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Persistent Na\(^+\) and K\(^+\)-Dominated Leak Currents Contribute to Respiratory Rhythm Generation in the Pre-Bötzinger Complex \textit{In Vitro}

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A central problem in analyzing neural network circuit conductances is establishing how intrinsic neuronal conductances contribute to the generation of network activity. We used real-time calcium activity imaging combined with whole-cell patch-clamp recording to analyze contributions of subthreshold conductances in the excitatory rhythm-generating network in the respiratory pre-Bötzinger complex (pre-BötC) of neonatal rat \textit{in vitro} brainstem slice preparations. Voltage-clamp ramp recordings from imaged pre-BötC neurons revealed that persistent sodium (NaP) and K\(^+\)-dominated leak currents primarily contribute to subthreshold \(I-V\) relations. We quantified NaP and leak conductance densities \((g/C_m)\) in intrinsic oscillatory bursters and intrinsically nonbursters, the two main electrophysiological phenotypes of inspiratory neurons within the pre-BötC. Densities of \(g_{\text{NaP}}\) were significantly higher for intrinsic bursters, whereas leak conductance densities were not significantly different between intrinsic bursters and nonbursters. By pharmacologically manipulating \(g_{\text{NaP}}\) and/or \(g_{\text{Leak}}\) directly within the pre-BötC, we could modulate network oscillation frequency over a wide dynamic range and cause transitions between oscillatory and quiescent states. These results were consistent with models of the pre-BötC excitatory network consisting of heterogeneous mixtures of intrinsic bursters and nonintrinsic bursters incorporating \(g_{\text{NaP}}\) and \(g_{\text{Leak}}\) with parameter values found experimentally. We propose a paradigm whereby NaP and Leak represent a functional set of subthreshold conductances that endow the pre-BötC with rhythmicogenic properties and represent targets for modulatory control of inspiratory rhythm generation.

\textit{Key words:} oscillations; brainstem; breathing; bursting neurons; membrane conductances; neuromodulation

Introduction

Neural network activity results from intrinsic neuronal conductances generating cellular electrophysiological behavior and from synaptic connections that give rise to collective network dynamics (Llinas, 1988; Grillner et al., 2005). In vertebrate rhythmic motor pattern generation networks, such as brainstem circuits generating respiratory movements, a major problem is to establish how intrinsic neuronal conductances are functionally specialized within different populations of circuit neurons for generation of network activity. In the present study, we have investigated how rhythmic activity is generated and controlled in the pre-Bötzinger complex (pre-BötC) (Smith et al., 1991; Gray et al., 1999, 2001), a spatially localized and functionally specialized region of the medulla containing a heterogeneous excitatory network with autorythmic properties (Koshiya and Smith, 1999; Johnson et al., 2001). The pre-BötC network is the substrate for inspiratory rhythm generation and the source of rhythmic excitatory drive to premotor transmission circuits in the brainstem respiratory network.

Despite intensive investigation, the biophysical mechanisms underlying rhythm generation in the pre-BötC have not been resolved (Smith et al., 1991, 2000; Rekling and Feldman, 1998; Richter and Spyer, 2001; Ramirez et al., 2004; Feldman and Del Negro, 2006). We focused our analysis on the roles of two specific neuronal subthreshold conductances that we show are prevalent in pre-BötC neurons: a subthreshold voltage-activated persistent sodium current (NaP) and a K\(^+\)-dominated leak current (Leak). We have hypothesized that these conductances play a fundamental role in rhythogenesis in combination with excitatory synaptic interactions (Butera et al., 1999a,b; Smith et al., 2000) (see also Rybak et al., 2003). Previous experimental studies investigating contributions of NaP, however, have suggested that rhythm generation in the pre-BötC in slice preparations \textit{in vitro} normally does not depend on NaP, despite observations establishing that this conductance is a ubiquitous property of pre-BötC neurons and endows a subset of these cells with intrinsic oscillatory bursting properties (Del Negro et al., 2002a, 2005; Pena et al., 2004; Feldman and Del Negro, 2006; Pace et al., 2007). Furthermore, regulation of pre-BötC network activity by Leak has not been thoroughly analyzed experimentally. Particular types of K\(^+\)-
dominated leak or background conductances play fundamental roles in neuronal excitability control (North, 2000; Bayliss et al., 2003; Lesage, 2003), and our previous computational modeling and experimental studies point to a potential major role (Butera et al., 1999a,b; Del Negro et al., 2001). We therefore reexamined the contributions of NaP with novel experimental approaches and extended our analysis of contributions of Leak.

In contrast to previous studies (Del Negro et al., 2002a, 2005; Pena et al., 2004; Pace et al., 2007), we find that NaP can be pharmacologically manipulated within the pre-BoTc in vitro to change network activity state and regulate oscillation frequency over a wide dynamic range. Furthermore, we find that Leak can regulate cellular and network oscillatory activity, either independently or coordinately with NaP. These results support our hypothesis that NaP and Leak represent a functional set of subthreshold conductances that endow the pre-BoTc network with rhythmogenic properties and represent potential targets for neuromodulatory control of inspiratory rhythm generation.

Materials and Methods

Thin slice and pre-BoTc island preparations. Transverse slices (250 –350 μm thick) of medulla oblongata were cut from Sprague Dawley neonatal [postnatal day 0 (P0)–P3] rats to contain the pre-BoTc and rostral end of the hypoglossal (XII) motor nucleus including XII nerve rootlets as previously described (Smith et al., 1991; Koshiya and Smith, 1999). The caudal end was cut through the caudal pre-BoTc for imaging and recording neuronal activity. Pre-BoTc island preparations, which enabled us to study activity of part of the pre-BoTc network isolated from the slice, were obtained by excising the pre-BoTc and immediately surrounding tissue from one side of the slice as previously described (Johnson et al., 2001). The slices or pre-BoTc islands were mounted in a recording chamber (0.2 ml) on a microscope stage and superfused (4 ml/min) with artificial CSF (ACSF) containing (in mM) 124 NaCl, 25 NaHCO3, 3 KCl, 1.5 CaCl2, 1.0 MgSO4, 0.5 NaH2PO4, 30 n-glucose, and antibiotics (500 U/L penicillin, 0.5 mg/L streptomycin, and 1 mg/L neomycin), equilibrated with 95% O2 and 5% CO2 (pH 7.35–7.40 at 27°C). Rhythmic respiratory network activity recorded from XII nerves in slices, or directly from the pre-BoTc in island preparations, was maintained by elevating the superfusate K+ concentration (7–9 ms).

Pharmacological procedures. Pharmacological agents [tetrodotoxin (TTX; Sigma), substance P (SP; Sigma), and sodium cyanide (NaCN; Sigma)] were dissolved in the standard perfusion solution with elevated K+ concentration. The NaP blocker riluzole [Sigma or Tocris (Ellisville, MO)] (Urbani and Belluzzi, 2000; Del Negro et al., 2002a; Spadoni et al., 2002; Ptak et al., 2005) was dissolved in the superfusate K+ concentration (7–9 ms). Low pressure (~10–20 mmHg) was continuously applied to the pipette during infusion with a precision pressure control and valve system [picospritzer-IIID (General Valve, Fairfield, NJ); 2PK- pressure controller (ALA Scientific Instruments, Westbury, NY)]. This system allowed us to precisely control convective microdelivery of pharmacological probes locally within the imaged locus (below) of pre-BoTc network activity.

Electrophysiological recording and data analysis. Voltage- and current-clamp data obtained from whole-cell patch-clamp recording was recorded with a HEKA EPC-9 amplifier (HEKA, Lambrecht/Pfalz, Germany) or an Axopatch-1D amplifier (Molecular Devices, Union City, CA) controlled by Pulse software (HEKA; 2.9 kHz low-pass filter). Re-
voltage dependence of whole-cell currents was analyzed from voltage-clamp data using Pulsefit (HEKA), Chart (AD-Instruments), and Igor Pro (WaveMetrics, Lake Oswego, OR) and hand-checked for accuracy. All data presented as burst frequency were first analyzed by computing burst period, defined as the interval from onset to onset of consecutive bursts. Voltage dependence of whole-cell currents was analyzed from voltage-clamp data using Pulsefit (HEKA), Chart (AD-Instruments), and Igor Pro (WaveMetrics) software. Voltage-clamp data were fitted to first-order Boltzmann functions: 

\[ g_{\text{max}} / g_{\text{leak}} = \left[ 1 + \exp(\Delta V_{\text{m}} - V_{1/2\text{max}}/k) \right]^{-1}, \]

where \( g_{\text{max}} \) and \( g_{\text{leak}} \) represent whole-cell conductance at \( V_{\text{m}} \) and the maximal conductance, respectively, \( V_{\text{m}} \) is membrane potential, \( V_{1/2\text{max}} \) is half-maximal activation voltage, and \( k \) is a slope factor. Linear regression analysis applied to the linear region of the quasi-static-state voltage-clamp ramp \( I-V \) relations was used to determine \( g_{\text{leak}} \). Cell capacitance (\( C_{\text{m}} \)) was determined from the integral of the transient capacity current (\( I_c \), leak subtracted) evoked by 15 ms hyperpolarizing voltage-step commands applied within \( -50 \) mV of resting potential, using \( \int I_c \, dt = -C_{\text{m}} \) at each command potential (\( V_c \)). \( C_{\text{m}} \) was determined from the slope of the plot of \( Q_{\text{m}} \) versus \( \Delta V_{\text{m}} \) for the series of step commands. Series resistance (\( R_S \)) was calculated from the decay-timeconstant of \( I_c \). Neurons failing to meet the criterion \( R_S > 10 \Omega \text{m} \) necessary to achieve space clamp were excluded from voltage-clamp analysis. Furthermore, neurons with clear evidence of poor space clamp such as unclamped action potential currents during the slow voltage-clamp ramps were excluded from the analysis. Statistical significance was determined by a Student’s paired \( t \) test on mean data. Data are presented as means ± SD unless indicated otherwise.

**Calcium activity and infrared differential interference contrast imaging.** We imaged Ca\(^{2+}\)-fluorescence activity of rhythmic inspiratory neurons to identify the location of the pre-Bo\'tC and individual neurons for whole-cell recording. Methods for pre-Bo\'tC neuronal labeling with Ca\(^{2+}\)-sensitive dye and imaging were as previously described (Koshiya and Smith, 1999; Johnson et al., 2001). Briefly, membrane-permeant Ca\(^{2+}\)-sensitive dye, Calcium Green-1 AM (CaG; Invitrogen, Carlsbad, CA; 50 \( \mu \)g), dissolved in 5 \( \mu \)l of DMSO containing 25 \( \mu \)g of pluronic F-127 (BASF, Florham Park, NJ) and dispersed in 10 \( \mu \)l of ACSF, was microinjected with a glass pipette (\( -10 \mu \text{m} \) tip diameter) into the slice near the midline to retrogradely label bilaterally pre-Bo\'tC neurons overnight (8–12 h). CaG-labeled inspiratory neurons were visualized with a fixed-stage upright videomicroscope (Axioskop-FS1; Zeiss, Thornwood, NY) with a 75 W xenon epi-illuminator, optical filters (excitation 485 nm, emission 530 and 505 nm beam splitter; Omega Optical, Brattleboro, VT), and water-immersion objectives (Zeiss Achromplan). Fluorescence images were captured with a CCD camera fiber-optically coupled to a fluorescence image-intensifying camera (ICCD-1000F; VidecoScope International, Sterling, VA) and recorded on videotape together with electrophysiological signals. Changes in fluorescence intensity (\( \Delta F/F \)) were detected in real time with an image processor (ARGUS 20; Hamamatsu Photonics, Hamamatsu, Japan) and quantified off-line after digitizing images using an audio–video board (Sony, Tokyo, Japan). Inspiratory population and single-neuron “flash” images were obtained by subtracting baseline CaG fluorescent images from images acquired dur-
ing peak inspiratory activity with an image processor in real time and displayed in pseudocolor. Simultaneous videomicroscopic infrared differential interference contrast (IR-DIC) imaging was done with an extended IR Newvicon camera (Hamamatsu Photonics) and enhanced with the ARGUS 20 in real time. Infrared (900 nm) transmission was optimized with a polarizer (wide spectrum; Melles Griot, Irvine, CA) and analyzer (850–950 nm; Polarcor; Corning, Midland, MI). For dual imaging, the Newvicon and ICCD cameras were mounted on a dual-port imaging head incorporating a dichroic beam splitter (700 nm; Zeiss), and images were aligned by reverse scanning and scan range shifting of the Newvicon camera.

Modeling methods. We modeled the pre-BoÈtC excitatory network by 50 synthetically coupled (Butera et al., 1999b; Purvis et al., 2007), heterogeneous, single-compartment neurons incorporating a fast-activating, slowly inactivating NaP and a K +–dominated ohmic leak conductance for the two subthreshold currents, as well as Hodgkin-Huxley-like transient Na + current (NaT) and delayed-rectifier-like K + current for action potential generation, as previously described in detail [Model 1 (Butera et al., 1999a; Del Negro et al., 2001)]. Except for NaP activation properties, neuron capacitance values, and E K, all other Model 1 current parameters, as well as parameters for phasic excitatory synaptic currents, were identical to those used previously (Butera et al., 1999b; Butera et al., 1999a,b). Steady-state activation properties for NaP were based on Boltzmann function data obtained in the present study (V 1/2max = −47 mV; k = 5.0); similarly data-based C m (28 pF) and E K (−73 mV) values were used. Because of the adjustments to the NaP half-maximum activation voltage (hyperpolarized by 7 mV relative to the original Model 1), the NaP steady-state half-maximum inactivation voltage was hyperpolarized by 7 mV, similar to the adjustments described by Purvis et al. (2007). The networks also incorporated a constant background tonic synaptic excitatory conduc-
tance as previously described (Butera et al., 1999b; Purvis et al., 2007), which was set to 0.1 nS. All simulations used all-to-all synaptic coupling for the 50 cells [the smallest population that preserves the dynamics observed for populations an order of magnitude larger (Butera et al., 1999b)]. To incorporate parameter heterogeneity, maximum conduc-
tances of NaP and Leak within the heterogeneous population and phasic excitatory synaptic conductance (g NaP) were randomly assigned from normal distributions with ±30% SD for each parameter to mimic our experimental data as described in detail in Purvis et al. (2007). The heterogeneity of NaP and Leak resulted in networks composed of variable numbers of intrinsically bursting and nonintrinsically bursting neurons [depending on the population mean values of NaP maximum conductance (G NaP) and maximum Leak conductance (G Leak)], when excitatory synaptic transmission was eliminated (g NaP = 0). Total Leak was partitioned into a dominant K +–component (e.g., population G Leak in Fig. 13) and a small fixed Na +–leak component as previously described (Del Negro et al., 2001). To model perturbations of network activity with changes in neuronal g NaP or g Leak, we varied G NaP and/or G Leak for the heterogeneous population of bursters and nonbursters with the SD for parameters fixed at ±30%. Numerical methods were identical to those described previously (Butera et al., 1999b; Purvis et al., 2007). The initial 20 s of simulated time (90 s total) was discarded. Net-
work activity was displayed and analyzed from running histograms (adjustable bin size, 10–100 ms) of average spike times computed across the population. Raster plots of neuron spiking were used to assess neuronal burst synchronization and analyze the composition of intrinsic burster and nonburster neurons within the heterogeneous network.

Results
NaP and Leak in pre-BoÈtC neurons in vitro
We imaged pre-BoÈtC neuronal Ca ++ activity at population and single-neuron levels (Fig. 1) to identify inspiratory neurons for whole-cell voltage- and current-clamp recording. Our objective was to characterize electrophysiological phenotypes, determine subthreshold conductance properties, and determine whether these properties were functionally correlated with phenotypic differences.

All neurons analyzed electrophysiologically exhibited Ca ++ transients during the inspiratory phase of network activity, as monitored by nerve recordings of the XII population discharge. We confirmed by current-clamp recording that these cells exhibited spike discharge in synchrony with inspiratory network activity (Fig. 1C). Consistent with our previous observations (Koshiya and Smith, 1999; Del Negro et al., 2002), these inspiratory neu-
rons (Fig. 1) had two distinct electrophysiological profiles. Approx-
imately 60% of the labeled neurons exhibited intrinsic voltage-dependent oscillatory bursting (intrinsically bursters; n = 30) after blocking excitatory synaptic transmission by either bath applying the non-NMDA glutamate receptor antagonist CNQX (20 μM) (Koshiya and Smith, 1999; the Ca ++ channel blocker Cd ++ (200 μM; n = 8). The other cells (intrinsically nonbursters; n = 20) exhibited only tonic spiking when the baseline potential was depolarized (Koshiya and Smith, 1999; Del Negro et al., 2002). Spatial reconstruction of the location of individual cells used in our analysis (n = 50 total) (Fig. 1), which were located in superficial layers of the slices at depths up to limits of optical resolution (−70 μm deep), indicated that these two cell types were interspersed within the main locus of imaged pre-BoÈtC pop-
ulation activity.

Subthreshold currents in the two types of inspiratory neurons were analyzed by applying slow voltage ramps (30 mV/s; −100 to +10 mV) under voltage-clamp conditions. All neurons exhibited N-shaped I–V relationships (Fig. 2A,B) that could be decom-
posed into two primary subthreshold conductances (Fig. 2A): an ohmic-like conductance, evident by the essentially linear region of the I–V curve below −65 mV (with little if any rectification at hyperpolarized voltages), and a voltage-activated, riluzole- and TTX-sensitive NaP, generating the negative slope region in the approximately −65 mV to −40 mV membrane voltage range. The linear region defining the total Leak conductance had a 0 current intercept (i.e., E Leak) near −65 mV (−65.1 ± 2 mV; n = 30 bursters; −64.7 ± 2.6 mV; n = 20 nonbursters), which was ∼8 mV depolarized from the calculated Nernst K + equilibrium potential (E K = −73 mV) for the extracellular and intracellular recording solutions used. Thus Leak was dominated by a K + current with a small contribution from an inward cationic cur-
rent (Na + and/or Ca ++ ) that caused the depolarized shift in E Leak from E K.

NaP was completely blocked in burster and intrinsically non-

![Figure 3](image-url)

**Figure 3.** Pharmacological profile of effects of TTX on NaP and NaT. A. Effect of 20 nM TTX on NaT from a representative nonburster neuron. Peak amplitude of NaT measured by voltage-clamp step (−70 to +10 mV; 10 ms duration) is only slightly affected by 20 nM TTX. NaT is completely blocked in this neuron by 1 μM TTX. B. Peak NaP and NaT amplitude versus TTX concentration, indicating relatively selective NaP attenuation at ≤20 nM TTX (n = 12; pooled intrinsic burster and nonburster pre-BoÈtC neurons) with E GNaP = 6.2 nM TTX concentration for NaP and 112 nM for NaT.
burster pre-Bo¨tC neurons by bath-applied riluzole (20 µM) or TTX at concentrations as low as 20 nM (Figs. 2A, 3B); lower concentrations of these blockers attenuated peak NaP amplitude in a concentration-dependent manner (Fig. 3) (EC50 = 5.1 µM for riluzole; n = 22 neurons; EC50 = 6.2 nM for TTX; n = 12). To characterize voltage dependence of quasi-steady-state NaP activation, we fit a first-order Boltzmann function to the computed NaP conductance versus voltage data derived from difference currents before and after block of NaP by riluzole (n = 12 bursters; n = 10 nonbursters) or TTX (n = 18 bursters; n = 10 nonbursters) (Fig. 2C,D). Essentially superimposable Boltzmann functions were obtained for intrinsically bursting versus nonbursting cell types, whether the NaP activation characteristics were obtained by the TTX or riluzole NaP-blocking protocols (compare Fig. 2C,D); the pooled group mean V1/2max = −46.6 ± 2.3 mV versus −46.9 ± 2.1 mV, and k = 5.0 ± 0.2 versus 5.0 ± 0.3, for bursters and nonbursters, respectively; values of V1/2max or k were not statistically different. Thus, the NaP identified from our voltage-clamp measurements was identical for all neurons. The half-maximum activation voltages obtained from this analysis were hyperpolarized by ~7 mV from values that we have previously reported (Del Negro et al., 2002b) for a small sample of intrinsic burster and nonburster neurons; these new values were used in our modeling studies (below).

In characterizing blocking actions of TTX on NaP, we also compared actions on the NaT in burster (n = 7) and nonburster (n = 5) neurons; this analysis was critical for our studies (below) evaluating the role of NaP in cellular and network bursting, which required specificity of low TTX for NaP. Effects on NaT were determined from voltage-clamp steps (~70 to +10 mV, 10 ms duration) (Fig. 3A). Whereas NaP was completely blocked by bath application of 20 nM TTX within 3.2 ± 1.2 min in all neurons (Fig. 3C), the peak amplitude of NaT (2.4 ± 0.6 nA, pooled data; n = 12) was only slightly reduced (by 4.4 ± 1.0%; n = 12) (Fig. 3A). The rise time, median width, and duration of NaP were not affected (Fig. 3A). Concentration–response curves (Fig. 3B) indicated a broad separation of EC50 values (6.4 nM for peak NaP current amplitude measured at ~40 mV vs 112 nM for NaT) (Fig. 3B).

Conductance densities in intrinsically bursting versus nonbursting neurons NaP conductance densities (gNaP/Cm) were significantly higher in intrinsically bursting neurons (92.6 ± 33 pS/pF; n = 30; p < 0.01, one-way factorial parametric ANOVA) compared with nonbursting neurons (42.1 ± 11.9; n = 20), whereas gLeak densities of the same bursting neurons (119.3 ± 44.1 pS/pF) and nonbursting neurons (134.3 ± 45.4 pS/pF) were not significantly different (p > 0.05). Thus, a higher gNaP density relative to gLeak density distinguishes the subpopulation of intrinsic bursters from all other circuit neurons within the pre-Bo¨tC, as we have previously suggested from modeling (Butera et al., 1999a; Purvis et al., 2007) and measurements in a small set of pre-Bo¨tC neurons (Del Negro et al., 2002b). According to our models, the intrinsic activity state (i.e., silence, bursting, or tonic spiking) for neurons with a subthreshold I–V profile similar to that found here is described by a gNaP versus gLeak Parameter space [for detailed analysis of the data set obtained in this study, see Purvis et al., (2007)], where specific combinations of gNaP and gLeak yield intrinsic oscillatory bursting, nonbursting, or tonically spiking behavior. In our modeling studies (below), we take advantage of this to construct heterogeneous networks of intrinsic bursters and nonintrinsic bursters. Our data exhibited considerable heterogeneity for conductance densities (~30% SD; CV = 0.41 and 0.38 of gNaP and gLeak, respectively, for intrinsic bursters; 0.27 and 0.34, respectively, for nonintrinsic burster populations), which we also mimic in our models.
Preparations. To block NaP (riluzole and TTX), whose pharmacology we check for consistency of results, we also tested the two different isolated pre-Bötz network for comparison. Furthermore, to attenuate NaP within the pre-Bötz network. Experiments in the pre-Bötz island preparations (Pace et al., 2007) that provide inputs controlling excitability of pre-Bötz neurons located in superficial layers of the slice (compare with Fig. 4B). Population burst amplitude and duration were also reduced in a concentration-dependent manner (33.8 ± 6.9% decrease in amplitude at 5–20 μM; 8.6 ± 3.9% decrease at 2 μM), before network activity stopped (Fig. 5C). All perturbations were rapidly reversible when microinfusion was terminated (Fig. 5A). We also verified site specificity of these disturbances of network activity with our microinfusion approach. Microinfusion of 20 μM riluzole (or 20 nM TTX below) after repositioning the pipette tips bilaterally at the same depth in the tissue, but 75–100 μM outside of the region of imaged pre-Bötz activity (i.e., ~175–200 μM from the center of imaged activity) failed to cause perturbations of network activity. We also confirmed that at the pipette tip locations that caused perturbations of inspiratory rhythm with the NaP blockers, microinfusion of control solution (standard slice perfusion solution) did not perturb network activity (Fig. 5B) with the microinfusion pressures applied (see Materials and Methods); higher perfusion pressures (≥30 mmHg) caused a depression of network activity, however. This site spec-

Perturbations and termination of network oscillations by attenuating NaP

We analyzed perturbations of inspiratory rhythm caused by attenuating NaP pharmacologically at the network level in slice and pre-Bötz island preparations. In slices, we microinfused riluzole or low concentrations of TTX directly into the core of the pre-Bötz bilaterally. The tips of the microinfusion pipettes were inserted into the tissue at a depth midway through the slice thickness (see Materials and Methods), in the center of the active pre-Bötz populations as determined in the plane of the slice by activity imaging (Fig. 5A). This approach of convective microdelivery of NaP blockers assured that perturbations of network activity could be attributed to local attenuation of NaP within the pre-Bötz network, in contrast to bath application of NaP blockers as used in previous studies (Del Negro et al., 2002a; Pena et al., 2004; Del Negro et al., 2005), where there is the possibility that any observed disturbances of respiratory network activity could result from perturbations of neuron activity in populations outside of the pre-Bötz [e.g., midline raphe neurons (Face et al., 2007)] that provide inputs controlling excitability of the pre-Bötz network. Experiments in the pre-Bötz island preparations also allowed us to analyze activity disturbances of an isolated pre-Bötz network for comparison. Furthermore, to check for consistency of results, we also tested the two different blockers of NaP (riluzole and TTX), whose pharmacology we have characterized in detail at the cellular level in our preparations.

Continuous microinfusion of riluzole (2–20 μM) reduced network oscillation frequency in a concentration-dependent manner with a monotonic time-dependent decrease of inspiratory burst frequency monitored by XII motoneuron discharge (n = 26 slices total: n = 6 at 2 μM, n = 6 at 5 μM, n = 6 at 10 μM, and n = 8 at 20 μM riluzole) (Fig. 5B). Riluzole concentrations as low as 5 μM reduced oscillation frequency and then completely blocked inspiratory rhythm generation (Fig. 5B). Lower concentrations (2 μM) consistently slowed (by 18.2 ± 6.8%), but did not eliminate network oscillations. The pharmacological profile for attenuation of network oscillations (Fig. 5B) with riluzole microinfusion resembled the profile for attenuation of NaP in individual pre-Bötz neurons located in superficial layers of the slice (compare with Fig. 4B). Population burst amplitude and duration were also reduced in a concentration-dependent manner (33.8 ± 6.9% decrease in amplitude at 5–20 μM; 8.6 ± 3.9% decrease at 2 μM), before network activity stopped (Fig. 5C). All perturbations were rapidly reversible when microinfusion was terminated (Fig. 5A). We also verified site specificity of these disturbances of network activity with our microinfusion approach. Microinfusion of 20 μM riluzole (or 20 nM TTX below) after repositioning the pipette tips bilaterally at the same depth in the tissue, but 75–100 μM outside of the region of imaged pre-Bötz activity (i.e., ~175–200 μM from the center of imaged activity) failed to cause perturbations of network activity. We also confirmed that at the pipette tip locations that caused perturbations of inspiratory rhythm with the NaP blockers, microinfusion of control solution (standard slice perfusion solution) did not perturb network activity (Fig. 5B) with the microinfusion pressures applied (see Materials and Methods); higher perfusion pressures (≥30 mmHg) caused a depression of network activity, however. This site spec-

Figure 5. Perturbations of network oscillatory activity accompanying attenuation of NaP. A, Continuous bilateral microinfusion of riluzole into the pre-Bötz imaged by Ca²⁺-sensitive dye (population flash images during inspiratory activity shown) slows and then stops network inspiratory oscillations (monitored by fXII). Spontaneous recovery always occurred with 5 min after terminating infusion. B, Time course of perturbation of network activity is dependent on infusate riluzole concentration. Both frequency (left) and amplitude (right) of activity are reduced, with frequency predominately affected (n = 26 slices). Microinfusion of control solution (slice bathing medium) does not disturb network activity.
Figure 6. Perturbations of network oscillations by low concentrations of TTX. A, B, Bath application of low concentrations of TTX (A) \((n = 10)\), or continuous bilateral microinfusion (B) \((n = 12\) slices) of low concentrations of TTX into the core of the imaged pre-Bo¨tC (B), slows and then stops network oscillations with time course dependent on TTX concentration. Immediate recovery occurs on bath washout or discontinuation of local microinfusion. C, Frequency (left) and amplitude (right) of network activity are both reduced, but frequency is predominately affected by local microinfusion or bath application of TTX.

Perturbations of frequency and inspiratory burst amplitude similar to those caused by riluzole were obtained during bilateral TTX as low as 5 nM. There were comparatively small perturbations of the XII inspiratory burst amplitude (Fig. 6B,C) \((n = 12\) slices for each TTX concentration shown in Fig. 6C). Microinfused TTX rapidly and reversibly slowed network oscillations with a concentration- and time-dependent decrease in frequency (Fig. 6B), resulting in termination of network oscillations. The oscillations were rapidly and completely stopped by concentrations of TTX as low as 5 nM. There were comparatively small perturbations of the XII inspiratory burst amplitude (Fig. 6B,C). For comparison, we also analyzed perturbations of network activity by low concentrations of bath-applied TTX; these experiments also provided a basis for the studies (below) using pre-Bo¨tC island preparations as well as studies in slices where both bath-applied TTX and local manipulations of NaP or Leak in the pre-Bo¨tC were used. Bath-applied TTX also reversibly slowed and blocked network oscillations with relatively small changes in activity amplitude \((n = 10\) slices) (Fig. 6A,C), but the average time to attenuate/block rhythmic activity (at a given concentration) was slightly longer (e.g., 3 min vs 1.0 min for 50% reduction in burst frequency at 20 nM TTX) (Fig. 6C). Imaging of pre-Bo¨tC population activity verified complete loss of cellular activity (data not shown) when network activity stopped. The differences in time courses for disturbances of the inspiratory activity reflects the time differences for drug penetration into the tissue by diffusion with bath application (despite the thin slices used) versus convective delivery into the core of the pre-Bo¨tC, which appears to be more efficacious for rapidly establishing relatively uniform local drug concentrations in the tissue close to those in the microinfusion pipettes [e.g., see Lonser et al. (1999) and references therein for general convective microinfusion theory]. The end result of termination of rhythmic activity at steady state is the same for both approaches, however.

In pre-Bo¨tC island preparations \((n = 6)\), bath-applied TTX (20 nM) also slowed and then stopped pre-Bo¨tC network oscillations as monitored by direct electrophysiological recordings of pre-Bo¨tC network activity (Fig. 7A). The time courses of the reductions in oscillation frequency and amplitude of population activity (Fig. 7B) were on average faster than with bath application in the intact slices [e.g., 1.5 min vs 3 min for 50% reduction in frequency (compare Figs. 6C, 7B)].

For comparison with experimental results, we modeled effects of progressive, uniform attenuation of \(G_{NaP}\), in a heterogeneous excitatory network, consisting of both intrinsic bursters and nonintrinsically bursting neurons, both with NaP and Leak and subthreshold \(I–V\) relationships closely matching those found here experimentally (see Materials and Methods, Modeling methods). As \(G_{NaP}\) was reduced, the model network exhibited reductions in oscillation frequency, with relatively small perturbations of population activity amplitude, and ultimately termination of network oscillations (Fig. 8). Thus, our modeling results indicated that a hallmark of a heterogeneous excitatory network incorporating NaP and Leak is that reductions in population mean NaP conductance result predominately in large perturbations of frequency with smaller amplitude perturbations, as displayed by the \textit{in vitro} network.

**Augmentation of network oscillations by augmenting NaP**

The modeling results (Fig. 8) indicated that augmenting NaP conductance independently of Leak conductance should increase oscillation frequency. We tested this prediction. From studies in other CNS neurons, cellular hypoxia is known to augment NaP (Hammarstrom and Gage, 1998, 2002; Horn and Waldrop, 2000). We therefore postulated that cellular hypoxia induced by NaCN (1 mM) would augment NaP. We examined this at the level of individual pre-Bo¨tC intrinsically bursting and nonintrinsically bursting inspiratory neurons. In bursting neurons, NaCN caused a small but statistically insignificant increase of peak NaP conductance (mean increase of 14.3 ± 8.8%; \(p > 0.05; n = 4\)) without affecting \(g_{\text{Leak}}\) as measured from ramp \(I–V\) curves, whereas there was a statistically significant increase (mean increase of
In another group of intrinsically bursting neurons (we bilaterally microinfused NaCN into the pre-Bo¨tC at the con-
prehension. Integrated pre-Bo¨tC population activity (stopping network oscillations as recorded electrophysiologically from the pre-Bo¨tC population. After NaCN) (Fig. 9A). Figure 8. Model pre-Bo¨tC network simulations examining effects of uniform reductions in NaP within the network. A. Examples of simulated network activity represented by population spike frequency histograms (average spikes per second; 30 ms bin size) at values of GNaP density indicated; maximum conductance density of Leak was set at 118 pS/pF (refer also to point *1 in Fig. 13). B. Reducing GNaP, monotonically reduces and blocks primarily network oscillation frequency; amplitude of population activity is less sensitive to GNaP attenuation. Simulations were performed with a heterogeneous population of excitatory neurons (n = 50), with I–V relationships and GNaP and GLeak distributions (see Materials and Methods, Modeling methods) within ranges found experimentally (see Results).

19 ± 3.5%; p < 0.05) in a group (n = 4) of nonbursting neurons. In another group of intrinsically bursting neurons (n = 8), we found that NaCN (1 mM) could significantly augment GNaP after partial block of NaP by 5 nM TTX (34 ± 12.6% increase of GNaP after NaCN) (Fig. 9A,B). This also occurred without affecting GLeak (Fig. 9B).

To test for a corresponding augmentation of network activity, we bilaterally microinfused NaCN into the pre-Bo¨tC at the concentration (1 mM) found to augment neuronal NaP, which reversibly increased network oscillation frequency (n = 8 slices) (Fig. 10A). We next continuously superfused the slices with TTX at concentrations (5 nM) sufficient to stop network oscillations (Fig. 10B,D), which were then rapidly reactivated by NaCN microinfused directly into the pre-Bo¨tC. However, at higher concentrations of TTX (20 nM), at which neuronal NaP should be completely blocked (Fig. 3), network oscillations could not be reactivated (n = 6) (Fig. 10C).

Independent and cooperative modulatory actions of Leak on network rhythm
Our modeling results also suggest that GLeak can regulate pre-Bo¨tC network oscillatory activity either independently or cooperatively with NaP (see Fig. 13). We tested these predictions experimentally by (1) analyzing how altering GLeak modulates pre-Bo¨tC neuronal and network oscillation frequency; and (2) determining whether reducing GLeak (relative to GNaP) after attenuating GNaP can cooperatively restore network activity. This latter type of network reactivation experiment has been previously attempted with variable results (Del Negro et al., 2005), and has not been analyzed from the viewpoint of GLeak–GNaP (or any other set of membrane current) interactions.

Modulation of rhythm by Leak
To alter GLeak, we used SP, which is known to potently augment inspiratory oscillation frequency in slices (Johnson et al., 1996; Gray et al., 1999; Pena and Ramirez, 2004), and has been shown to modulate a K+ leak in other CNS neurons (Lepre et al., 1996; Ptak et al., 2000; Talley et al., 2000). We verified for a group of pre-Bo¨tC intrinsically bursting (n = 6) and nonbursting neurons (n = 6) that a K+ Leak conductance is reduced by SP (Fig. 11). Local application of SP (1 µM), via a pipette positioned just above the slice surface over the imaged pre-Bo¨tC and recorded neuron, significantly reduced GLeak (by 15–30% range; mean decrease of 21 ± 6.4%, p < 0.01, for intrinsic bursters, and 19 ± 5.7%, p < 0.01, for nonbursters) without significantly affecting GNaP (Fig. 11A). Ramp I–V relations obtained with SP intersected pre-SP I–V curves at −72.5 ± 2 mV (n = 12) (Fig. 11A), close to the calculated EK (−73 mV) for our recording conditions. Consistent with these results, SP depolarized nonbursters (5.5 ± 2.4 mV; n = 6) and NaP-dependent intrinsic bursters (6.8 ± 4.1 mV; n = 6) and increased bursting frequency (by maximally a factor of three) (Fig. 11B) of intrinsic bursters after blocking synaptic transmission (see also Pena and Ramirez, 2004). We also confirmed that bilateral microinfusion of SP (1 µM) into the pre-Bo¨tC increased network oscillation frequency (Fig. 12A), consistent with previous observations (Johnson et al., 1996; Gray et al., 1999).

Interactions of GLeak and GNaP in controlling network and cellular oscillations
We first superfused the slice with 5 nM TTX, which terminated network oscillations, and subsequently microinfused SP (1 µM) into the pre-Bo¨tC, which restored network oscillations in all cases (n = 10) (Fig. 12B,D). Our previous modeling studies have demonstrated the interplay between NaP and Leak in controlling intrinsinc oscillatory bursting activity at the single-neuron level (Butera et al., 1999a; Purvis et al., 2007); furthermore, network simulations for a heterogeneous excitatory network consisting of a mixture of intrinsic bursters and nonintrinsic bursters (Fig. 13) show that relatively small reductions of the population mean Leak conductance, similar to those found experimentally with SP for individual neurons, should be sufficient to restore network oscillations after partial attenuation of population mean NaP and GNaP.
conductance. However, network oscillations should theoretically not occur after complete block of NaP (Fig. 13). To test this, we continuously superfused the slice with TTX at concentrations (20 nM) into the pre-BoTc did not restore network activity in any experiment (n = 7 experiments) (Fig. 12C).

Our modeling results also indicated that with reductions in Leak conductance at a fixed gNaP, as during reactivation experiments, the composition of intrinsic burster and nonburster neurons within the network changes. Some intrinsic bursters are converted to tonically spiking neurons, whereas a small fraction (e.g., 8/46 in supplemental Fig. 1C, available at www.jneurosci.org as supplemental material) of nonintrinsic bursters are converted to intrinsic bursters, depending on the magnitude of the reduction in gLeak relative to gNaP and the initial conditions of a given neuron [i.e., whether or not the neuron has a high enough gNaP to support intrinsic bursting under any conditions (Butera et al., 1999a)]. This shift in network composition may explain network reactivation by SP under our experimental conditions. The concept that the extent of intrinsic bursting activity in the pre-BoTc network can be regulated by neuromodulators such as SP has been described (Pena and Ramirez, 2004), and changes in network dynamics by the fractional composition of intrinsic NaP-dependent bursters and nonintrinsic bursters have been explored theoretically in detail (Purvis et al., 2007). We therefore examined a group of intrinsic and nonintrinsic burster neurons to determine whether a change in electrophysiological phenotype occurs as gLeak was reduced by SP (1 µM) under conditions in which synaptic transmission was blocked. A few intrinsic bursters (2 of 8 tested) were converted to tonically spiking neurons, whereas we could not convert nonintrinsic bursting cells (n = 10) to intrinsic bursting behavior with the small SP-induced reduction in gLeak (18.4–23.7% range). This was likely a result of the low initial values of gNaP density in these nonbursters (41.1 ± 9.5 pS/µF) relative to gLeak densities (131.0 ± 43.2 pS/µF); indeed, the majority of the nonbursters that we have analyzed in the present study have values of gNaP relative to gLeak densities that are too low to support intrinsic bursting even with much larger changes in gLeak as indicated by plots of gNaP versus gLeak for this data as presented in Purvis et al. (2007). This question of the possible conversion of electrophysiological phenotypes by neuromodulators requires more extensive experimental investigation.

**Discussion**

Our results indicate that generation and control of inspiratory rhythm in the pre-BoTc in vitro can to an important extent be understood from contributions of NaP and Leak operating within a heterogeneous excitatory network (Butera et al., 1999b). Subthreshold I–V relations are dominated by gNaP and gLeak, where gNaP conductance densities distinguish intrinsic bursters from nonintrinsic bursters, as we previously postulated (Del Negro et al., 2002b) but now demonstrate for a large sample of neurons (see also Purvis et al., 2007). Manipulations of gNaP and/or gLeak within the pre-BoTc upregulated or downregulated network oscillations over a wide dynamic range. Importantly, attenuating NaP reduced oscillation frequency and could terminate rhythmogenesis. These results are the first direct evidence for the concept (Butera et al., 1999a,b; Smith et al., 2000) that NaP and Leak are fundamental for generation and control of rhythmogenesis in the pre-BoTc in vitro.

Pre-BoTc neurons have voltage- and calcium-activated currents (Smith et al., 2000; Richter and Spyer, 2001; Ramirez et al., 2004) contributing to repetitive spiking properties and inspiratory burst generation. Specifically, h-current (Mironov et al.,
threshold conductance densities in the neurons studied here. We did not find pronounced rectification of I–V relations at hyperpolarized voltages that would indicate a major h-current contribution. We also did not find residual subthreshold inward currents after block of Na⁺ currents, consistent with observations that pre-BötC neuron Ca²⁺ currents are primarily high-voltage activated (Elsen and Ramirez, 2005). NaP-dependent intrinsic bursting is also not substantially affected by attenuating Ca²⁺ currents (Del Negro et al., 2001, 2005; Pena et al., 2004). The neuron models used here are biophysically minimal models. Sorting out how these other cationic currents may operate dynamically with NaP and Leak, including CAN, which has not yet been biophysically characterized in pre-BötC neurons, remains an important problem.

**NaP as a ubiquitous pre-BötC neuronal property contributing to rhythm generation**

Many CNS neurons have NaP that underlies spontaneous persistent spiking (Taddese and Bean, 2002; Do and Bean, 2003) and oscillatory activity (Llinás, 1988; Agrawal et al., 2001; Darbon et al., 2004; Wu et al., 2004). The contribution to respiratory network oscillations, however, has not been clearly resolved. In contrast to our results and an earlier report (Rybak et al., 2003), it has been suggested (Del Negro et al., 2005; Pace et al., 2007) that NaP does not play a role in generating inspiratory oscillations in neonatal mouse slices, whereas others (Pena et al., 2004) propose that NaP-dependent and CAN current-dependent bursting mechanisms collectively contribute to rhythm generation (but not NaP mechanisms alone) in mouse slices.

We suggest several explanations for these discrepancies. First, large perturbations of oscillation frequency would theoretically occur only when a uniform attenuation of NaP is achieved throughout the pre-BötC network. Perturbations of network activity amplitude without frequency perturbations (Del Negro et al., 2002a, 2005) can result from spatially and/or temporally nonuniform pharmacological attenuation of NaP (see supplemental Fig. 2, available at www.jneurosci.org as supplemental material); this may occur with bath-applied blockers in thick (500–600 μm) mouse slices used in these previous studies. We have convectively delivered NaP blockers by microinfusion into the pre-BötC to minimize local tissue concentration gradients. This approach has also been used recently in neonatal mouse slices [but without activity imaging for placement of pipette tips in the pre-BötC (Pace et al., 2007)], in which no disturbances of rhythm generation were found with NaP blockers. These results differ from ours in neonatal rat slices and isolated pre-BötC islands. Possibly rhythmogenic mechanisms operating in mice are different from rats; this issue needs to be resolved.

Second, contributions of NaP and rhythm generation mechanisms depend on network state. We have shown that, in a more intact brainstem network, only small perturbations of inspiratory rhythm occur after NaP block (Paton et al., 2006; Smith et al., 2007). We postulate that this insen-
in general stop for any initial conditions when bound estimate for the combined population of bursters and nonbursters). Network oscillations reduction by 5 nM TTX [estimated from single-neuron-level data (Fig. 3)] stops network oscillation by a percentage (when “gasping” inspiratory oscillations occur, does rhythm generation depend critically on NaP (Paton et al., 2006). Thus regardless of whether or not rhythm generation \textit{in vitro} can be attributed to cellular “pacemaker” properties per se, it is clear from our results that NaP, as a common conductance in pre-Bo\textsuperscript{t}C neurons, contributes to rhythm generation, at least in the isolated neonatal rat pre-Bo\textsuperscript{t}C network \textit{in vitro} and in certain states in the more intact, rat respiratory network (Paton et al., 2006). This conclusion is supported by our results that once \(g_{NaP}\) is reduced to low enough levels, network oscillations cannot be generated in reactivation experiments. The augmentation of NaP and network oscillations by cellular hypoxia shows that NaP can be modulated, consistent with a homeostatic role of NaP in maintaining network activity during CNS hypoxia (Pena et al., 2004; Paton et al., 2006).

**Nature of Leak currents**

Leak is a \(K^+\)-dominated conductance that can both promote and terminate oscillatory activity at cellular and network levels. We show that an excitatory neuromodulator (SP) implicated in rhythm generation control (Gray et al., 1999) operates through \(g_{Leak}\). We have other evidence (S. Smerin, H. Koizumi, and J. C. Smith, unpublished observations) that TASK channels (North, 2000; Bayliss et al., 2003) mediate a major \(K^+\) current component of Leak in pre-Bo\textsuperscript{t}C neurons. TASK channels, as background conductances, are modulated in other neurons (Bayliss et al., 2003) by a variety of neurochemical signals (Talley et al., 2000), including SP and \(H^+\). A \(Na^+\)-dependent leak current has been implicated in SP-induced depolarization of mouse inspiratory neurons (Pena and Ramirez, 2004). Our data indicate that there is a relatively small \(Na^+\) or mixed cationic inward current that opposes the outward \(K^+\) current in establishing \(E_{Leak}\). Our voltage-clamp analysis indicates that SP reduces the dominant \(K^+\) current component in some pre-Bo\textsuperscript{t}C neurons. In recent work (T. Yamanishi, K. Ptak, and J. C. Smith, unpublished observations), however, we found that SP also altered \(g_{Leak}\) in a group of pre-Bo\textsuperscript{t}C neurons, but caused a several millivolt depolarized shift of neuronal \(I-V\) relations, causing a more depolarized value of \(E_{Leak}\) than can be accounted for by reduction of a \(K^+\) current alone. This suggests that SP may also simultaneously augment the inward cationic component of Leak in some neurons (Shen and North, 1992) (see discussions in Pena and Ramirez, 2004).
2004). The nonselective, Na⁺ leak channel [NALCN (Lu et al., 2007)] is a possible candidate for this component. Furthermore, additional G-protein coupled K⁺ channels (e.g., GIRK channels) may also contribute to Leak and neuromodulatory control of pre-Botziní neurons (Johnson et al., 1996). Our model predicts that regardless of the how total ßmax is changed, the general trends shown for modulation of inspiratory rhythm in Figure 13 (in that case for changes in the K⁺ component) would apply. Dissection of Leak into discrete channel components remains an important problem.

In general, our experimental and modeling results suggest that Leak and NaP are key conductances that can be targeted for modulation of inspiratory rhythm, either independently or coordinately, by neurochemical signals that control generation of pre-Botziní oscillatory activity.

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