena and Ramirez 2004). Its reversal potential ($E_{SP}$) is ~19 mV, so we conclude that K+ is also a charge carrier. $E_{SP}$ was the same with K-glutamate patch solution and Cs+-based patch solution that substantially raised the Cl− reversal potential, so Cl− is not a charge carrier for $I_{SP}$. We observed $I_{SP}$ in the presence of combined Na+, Ca2+, and K+ blockers, which suggests that $I_{SP}$ arises from a single type of mixed cation channel.

Tail current analysis would enable detection of any component of $I_{SP}$ that slowly inactivates during the steady-state I-V protocol. Because $I_{SP}$ tail currents and the steady-state $I_{SP}$ were identical (Fig. 4C, inset), we concluded that there was no significant voltage-dependent component of $I_{SP}$ that inactivates on the time scale of 100–500 ms. This contradicts the hypothesis that $I_{SP}$ is a TTX-insensitive Na+ current (Pena and Ramirez 2004) that can give rise to negative slope resistance and bursting properties (Delmas et al. 1997).

SP increases excitability through the closure of K+ channels in hypoglossal motoneurons (Yasuda et al. 2001) and C1 neurons that are situated at the ventral border of the preBoëtC (Blessing 1997; Li and Guyenet 1997). Our data set did not contain C1 neurons because $I_{SP}$ never reversed at $E_K$ and was unaffected by intracellular and extracellular K+ channel blockers.

SP has widespread excitatory effects on inspiratory neurons in vitro (Gray et al. 1999; Pena and Ramirez 2004; Yamamoto...
et al. 1992), and we evoked $I_{SP}$ in 86.7% of inspiratory neurons in the absence of CBX. However, NKR expression seems to be much less prevalent: ~42% of both early and late inspiratory neurons in neonatal mice were TMR-SP$^+$, 34% of pre neurons identified in adult rats in vivo were NK1R-ir (Guyenet and Wang 2001), and 31% of preBo$\ddot{\text{b}}$tC neurons were NK1R$^+$ as identified with single-cell RT-PCR (Manzke et al. 2003). The latter measurements are consistent with our ability to evoke $I_{SP}$ in only 50% of inspiratory neurons after blocking gap junctions, suggesting that gap junctions are involved in evoking $I_{SP}$ in NKR$^-$ neurons. It is conceivable that the slightly higher (but not statistically significant) difference between our TMR-SP$^+$ fraction and the meta-analyzed NK1R-expression data can be attributed to other tachykinin receptors that can bind TMR-SP but do not show NK1R immunoreactivity. However, this is unlikely because respiratory-related neurons in NK1R$^{-/-}$ mice do not respond to SP (Ptak et al. 2000).

A cationic current that reverses at ~11 mV (in ACSF with 9 mM external [K$^+$]) is coupled to muscarinic receptor activation in preBo$\ddot{\text{b}}$tC neurons (Shao and Feldman 2000). This current is very similar to $I_{SP}$; it is TTX-insensitive, its activation is voltage- and Ca$^{2+}$-independent, and Na$^+$ and K$^+$ are the principal charge carriers. This suggests that muscarinic and neurokinin receptors may open the same underlying class of cation channels (Pena and Ramirez 2004; Shao and Feldman 2000), but this remains to be tested.

**Putative roles of NKR$^+$ and NKR$^-$ inspiratory neurons in respiratory rhythogenesis**

The majority of our neurons showed early inspiratory activity patterns and small $C_M$. The early latency, small size, and incremental discharge trajectory are characteristic of pro-primodulatory glutamatergic interneurons that putatively serve in a rhythmic capacity (Guyenet and Wang 2001; Stornetta et al. 2003a; Wallen-Mackenzie et al. 2006). Thirty-six percent of these early inspiratory neurons were NKR$^+$. Given their discharge pattern and NKR expression, these neurons are probably glutamatergic and are unlikely to be GABAergic or glycinergic (Stornetta et al. 2003a,b; Wang et al. 2001), although we cannot rule out some of these NKR$^+$ neurons belonging to a class of GABAergic neurons involved in sympathetic control of blood pressure (Wang et al. 2002). NKR$^+$ early inspiratory neurons are unlikely to contain cardiovagal preganglionic motoneurons in the external division of the nucleus ambiguous because choline acetyl-transferase was never co-detected with NK1R expression in adult rat preBo$\ddot{\text{b}}$tC neurons (Wang et al. 2001). The fraction of NKR$^+$-ir early inspiratory-like neurons (called pre$\ddot{\text{b}}$ by the authors) in adult rats in vivo is also near 36% (Guyenet and Wang 2001), so we conclude that the fraction of NKR$^+$ rhythmicogenic neurons in the preBo$\ddot{\text{b}}$tC is consistent in neonates and adults.

Large $C_M$ and late inspiratory discharge pattern are characteristics consistent with glutamatergic bulbospinal neurons that putatively serve in a premotor capacity (Guyenet et al. 2002; Rekling and Feldman 1997; Rekling et al. 1996), as well as respiratory premotoneurons (Guyenet et al. 2002). Inspiratory neurons within the preBo$\ddot{\text{b}}$tC may also be GABAergic (Kuwana et al. 2006) or glycinergic (Shao and Feldman 1997). Therefore some NKR$^-$ neurons are either inhibitory or motor-related neurons that presumably do not directly contribute to rhythmogenesis.

Nevertheless, because of their discharge phenotype and sensitivity to SP (with gap junctions intact), we propose that many early inspiratory NKR$^-$ neurons are also rhythmicogenic interneurons analogous to NKR$^+$ glutamatergic early inspiratory interneurons (Guyenet et al. 2002; Stornetta et al. 2003a). However, we cannot be certain of the transmitter type in NKR$^+$ early inspiratory neurons and thus cannot exclude the possibility that some of these neurons have nonrhythmicogenic functions.

Furthermore, it is difficult to ascertain how our early and late inspiratory neurons map to respiratory phenotypes in larger brain stem preparations or in vivo, which is problematic from the standpoint of nomenclature, because the pattern of activity may change with further levels of embedded neural circuitry. However, we provide simple names for distinct phenotypes in slices, and our dichotomy may be useful to distinguish putatively rhythmicogenic and premotor neurons in this context.

**Estimating the size and composition of the neonatal preBo$\ddot{\text{b}}$tC**

The preBo$\ddot{\text{b}}$tC in rats is remarkably constant in size during early neonatal development and extends for ~200 μm in the rostral-caudal axis of rats (Ruangkittisakul et al. 2006; Smith et al. 1991). Given somatic diameter of ~10 μm for preBo$\ddot{\text{b}}$tC neurons (Stornetta et al. 2003a; Wang et al. 2001), we can offer a rough estimate of the population size of inspiratory neurons in the preBo$\ddot{\text{b}}$tC. If we assume one neuron-layer per 10 μm of tissue in the sagittal plane, account for a bilaterally distributed preBo$\ddot{\text{b}}$tC, and use our measured average of six inspiratory neurons per plane, then the neonatal rodent preBo$\ddot{\text{b}}$tC contains ~240 inspiratory neurons. This assumes that the rostro-caudal extent of the neonatal mouse preBo$\ddot{\text{b}}$tC matches that of the rat.

We counted 22/28 (78.6%) neurons with early inspiratory discharge pattern and small $C_M$, in which 8/22 (36.3%) were TMR-SP$^+$. We found 6/28 (21.4%) neurons with late inspiratory pattern and large $C_M$ in which 4/6 (66.7%) were TMR-SP$^+$. We thus estimate that the preBo$\ddot{\text{b}}$tC contains ~189 early inspiratory neurons, of which 69 are NKR$^+$ and 120 are NKR$^-$, and 51 late inspiratory neurons, of which 34 are NKR$^+$ and 17 are NKR$^-$.

**Physiological significance: a prediction for recovering respiratory function after NKR$^+$ neuron loss**

In neonatal mice (our results) and adult rats (Guyenet and Wang 2001), ~36% of rhythmicogenic-like neurons showed...
evidence of NKR expression. We postulate that ~64% of putative rhythmogenic inspiratory neurons and ~33% of premotor-like neurons may survive SP-SAP lesions or diseases that ablate NKR+ neurons and impair breathing (Gray et al. 2001; McKay et al. 2005). Our estimates for population sizes will facilitate graded cell-destruction simulations in mathematical models of the preBo¨tzinger that reflect the approximate numbers of NKR+ and NKR− neurons with respective rhythmogenic-like and premotor-like phenotypes. Models of this type may elucidate the mechanism by which graded neuron destruction perturbs rhythmonogenesis and may help clarify the different effects of destroying rhythmogenic versus premotor neurons.

Stable breathing behavior is impaired by NKR+ neuron loss in the preBo¨tzinger and may be a result of a breakdown in fundamental rhythmogenic mechanisms. However, strengthening the excitatory synaptic transmission between NKR− preBo¨tzinger neurons may restore respiratory function, assuming that NKR− preBo¨tzinger neurons are glutamatergic and interconnected (Guyenet et al. 2002; Rekling et al. 2000; Stornetta et al. 2003a,b). Augmenting excitatory synaptic strength could be accomplished using cyclohexizide (Funk et al. 1995) or amnokines (Ren et al. 2006) that enhance ionotropic glutamate receptors or by enhancing the role of metabolotropic glutamate receptors by targeting specific intracellular signaling cascades coupled to their activation. This prediction arises from the hypothesis that a limited number of synthetically interconnected constituent neurons in the preBo¨tzinger can maintain rhythmic function by periodically evoking burst-generating intrinsic membrane properties that are only available in the context of behavior through ionotropic and metabolotropic glutamate receptors (Feldman and Del Negro 2006; Rekling and Feldman 1998; Rekling et al. 1996; Wallen-Mackenzie et al. 2006).

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REFERENCES


Rekling JC, Shao XM, Feldman JL. Electrical coupling and excitory


**Wang H, Germanson TP, Guyenet PG.** Depressor and tachypneic responses to chemical stimulation of the ventral respiratory group are reduced by ablation of neurokinin-1 receptor-expressing neurons. *J Neurosci* 22: 3755–3764, 2002.

