Published in final edited form as:

Eur J Neurosci. 2008 December ; 28(12): 2423. doi:10.1111/j.1460-9568.2008.06537.x.

Background Sodium Current Underlying Respiratory Rhythm Regularity

Marc Chevalier¹, Faiza Ben Mabrouk¹, and Andrew Kieran Tryba^{1,*}

¹Dept of Physiology, Medical College of Wisconsin, 8701 Watertown Plank Rd., Milwaukee, WI 53226

Abstract

Rhythm generating neural circuits underlying diverse behaviors such as locomotion, sleep states, digestion and respiration play critical roles in our lives. Irregularities in these rhythmic behaviors characterize disease states - thus, it is essential that we identify ionic and/or cellular mechanisms that are necessary for triggering these rhythmic behaviors on a regular basis. Here, we examine which ionic conductances underlie regular or “stable” respiratory activities, proposed to underlie eupnea, or normal quiet breathing. We used a mouse *in vitro* medullary slice preparation containing the rhythmogenic respiratory neural circuit, called the preBötzinger Complex (preBötC) that underlies inspiratory respiratory activity. We varied either $[K^+]_o$, $[Na^+]_o$, or blocked voltage gated calcium channels (VGCC) while recording from synaptically isolated respiratory pacemakers and examined which of these manipulations resulted in their endogenous bursting to become more irregular. Of these, lowering $[Na^+]_o$ increased the irregularity of endogenous bursting by synaptically isolated pacemakers. Lowering $[Na^+]_o$ also decreased the regularity of fictive eupneic activity generated by the ventral respiratory group (VRG) population and hypoglossal motor output. Voltage clamp data indicate that lowering $[Na^+]_o$, in a range that results in irregular population rhythm generation, decreased persistent sodium currents, but not transient sodium currents underlying action potentials. Our data suggest that background sodium currents play a major role in determining the regularity of the fictive eupneic respiratory rhythm.

Introduction

Irregular rhythmic electrical activity characterizes epilepsy (Dudek et al., 1995; Topolnik et al., 2003; van Drongelen et al., 2003), aberrant sleep patterns (Steriade et al., 2001) and breathing problems (Feldman et al., 2003a). In contrast to sustained cessation of breathing that requires urgent medical attention, irregular breathing patterns are the most prevalent and clinically relevant issue in respiratory physiology and diseases such as sudden infant death syndrome (SIDS), Rett syndrome (RS) and central sleep apnea (Feldman et al., 2003a; Viemari et al., 2005b; Tryba et al., 2006; Hunt et al., 2008).

In many rhythmic neural networks underlying locomotion, or breathing, synaptic and/or intrinsic mechanisms are proposed to play critical roles in rhythmic oscillatory behavior (Tryba and Ritzmann, 2000; Pena et al., 2004a; Ramirez et al., 2004a; Del Negro et al., 2005). Additionally, neuromodulators, such as serotonin, norepinephrine, acetylcholine and dopamine all play major roles in triggering intracellular second messenger pathways that can strongly influence the regularity of rhythmic activity (Huey et al., 2000; Fuller et al., 2001; Feldman et al., 2003b; Pearlstein et al., 2005; Viemari et al., 2005a; Tryba et al., 2006; Viemari and

*Corresponding author: Andrew K. Tryba Medical College of Wisconsin Dept. of Physiology 8701 Watertown Plank Rd. Milwaukee WI 53051 Phone : 414.456.4975 FAX: 414.456.6546 e: atryba@mcw.edu.

Ramirez, 2006; Tryba et al., 2008). While varied and complex intracellular pathways dramatically alter rhythmicity, they must ultimately change ionic conductances that give rise to regular rhythmic cycling. Thus, understanding which ionic mechanisms underlie rhythm regularity may provide useful insights into which currents could be a target of neuromodulators, or theoretically pharmacologically targeted to restore, for example, regularity to irregular breathing patterns.

Several studies suggest that background sodium and potassium currents play important roles and differ in their contribution to respiratory pacemaker and non-pacemaker excitability (Tryba et al., 2003; Tryba and Ramirez, 2004b; Koizumi and Smith, 2008). However, the functional consequences of these or other such currents on respiratory network and pacemaker rhythmicity remain largely unknown. Here, we sought to examine which ionic conductances underlie regular or “stable” respiratory bursting activities, proposed to underlie eupnea, or normal quiet breathing. We used a mouse *in vitro* brain slice preparation containing the rhythmogenic respiratory neural circuit, called the preBötzinger (preBötC) that underlies inspiratory respiratory activity (Smith et al., 1991). We varied either $[K^+]_o$, $[Na^+]_o$, or blocked voltage gated calcium channels (VGCC) while recording from synaptically isolated respiratory pacemakers and examined which of these manipulations resulted in their endogenous bursting to become more irregular. Of these, lowering $[Na^+]_o$ increased the irregularity of endogenous bursting by synaptically isolated pacemakers. Lowering $[Na^+]_o$ also decreased the regularity of fictive eupneic activity generated by the ventral respiratory group (VRG) population. These data suggest that, low threshold, background sodium currents play a major role in determining the regularity of the fictive eupneic respiratory rhythm.

Materials and Methods

All experiments conformed to the guiding principles for the care and use of animals approved by the National Institutes of Health (U.S.A.) and the Animal Care and Use Committees at the Medical College of Wisconsin.

Respiratory medullary brain-slice preparation

All experiments used the transverse, rhythmic 400–600µm thick medullary brain-slice obtained from 8–13 day old, CD-1 outbred mice (Charles River Laboratories, Wilmington, MA) (Smith et al., 1991; Thoby-Brisson and Ramirez, 2001). The 600µm thick medullary brain-slices were used in current clamp studies, whereas 400µm thick medullary brain-slices were used in visual voltage clamp studies as the thinner slices allow for more light penetration, facilitating visualization of neurons. CD-1 mice were quickly decapitated at the C3/C4 spinal level and the brain-stem was dissected in ice cold artificial cerebral spinal fluid (ACSF) that was equilibrated with carbogen (95% O₂ and 5% CO₂, pH=7.4). Rhythmic slice preparations containing the ventral respiratory group (VRG), including the preBötzinger Complex (preBötC), were obtained by slicing the medulla using a microslicer (Leica, VT1000S, Nussloch, Germany) as described in detail elsewhere (Thoby-Brisson and Ramirez, 2001). Briefly, the brainstem was glued onto an agar block with the rostral end up, mounted into a microtome and serially sliced from rostral to caudal until the rostral boundary of the preBötC was visible. This area is recognized by landmarks such as the inferior olive (IO), the nucleus ambiguus (NA) and the hypoglossal nucleus (XII) (Fig. 1a). For both 600µm and 400µm thick medullary brain-slice, the slice was taken so that the caudal surface was approximately ~30µm rostral to obex. Slices containing the preBötC were submerged in a recording chamber (6 mL) under circulating ACSF (30°C; flow rate 17 ml/min, total circulating volume = 200mL).

ACSF contained in mM: 118 NaCl, 3 KCl, 1.5 CaCl₂, 1 MgCl₂·6H₂O, 25 NaHCO₃, 1 NaH₂PO₄ and 30 D-glucose, equilibrated with carbogen (95% O₂ and 5% CO₂, pH = 7.4). All ACSF chemicals were obtained from Sigma (St. Louis, MO) Extracellular KCl was elevated

from 3mM to 8mM over a span of 30 minutes before commencing recordings, to maintain rhythmic population activity (Tryba et al. 2003). Note that raising ACSF $[K^+]_o$ does not artificially introduce pacemaker bursting properties; pacemakers show similar bursting properties in 3mM versus 8mM $[K^+]_o$ ACSF (Tryba et al., 2003). Further, it should be noted that $[K^+]_o$ changes on a breath by breath basis and changes in $[K^+]_o$ do not obviously alter the form of respiratory activity generated, as both eupneic and gasping activities can be recorded in hypokalemic and hyperkalemic conditions *in situ* (St-John et al., 2005). As bath temperature can alter respiratory slice activity (Tryba and Ramirez, 2004a), it was monitored and maintained at $30^\circ\text{C} \pm 0.7^\circ\text{C}$ using a Warner Instrument Corp. (Hamden, CT) TC-344B temperature regulator with an in-line solution heater (SH-27B); bath temperature at various locations within the bath was uniform.

Electrophysiology- Population activity and identification of inspiratory neurons

Extracellular recordings were obtained with glass suction electrodes positioned on the slice surface in the ventral respiratory group (VRG) near or on top of the preBötC (Figs. 1A, 1B) (Tryba et al., 2003). The VRG population bursting is dominated by inspiratory neurons such that integrated VRG ($\int\text{VRG}$) activity is in-phase with integrated XII ($\int\text{XII}$) activity (Tryba et al., 2006). Thus, VRG population bursts serve as a marker of fictive inspiration (Tryba et al., 2006; Tryba et al., 2008). This population activity was rectified and integrated and the data were digitized with a Digidata acquisition system (Molecular Devices, CA), stored on an IBM compatible PC using Axoscope 10 (Molecular Devices, CA) software and analyzed off-line using Igor Pro (WaveMetrics, Lake Oswego, OR). The raw extracellular population VRG and XII data was integrated using a custom built electronic integrator (JFI electronics, The University of Chicago, Chicago), with an integration time constant was 70ms. The $\int\text{VRG}$ population burst amplitude was measured as baseline to peak height, while frequency was calculated based on the burst intervals. To minimize the potential influence of baseline fluctuations and differences in burst peak trajectories, the $\int\text{VRG}$ or $\int\text{XII}$ burst duration was calculated as the duration of the burst at half-maximal burst amplitude. To show the relationship between the onset of the $\int\text{VRG}$ or $\int\text{XII}$ bursts, we used the clampfit 10 (Molecular Devices, CA) software thresholding feature to select bursts and acquire a window of data for a fixed time before and after the peak of the $\int\text{VRG}$ or $\int\text{XII}$ bursts. We then averaged the averaged the $\int\text{VRG}$ or $\int\text{XII}$ bursts, with clampfit 10 and aligned the averaged bursts by the onset of the data point windows.

Intracellular whole cell current-clamp recordings were obtained with a MultiClamp 700B amplifier (Molecular Devices, CA), applying the blind-patch technique to VRG neurons in 600 μm brainstem slice preparations (Thoby-Brisson and Ramirez, 2001). Patch electrodes were manufactured from filamented borosilicate glass tubes (Clark G150F-4; Warner Instruments Corp., Hamden, CT, USA) and filled with an intracellular solution containing (in mM): 140 K-gluconic acid, 1 $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 10 EGTA, 2 $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 4 Na_2ATP , 10 HEPES.

Only inspiratory VRG neurons active throughout and in-phase with the $\int\text{VRG}$ population burst were recorded in this current-clamp study. The discharge pattern of each inspiratory neuron was first identified in the cell-attached mode and remained similar in whole-cell configuration (Pena et al., 2004b). Experiments were then performed in the whole cell patch-clamp mode. The V_m values were corrected for the liquid junction potential as calculated using pClamp 10 software (Molecular Devices). In current-clamp, neurons were isolated from ionotropic chemical synaptic input using a mixture of glutamatergic, GABAergic and glycinergic antagonists. These drugs were bath applied at the final concentrations of: 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX (Tocris, Ellisville MO, USA)), 10 μM (RS)-3-(2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid ((RS)-CPP)(Tocris), 1 μM Strychnine (Sigma) and 20 μM bicuculline free-base (Sigma). Note that unlike bicuculline methiodide,

the bicuculline free base derivative is a specific GABA receptor antagonist that does not block apamin-sensitive Ca^{2+} -activated K^{+} currents (Johnson and Seutin, 1997; Debarbieux et al., 1998).

A subset of inspiratory neurons, considered pacemakers, continued to generate voltage-dependent intrinsic bursting properties following blockade of ionotropic glutamate (both AMPAR and NMDAR antagonists were co-applied), GABA and glycinergic receptors. These pacemakers met several criteria before being classified as inspiratory pacemaker neurons, the criteria used here are provided in detail elsewhere (Thoby-Brisson and Ramirez, 2001; Tryba et al., 2003). Briefly, after isolation of the neuron from chemical synaptic input with bath applied CNQX, CPP, strychnine and bicuculline, pacemakers continued to burst in absence of VRG population bursts. Second, isolated pacemakers exhibited voltage-dependent bursting properties. That is, brief depolarizing current injection could evoke a burst, or hyperpolarizing current could terminate an ongoing burst; either of these reset the ongoing pacemaker bursting rhythm. Finally, depolarizing current injection increased, and injected hyperpolarizing current decreased, the bursting frequency. After synaptic isolation with CNQX, CPP, bicuculline and strychnine, in some cases, cadmium (Cd^{2+}), a broad-spectrum calcium channel blocker (Elsen and Ramirez, 1998), was used as a tool to discriminate between pacemaker neurons whose endogenous bursting mechanism is dependent on calcium versus sodium (Thoby-Brisson and Ramirez, 2001). Synaptically isolated pacemakers that continue to burst in the presence of voltage-gated calcium channel blockade with $200\mu\text{M}$ Cd^{2+} are described as Cd^{2+} -insensitive (CI-) pacemakers (Thoby-Brisson and Ramirez, 2001) and are typically riluzole-sensitive (Pena et al., 2004b). Note that $200\mu\text{M}$ Cd^{2+} ensures blockade of voltage activated calcium currents in VRG neurons (Elsen and Ramirez, 1997). Thus, following bath application of $200\mu\text{M}$ Cd^{2+} calcium-dependent chemical synaptic transmission should be blocked. The endogenous bursting of CI-pacemakers is also sensitive to ketanserin, a serotonin type 2a (5HT_{2a}) receptor antagonist, that reduces low threshold sodium currents (Pena and Ramirez, 2002; Tryba et al., 2006). Thus, in CI pacemakers, we additionally added ketanserin as a tool to test if modulating low threshold sodium currents alters CI bursting regularity (Pena and Ramirez, 2002; Tryba et al., 2006). In contrast, isolated pacemakers that cease bursting in Cd^{2+} , are Cd^{2+} -sensitive (CS-) pacemakers (Thoby-Brisson and Ramirez, 2001) and have been shown to be FFA-sensitive (FFAS) (Pena et al., 2004b).

To determine the effect of lowering $[\text{Na}^{+}]_o$ on VRG population and inspiratory neuron rhythm regularity, yet maintain ionic balance, we substituted ACSF $[\text{Na}^{+}]_o$ with equimolar N-methyl-D-glucamine (NMDG). Thus, for example, ACSF with a 20% reduction in $[\text{Na}^{+}]_o$ contained a 20% equimolar equivalent of NMDG. Here, we refer to such an ACSF, with a 20% reduction in $[\text{Na}^{+}]$ as 80% $[\text{Na}^{+}]_o$ ACSF - as it contains 80% of normal $[\text{Na}^{+}]_o$. Likewise, ACSF with 75% normal $[\text{Na}^{+}]_o$ contains a 25% reduction in mM than control ACSF, etc.

To exchange solutions, in each case we washed-on the carbogen saturated ACSF containing (8mM $[\text{K}^{+}]_o$ at 30°C) and the tested low- $[\text{Na}^{+}]_o$ (either 87%, 80%, 75%) over a 10min. period. The bath solution was then recycled over an additional 10 min. period. Data collected over the subsequent 10 min. period was used for measurements and comparison of rhythm regularity. After the response to low- $[\text{Na}^{+}]_o$ ACSF was recorded, in some cases, we then washed-on and recycled control ACSF and examined recovery of respiratory activity.

To measure VRG or pacemaker bursting regularity, we calculated an irregularity score (S), by applying a formula for consecutive cycle length values: $S_n = 100 * \text{ABS}(\text{P}_n - \text{P}_{n-1}) / \text{P}_{n-1}$, where S_n = score of the nth cycle, P_n being its period, P_{n-1} the period of the preceding burst and ABS the absolute value (Barthe and Clarac, 1997; Telgkamp et al., 2002; Viemari et al., 2005c; Tryba et al., 2006). Biological rhythms are not without variability, thus after this calculation, we statistically compare irregularity scores from control versus experimentally manipulated

conditions (e.g. control versus low- $[Na^+]_o$ ACSF application); $p \leq 0.05$ is considered to reach significance. Regular rhythms have lower irregularity scores while irregular rhythms have higher irregularity scores (Telgkamp et al. 2002, Tryba et al. 2006). For comparison, we calculated the irregularity score of the VRG and isolated pacemaker rhythmic bursts over a period of 10 mins. in control and following experimental manipulation. However, in isolated CI pacemakers where ketanserine was added, their bursting mechanism is sensitive to (and eventually blocked by) ketanserine (Tryba et al. 2006). Thus, in those cases where ketanserine was added, we compared the irregularity score over a 5 min period in control versus a 5 min period following ketanserine addition, that began 7 mins before and ended 2 mins before we could no longer evoke voltage dependent bursting by depolarizing current injection.

Voltage Clamp Studies - Sodium Currents—To investigate which sodium currents are effected by reducing ACSF $[Na^+]_o$, we used visual voltage patch-clamping of medullary brainstem neurons in the region of the VRG, using a Multiclamp 700B amplifier. Data were collected with a personal computer using pClamp 10 software combined with a Digidata 1440A interface (Molecular Devices, CA, USA). Visual targeting of neurons was aided with a Zeiss Axioscop 2FS (Thornwood, NY), using infrared filtering and images captured with a DMK 31AF03 firewire camera and software (The Imaging Source, Charlotte, NC, USA). Slices were submerged in a recording chamber (2.5 mL) under circulating ACSF (30°C; flow rate 8 ml/min). The voltage clamp electrodes had a resistance of 3-5 M Ω when filled with the whole-cell patch clamp pipette solution containing chemicals obtained from Sigma (St. Louis, MO, USA) in mM: 110 CsCl, 30 TEA-Cl, 1 CaCl₂, 10 EGTA, 2 MgCl₂, 4 Na₂ATP, 0.3 Na-GTP, 10 HEPES (pH = 7.2).

For visual voltage clamp studies, we targeted medullary neurons 2-4 cell layers from the slice surface, as those located on or near the surface of a 400 μ m respiratory brain slice could be more likely damaged during the preparation of the slice than neurons located deeper within the slice. Current response traces were recorded with online leak subtraction, eliminating the linear leak current and residual capacity currents. After establishing the whole cell configuration the series resistance was compensated by 80% and regularly corrected throughout the experiments.

We should emphasize that whole-cell voltage clamp recordings from neurons embedded in a functional network are accompanied by difficult clamp control that could lead to incorrect measurements of current amplitudes. Thus, recordings with obvious space clamp problems (Armstrong CM, 1992; Cepeda et al., 2003) were discarded. Poor space clamping was indicated by rebound spikes (rapid, fast inactivating inward currents, which were induced by steps from depolarizing test potentials to the former holding potential) or an increase in the delay to onset of an inward current with increasing magnitude of test pulse. Steps to higher test potentials were typically associated with a reduction in delay to current onset. We also discarded neurons with insufficiently blocked K⁺ currents. This was evident in outward currents typically commencing at voltage steps to 10 mV under control conditions. Voltage-activated inward currents were pharmacologically isolated by intracellular blockade of voltage-activated potassium currents with 110 mM CsCl and 30 mM tetraethylammonium (TEA) chloride (Elsen and Ramirez, 2005). To isolate the voltage-activated sodium currents ($I_{Na^+ (f)}$ and $I_{Na^+ (p)}$) we blocked the voltage-activated calcium currents by bath application of 200 μ M cadmium.

Data analysis

The data were analyzed off-line with ClampFit 9.2 (Molecular Devices), Igor Pro (WaveMetrics, Lake Oswego, OR) and further statistical analysis was performed with Graph Pad Prism software v4.03 (San Diego, CA). All quantitative data are given as mean $\pm$ standard error, significance was assessed with Students t-tests and $p \leq 0.05$ are considered to reach significance.

Results

Lowering $[Na^+]_o$ reduces VRG fictive eupneic rhythm regularity—Several studies indicated that subthreshold sodium and potassium currents may be important neuromodulatory targets and play important roles in respiratory rhythm generation (Tryba et al., 2003; Tryba and Ramirez, 2004b; Koizumi and Smith, 2008). To begin to investigate whether sodium conductances play a role in respiratory regularity, we recorded fictive eupneic inspiratory bursting in the *in vitro* transverse medullary slice preparation from mice, containing the respiratory network (VRG) (Fig. 1a). After recording VRG inspiratory bursting in control ACSF for 10 mins., we tested whether lowering ACSF $[Na^+]_o$ from control (144 mM $[Na^+]_o$) to either 87%, 80% or 75% of control alters VRG population bursting regularity, amplitude and duration (Figs. 1b, 1c, 1d; Fig. 2a, 2b, 2c). In VRG slice preparations (n=36 total), lowering ACSF $[Na^+]_o$ to 87% (n=9), 80% (n=12) or 75% (n=15) of control significantly reduces the regularity of VRG fictive eupneic bursting; $p=0.026$ (paired Student's T-test) for 87% (n=9), $p=0.003$, (paired Student's T-test) for 80% (n=12), $p=0.014$ (paired Student's T-test) for n=6/15 in 75%; n=9/15 slice preparations ceased bursting and became silent in 75% $[Na^+]_o$ ACSF, so no irregularity score could be calculated in these n=9 preparations) (Fig. 2a). In n=5 preparations, we tested if and found that VRG bursting regularity recovered to control levels ($p=0.47$; paired Student's T-test) after lowering ACSF $[Na^+]_o$ to 80% and returning to control ACSF (100% $[Na^+]_o$) (Fig. 2a).

The VRG fictive eupneic bursts provide excitatory drive to the hypoglossal (XII) motor neurons. As shown in previous studies (Tryba and Ramirez 2004a; Tryba et al., 2006), each inspiratory VRG burst is preceded and is synchronized to a XII burst (Figs. 1a, 1b). In n=4 preparations, we simultaneously recorded VRG and hypoglossal (XII) motor neuron activity while lowering ACSF $[Na^+]_o$. Reducing ACSF to 80% simultaneously reduced VRG and XII population bursting in parallel, suggesting that the reduction in VRG bursting regularity following lowering ACSF $[Na^+]_o$ is likely to have a behaviorally relevant impact on inspiratory motor outflow (Fig. 1b, 1c). Note the data are shown at different time scales in Figs. 1b, 1c. Overlaying averaged $\int$ VRG or $\int$ XII bursts, on the same expanded time-scale, indicates their burst durations are similar to controls (black line, n=30 bursts averaged) and after lowering ACSF $[Na^+]_o$ to 80% (Figs. 1d) (red lines, n=51 bursts averaged, $p=0.074$, unpaired T-test of $\int$ VRG burst duration; $p=0.099$ unpaired t-test of $\int$ XII burst duration, n=55 bursts averaged). Additionally, our data suggest the observed VRG bursting irregularity is unlikely due to a global reduction in synaptic transmission, since the VRG drives XII motor pool bursting without loss of synchronized fidelity.

As another indication that lowering ACSF $[Na^+]_o$ did not simply reduce VRG network synchronization and in turn fictive eupneic regularity, there was no reduction in VRG burst amplitude (Fig. 2b; $p=0.22$ (paired Student's T-test) for 87%, n=9, $p=0.06$ (paired Student's T-test) for 80% (n=12), $p=0.15$ (paired Student's T-test) for in 75% (n=6); $p=0.32$ (paired Student's T-test) following recovery from 80%) or duration (Fig. 2c; $p=0.094$ (paired Student's T-test) for 87%, $p=0.38$ (paired Student's T-test) for 80%, $p=0.22$ (paired Student's T-test) for n=6/15 in 75%, $p=0.63$ following recovery from 80%).

Inspiratory pacemaker bursting becomes irregular in low- $[Na^+]_o$ ACSF—Two basic types of preBötC inspiratory pacemaker neurons were previously described that generate endogenous, voltage-dependent bursting after being synaptically isolated from ionotropic glutamatergic input (Thoby-Brisson and Ramirez, 2001; Pena et al., 2004b; Del Negro et al., 2005; Tryba et al., 2006). The bursting mechanism of one type of pacemaker is sensitive to cadmium (cadmium-sensitive, CS) and flufenamic acid (FFA-sensitive, FFAS), the other type is cadmium- and flufenamic- insensitive (cadmium-insensitive, CI) but riluzole-sensitive (RS) (Pena et al., 2004a; Tryba et al., 2006; Viemari and Ramirez, 2006). We refer to these cells as

pacemakers because following isolation from glutamatergic synaptic input with CNQX, CPP, and isolation from inhibitory transmission with bicuculline and strychnine, they exhibited endogenous, voltage-dependent pacemaker properties (Figs. 3a) (Thoby-Brisson and Ramirez, 2001; Tryba et al., 2003; Pena et al., 2004b). Previous studies showed that, in contrast to nonpacemakers, respiratory pacemakers exhibit a slow, long-lasting (minutes) depolarizing “sag” current upon hyperpolarization (Tryba et al., 2003) that is blocked by TTX and low- $[\text{Na}^+]_o$ ACSF, suggesting a background sodium current underlies the depolarizing sag. These data indicated that a background sodium current in part stabilizes the membrane potential of pacemaker neurons (Tryba et al., 2003; Tryba and Ramirez, 2004b) and here we examine these initial findings with respect to respiratory rhythm regularity.

While continuously applying the cocktail of synaptic antagonists, additionally lowering the ACSF $[\text{Na}^+]_o$ to either 87% ($n=8$; $p=0.003$, paired Student's T-test) or 75% ($n=5$; $p=0.009$, paired Student's T-test) of control ACSF, significantly increased the irregularity of synaptically isolated pacemaker bursting (Fig. 4a; 4b; 4c). Reducing ACSF $[\text{Na}^+]_o$, *via* NMDG substitution, did not significantly alter the baseline membrane potential ($p=0.31$, paired Student's T-test, Fig. 4d), action potential amplitude (AP Amp., $p=0.69$, paired Student's T-test; Fig. 4d), burst duration ($p=0.68$, paired Student's T-test, Fig. 4d) or burst frequency (Burst FRQ., $p=0.88$, paired Student's T-tests, Fig. 4d). However, the 13% reduction in ACSF $[\text{Na}^+]_o$ decreased the number of action potentials/burst (#AP/BRST, Fig. 4d; ($p=0.013$; paired Student's T-test), despite normalizing the number of action potentials to the burst duration (#AP/BD, Fig. 4d; ($p=0.03$; paired Student's T-test).

In contrast to lowering ACSF $[\text{Na}^+]_o$ (Fig. 4a; 4b) we found that blocking voltage gated calcium currents with Cd^{2+} (200 μM), decreased Cd^{2+} -insensitive (CI) pacemaker irregularity ($n=6$; Fig. 4e; $p=0.006$, paired Student's T-test). In addition to differential sensitivity to either Cd^{2+} or FFA, the two pacemaker types respond differently to antagonists of the 5-hydroxytryptamine type 2a (5HT2a) receptor (Pena and Ramirez, 2002; Tryba et al., 2006)(Fig. 4e). CS pacemaker bursting is blocked by the 5HT2a receptor antagonists, ketanserin or piperidine, whereas FFAS pacemaker bursting continues in their presence (Pena and Ramirez, 2002; Tryba et al., 2006)(Fig. 4e). The cessation of CI pacemaker bursting following application of 5HT2a receptor antagonists is through a signaling mechanism that reduces low threshold, persistent sodium current ($\text{INa}^+(p)$) (Pena and Ramirez, 2002; Tryba et al., 2006). In the presence of Cd^{2+} , additionally blocking low threshold sodium currents with the 5HT2a antagonist, ketanserin (40 μM) (Pena and Ramirez, 2002), increased the irregularity of Cd^{2+} -insensitive pacemaker bursting ($\text{Cd}^{2+} + \text{KET}$; $n=6$, $p=0.005$, paired Student's T-test ; Fig. 4e).

Both low threshold Na^+ and K^+ leak currents have been proposed to play an important role in respiratory neural network rhythm generation (Tryba et al., 2003; Tryba and Ramirez, 2004b; Koizumi and Smith, 2008). In contrast to manipulating sodium and calcium currents, in six other synaptically isolated pacemakers, raising $[\text{K}^+]_o$ from 3mM to 8mM did not significantly alter pacemaker bursting regularity (Fig. 4f; $p=0.29$, paired Student's T-test) (Tryba et al., 2003).

Nonpacemaker inspiratory neuron excitability following lowering ACSF

$[\text{Na}^+]_o$ —To examine if low- ACSF $[\text{Na}^+]_o$ altered respiratory nonpacemaker excitability, in $n=14$ brain slice preparations, we simultaneously recorded both VRG network activity and nonpacemaker inspiratory neuron activity using whole-cell current clamp, while reducing ACSF $[\text{Na}^+]_o$ to either 75% ($n=7$; Figs. 5a-i, 5a-ii, and 5a-iii) or 87% ($n=7$) of control ACSF. While the network bursting is irregular in low- $[\text{Na}^+]_o$ ACSF (Fig. 2a), nonpacemaker action potential amplitude ($p=0.12$ for 75%; $p=0.29$ for 87%, paired Student's T-test), number of action potentials per burst ($p=0.56$ for 75%; $p=0.26$ for 87%, paired Student's T-test), action potential frequency ($p=0.32$ for 75%; $p=0.35$ for 75%, paired Student's T-test) and membrane

potential (0.39 for 75%; $p=0.77$ for 87%, paired Student's T-test) are not significantly effected by low- $[\text{Na}^+]_o$ (Figs. 5a-5c). We did find a significant increase (Figs. 5a, 5b; $p=0.034$ for 75%, paired Student's T-test; $p=0.048$ for 87%, paired Student's T-test) in nonpacemaker burst duration in low- $[\text{Na}^+]_o$ ACSF, but this did not significantly impact VRG population burst duration (Fig. 2c).

Lowering ACSF $[\text{Na}^+]_o$ to 87% reduces persistent, not fast sodium currents—

To begin to examine which sodium currents underlie reduced respiratory rhythm regularity following a reduction in ACSF $[\text{Na}^+]_o$, we voltage clamped medullary neurons in the region of the VRG ($n=6$). At least four of the six neurons tested were inspiratory neuron as they received rhythmic inspiratory synaptic burst inputs typical of inspiratory VRG neurons, seen in other studies (Koizumi and Smith, 2008) (Figs. 6a; 6b). We then isolated the transient and persistent sodium currents and examined the effect of lowering ACSF $[\text{Na}^+]_o$ to 87% of control - which in both the above population and isolated pacemaker studies was sufficient to increase rhythm irregularity. Consistent with our current-clamp action potential measurements (Figs. 4d; 5), lowering ACSF $[\text{Na}^+]_o$ to 87% did not significantly reduce the fast sodium current underlying the transient action potential (Figs. 6c; 6d; $p=0.66$, paired Student's T-test, $n=4$). In contrast, this manipulation caused a significant reduction in the sustained or persistent sodium current (Figs. 6c; 6d; $p=0.008$, paired Student's T-test). We also found similar results from $n=2$ additional medullary neurons (not shown) in the region of the VRG, that were not receiving rhythmic synaptic drive during VRG bursts. In these neurons, washing on 87% $[\text{Na}^+]_o$ also did not alter the fast transient sodium current (black trace; $p=0.79$, Student's T-Test, $n=2$) but reduced the persistent sodium current (red trace; $p=0.005$, Student's T-test, $n=2$).

Discussion

Rhythmic electrical circuits underlying seemingly disparate activities such as nonrapid eye movement (NREM) sleep (Babcock and Badr, 1998; McNamara et al., 2002), locomotion (Zhong et al., 2007), gut peristalsis (Hooper and Moulins, 1989; Prinz et al., 2004), cardiac cycling (Dipolo and Beauge, 2006; Mangoni et al., 2006; Saint, 2006) and respiration have in common a requirement of coordinated rhythm regularity. For example, regular cardiac cycling maintains blood flow. NREM sleep is characterized by regular rhythmic discharges of the neocortex, whereas disruption of this rhythm can lead to sleep disturbances (Steriade et al., 1993; McNamara et al., 2002; Foldvary-Schaefer and Grigg-Damberger, 2006). With respect to the respiratory rhythm, breathing must be triggered at regular intervals to maintain adequate blood gasses whereas irregular respiratory patterns are typical of respiratory diseases such as central apneas, Rett Syndrome and Sudden Infant Death Syndrome (SIDS) (Issa and Porostocky, 1993; Carley et al., 1998; Viemari et al., 2005a; Tryba et al., 2006; Hunt et al., 2008).

Despite the apparent clinical importance of regularity of rhythms generated by neural circuits, relatively little information is available regarding which ionic mechanisms are critical to generate regular rhythmic cycling. Such information seems important to identify potential pharmaceutical targets to alleviate irregularities in rhythmogenesis underlying irregular sleep states, locomotory or breathing patterns (Tryba and Ramirez, 2004b; Tryba et al., 2006; Fengler et al., 2007; Koizumi and Smith, 2008). Similarly, identifying ionic mechanisms that can be targeted to disrupt pathologic, but regular rhythmic, or 'hypersynchronous' discharges during epileptic seizures could lead to new approaches to disrupt ongoing status epilepticus (Stafstrom, 2007). Surprisingly, despite its clinical relevance, very few studies have been focused on which ionic currents are critical to rhythm regularity (Wilders and Jongsma, 1993; Pena and Ramirez, 2002).

To our knowledge, this is the first data to indicate that background sodium currents play a critical role in the regularity of respiratory pacemaker bursting. Further, only few studies suggested an important role of background sodium currents on the respiratory pattern associated with fictive eupnea (Tryba et al., 2003; Tryba and Ramirez, 2004b; Koizumi and Smith, 2008). These data point to the possibility that neuromodulation, or pharmaceutical manipulation of low threshold sodium currents as a potential mechanism to correct breathing irregularities (Tryba et al., 2006; Koizumi and Smith, 2008) and epilepsy (Stafstrom, 2007).

Several studies suggest that the generation of the eupneic pattern results from a complex interaction between the emergent properties of a synaptically coupled network and intrinsically bursting pacemaker neurons (Del Negro et al., 2002; Pena et al., 2004a; Del Negro et al., 2005; Feldman and Del Negro, 2006; Pace et al., 2007; Koizumi and Smith, 2008; Tryba et al., 2008). Further, pacemakers likely play an important role to amplify and initiate the respiratory rhythm from cycle to cycle, while synaptic mechanisms are critical to synchronize and propagate the fictive eupneic bursting throughout the network (Ramirez et al., 2004b). Similar synaptic and intrinsic mechanisms have been proposed for other fictive respiratory patterns, including augmented breaths, called sighs and gasping during severe hypoxia (Tryba et al., 2008). Regardless of the underlying mechanisms of respiratory rhythm or pattern generation, this study serves to identify a specific ionic mechanism, background sodium currents, as likely key players in central breathing rhythm regularity, whose dysregulation may contribute to disturbed breathing in disease states (Tryba et al., 2003; Tryba and Ramirez, 2004b; Lu, 2007; Koizumi and Smith, 2008). While a lot more work, specifically *in vivo* studies, would need to be done to test this hypothesis further, our data additionally suggest the possibility that altered low-threshold sodium currents may underlie irregular breathing patterns seen in respiratory diseases such as Sudden Infant Death Syndrome (Tryba et al., 2006) and Rett Syndrome (Viemari et al., 2005a). That said, we have yet to identify whether subthreshold tetrodotoxin resistant (TTX-R) or sensitive (TTX-S) currents play a more important role, or whether they equally contribute to determining rhythm regularity (Massimo et al., 1998; Blair and Bean, 2003; Do and Bean, 2003; Tryba and Ramirez, 2004b; Koizumi and Smith, 2008).

Here, lowering ACSF $[Na^+]_o$ decreased fictive eupneic population and pacemaker regularity giving rise to irregular VRG and XII bursting. Reducing ACSF $[Na^+]_o$ reduced the regular initiation of respiratory pacemaker bursting that we hypothesize results in lowered regularity of network and motor output bursting. The sensitivity of the respiratory pacemaker and network rhythm regularity to altering background sodium currents may also be expected, as in other systems, background sodium currents represent the major current flowing between pacemaker potentials (Taddese and Bean, 2002; Do and Bean, 2003; Bean, 2004). That said, of course, in gut peristalsis, or other systems, ionic mechanisms, different than sodium, may play a more important role in rhythm regularity.

Interestingly, *during* fictive eupneic VRG bursts, the effectiveness of synaptic drive in synchronizing population activity is likely regulated by the inactivating A-current (I_A) (Hayes et al., 2008). Accordingly, blocking I_A with 4-aminopyridine (4-AP), increases the irregularity of fictive eupneic rhythm (Hayes et al., 2008). As indicated by Hayes et al. (2008), an important caveat to this hypothesis is that future studies will need to clarify whether this 4-AP effect is due to altered synchronization of the network, or potential effects of 4-AP on other potassium currents (Hayes et al., 2008). With regard to rhythm regularity, if synaptic effectiveness and network synchronization is, as proposed (Hayes et al., 2008), modulated by I_A , when taken together with the results of the present study, the data suggest that the interplay of intrinsic membrane currents regulating excitability and synaptic drive can play an important role in the propagation of the fictive eupneic respiratory pattern. We hypothesize that background sodium currents in pacemaker neurons play an important role in initiation of the respiratory rhythm, while network synaptic drive and synchronization is likely regulated by mechanisms such as

I_A (Hayes et al., 2008) after the rhythmic burst is initiated. This interplay of synaptic and intrinsic mechanisms in turn likely regulates the timing and propagation of the fictive eupneic burst throughout the network, disruption of which can alter rhythm regularity.

Previous studies indicated that persistent, background sodium currents play a more important role than potassium “leak” currents in the membrane excitability of respiratory pacemakers when compared with non-pacemakers (Tryba et al., 2003; Tryba and Ramirez, 2004b). Those studies indicated that background sodium currents serve to stabilize the baseline membrane potential of pacemaker neurons within a range of membrane potentials where they preferentially express their voltage-dependent bursting properties. In marked contrast to pacemakers, whose resting membrane potential did not significantly change when $[K^+]_o$ was increased from 3mM to 8mM, non-pacemaker respiratory neurons depolarized (Tryba et al., 2003). Accordingly, non-pacemaker excitability can be predicted by a Nernstian resting potential dominated by E_{K^+} , while this was not the case for respiratory pacemakers, whose resting membrane potential was previously shown to be dominated by persistent sodium currents (Tryba et al., 2003; Tryba and Ramirez, 2004b).

Recently, voltage clamp studies confirmed these current-clamp studies (Tryba and Ramirez, 2004b) indicating that subthreshold persistent sodium currents play a more important role in stabilizing the baseline membrane potential of pacemakers than non-pacemakers (Koizumi and Smith, 2008). Thus, current clamp and voltage clamp studies, using various pharmacological and ion substitution approaches, have independently confirmed that respiratory pacemaker excitability contrasts that of non-pacemakers and largely depends on persistent, background sodium currents while non-pacemaker excitability is relatively more dependent on potassium (leak) currents (Tryba et al., 2003; Tryba and Ramirez, 2004b; Koizumi and Smith, 2008). Here, we additionally hypothesize that the functional consequence of these differences can result in irregular central respiratory rhythm generation, whereby alterations in background sodium currents may lead to irregular initiation of fictive eupneic activity.

The enhanced role of background sodium currents in the excitability of pacemakers versus non-pacemakers suggests that there are likely differences in expression of both TTX-S and/or TTX-R channels underlying low threshold sodium currents in these neurons (Tryba and Ramirez, 2004b). The voltage-independent, nonselective cation channel, NALCN has been suggested to be a potential candidate channel for TTXR channels regulating the excitability of neurons (Lu, 2007). Accordingly, the background Na^+ leak current is absent in NALCN mutant hippocampal neurons (Lu, 2007). However, while NALCN mutant mice have respiratory dysrhythmias, further studies would be needed to determine if NALCN expression is (for example) up-regulated in respiratory pacemakers versus non-pacemakers; to better understand whether NALCN channels may contribute to the difference in background sodium currents among them and its potential role in modulating the regularity of respiratory rhythmogenesis (Lu, 2007).

During hypoxia, failure of regular gasping to autoresuscitate is proposed to underlie Sudden Infant Death Syndrome (SIDS) (Tryba et al., 2006). Central gasping rhythmogenesis has been proposed to depend on persistent sodium-dependent (cadmium-insensitive) pacemakers, whose bursting mechanism is sensitive to 5HT_{2a} receptor activation that enhances persistent sodium currents (Tryba et al., 2006). Blocking voltage gated calcium channels with cadmium enhanced CI pacemaker bursting regularity, but we did not investigate the mechanism for this effect here. In contrast, the 5HT_{2a} receptor antagonist, ketanserin, reduced CI pacemaker bursting regularity. Taken together, these data additionally suggest that low threshold sodium currents can play a major role in respiratory rhythm regularity, neuromodulation of which may help in understanding breathing irregularities.

Acknowledgments

Supported by: NIH R01-HL 079294 to AKT. Authors thank Bert Forster for editing the manuscript prior to submission.

Abbreviations

preBötC	preBötzinger complex
VRG	Ventral Respiratory Group
SIDS	Sudden Infant Death Syndrome

References

- Armstrong CMGW. Access resistance and space clamp problems associated with whole-cell patch clamping. *Methods Enzymol* 1992;207:100–122. [PubMed: 1528114]
- Babcock MA, Badr MS. Long-term facilitation of ventilation in humans during NREM sleep. *Sleep* 1998;21:709. [PubMed: 11286347]
- Barthe J-Y, Clarac F. Modulation of the spinal network for locomotion by substance P in the neonatal rat. *Exp Brain Res* 1997;115:485–492. [PubMed: 9262203]
- Bean BP. Ionic Currents Driving Pacemaking in Central Neurons. *Soc For Neurosci Abstr*. 2004
- Blair NT, Bean BP. Role of Tetrodotoxin-Resistant Na⁺ Current Slow Inactivation in Adaptation of Action Potential Firing in Small-Diameter Dorsal Root Ganglion Neurons. *J Neurosci* 2003;23:10338–10350. [PubMed: 14614093]
- Carley David W, Trbovic Sinisa M, Radulovacki M. Diazepam Suppresses Sleep Apneas in Rats. *Am J Respir Crit Care Med* 1998;157:917–920. [PubMed: 9517612]
- Cepeda C, Hurst RS, Flores-Hernández J, Hernández-Echeagaray E, Klapstein GJ, Boylan MK, Calvert CR, Jocoy EL, Nguyen OK, André VM, Vinters HV, Ariano MA, Levine MS, G.W. M. Morphological and electrophysiological characterization of abnormal cell types in pediatric cortical dysplasia. *Journal of Neuroscience Research* 2003;72:472–486. [PubMed: 12704809]
- Debarbieux F, Brunton J, Charpak S. Effect of bicuculline on thalamic activity: a direct blockade of IA_{HP} in reticularis neurons. *J Neurophys* 1998;79:2911–2918.
- Del Negro CA, Morgado-Valle C, Feldman JL. Respiratory Rhythm: An Emergent Network Property? *Neuron* 2002;34:821–830. [PubMed: 12062027]
- Del Negro CA, Morgado-Valle C, Hayes JA, Mackay DD, Pace RW, Crowder EA, Feldman JL. Sodium and Calcium Current-Mediated Pacemaker Neurons and Respiratory Rhythm Generation. *J Neurosci* 2005;25:446–453. [PubMed: 15647488]
- Dipolo R, Beauge L. Sodium/Calcium Exchanger: Influence of Metabolic Regulation on Ion Carrier Interactions. *Physiol Rev* 2006;86:155–203. [PubMed: 16371597]
- Do MTH, Bean BP. Subthreshold Sodium Currents and Pacemaking of Subthalamic Neurons: Modulation by Slow Inactivation. *Neuron* 2003;39:109–120. [PubMed: 12848936]
- Dudek FE, Wuarin J-P, Tasker JG, Kim YI, Peacock WJ. Neurophysiology of neocortical slices resected from children undergoing surgical treatment for epilepsy. *Journal of Neuroscience Methods* 1995;59:49–58. [PubMed: 7475250]
- Elsen FP, Ramirez JM. Hypoxia reduces the amplitude of voltage dependent calcium currents in the isolated respiratory system of mice. *Soc Neurosci Abstr* 1997;27:495.411.
- Elsen FP, Ramirez JM. Calcium currents of rhythmic neurons recorded in the isolated respiratory network of neonatal mice. *J Neurosci* 1998;18:10652–10662. [PubMed: 9852600]
- Elsen FP, Ramirez J-M. Postnatal Development Differentially Affects Voltage-Activated Calcium Currents in Respiratory Rhythmic Versus Nonrhythmic Neurons of the Pre-Botzinger Complex. *J Neurophysiol* 2005;94:1423–1431. [PubMed: 15888528]
- Feldman JL, Del Negro CA. Looking for inspiration: new perspectives on respiratory rhythm. *Nat Rev Neurosci* 2006;7:232–241. [PubMed: 16495944]

- Feldman JL, Mitchell GS, Nattie EE. BREATHING: Rhythmicity, Plasticity, Chemosensitivity. *Annual Review of Neuroscience* 2003a;26:239–266.
- Feldman JL, Mitchell GS, Nattie EE. Breathing: rhythmicity, plasticity, chemosensitivity. *Annu Rev Neurosci* 2003b;26:239. [PubMed: 12598679]
- Fengler BT, Brady WJ, Plautz CU. Atrial fibrillation in the Wolff-Parkinson-White syndrome: ECG recognition and treatment in the ED. *The American Journal of Emergency Medicine* 2007;25:576–583. [PubMed: 17543664]
- Foldvary-Schaefer N, Grigg-Damberger M. Sleep and epilepsy: What we know, don't know, and need to know. *J Clin Neurophys* 2006;23:4–20.
- Fuller DD, Zabka AG, Baker TL, Mitchell GS. Phrenic long-term facilitation requires 5-HT receptor activation during but not following episodic hypoxia. *J Appl Physiol* 2001;90:2001. [PubMed: 11299296]
- Hayes JA, Mendenhall JL, Brush BR, Del Negro CA. 4-aminopyridine-sensitive outward currents in preBotzinger Complex neurons influence respiratory rhythm generation in neonatal mice. *J Physiol* 2008;586(7):1921–36. [PubMed: 18258659]
- Hooper SL, Moulins M. Switching of a neuron from one network to another by sensory-induced changes in membrane properties. *Science* 1989;244:1587–1580. [PubMed: 2740903]
- Huey KA, Brown IP, Jordan MC, Powell FL. Changes in dopamine D(2)-receptor modulation of the hypoxic ventilatory response with chronic hypoxia. *Respir Physiol* 2000;123:177. [PubMed: 11007985]
- Hunt C, Corwin M, Lister G, Weese-Mayer DE, Davidson SL, Tinsley LR, Neuman MR, Willinger M, Ramanathan R, Rybin D, Group CHIMES. Precursors of cardiorespiratory events in infants detected by home memory monitor. *Pediatric Pulmonology* 2008;43:87–98. [PubMed: 18041078]
- Issa FG, Porostocky S. Effect of sleep on changes in breathing pattern accompanying sigh breaths. *Respir Physiol* 1993;93:175–187. [PubMed: 8210757]
- Johnson SW, Seutin V. Bicuculline methiodide potentiates NMDA-dependent burst firing in rat dopamine neurons by blocking Ca²⁺-activated K⁺ currents. *Neurosci Letts* 1997;231:13–16. [PubMed: 9280156]
- Koizumi H, Smith JC. Persistent Na⁺ and K⁺-Dominated Leak Currents Contribute to Respiratory Rhythm Generation in the Pre-Botzinger Complex In Vitro. *J Neurosci* 2008;28:1773–1785. [PubMed: 18272697]
- Lu BSY, Das S, Liu J, Xia J, Ren D. The neuronal channel NALCN contributes resting sodium permeability and is required for normal respiratory rhythm. *Cell* 2007;129:371–383. [PubMed: 17448995]
- Mangoni ME, Traboulsie A, Leoni A-L, Couette B, Marger L, Le Quang K, Kupfer E, Cohen-Solal A, Vilar J, Shin H-S, Escande D, Charpentier F, Nargeot J, Lory P. Bradycardia and Slowing of the Atrioventricular Conduction in Mice Lacking Ca_v3.1/ α 1G T-Type Calcium Channels. *Circ Res* 2006;98:1422–1430. [PubMed: 16690884]
- Massimo M, Francheschetti S, Avanzini G. Anemone toxin (ATX II)-induced increase in persistent sodium current: effects on the firing properties of rat neocortical pyramidal neurones. *J Physiol* 1998;507:105–116. [PubMed: 9490824]
- McNamara F, Lijowska AS, Thach BT. Spontaneous arousal activity in infants during NREM and REM sleep. *J Physiol* 2002;538:263–269. [PubMed: 11773333]
- Pace RW, Mackay DD, Feldman JL, Del Negro CA. Inspiratory bursts in the preBotzinger Complex depend on a calcium-activated nonspecific cationic current linked to glutamate receptors. *J Physiol* 2007;582(Pt 1):113–25. [PubMed: 17446214]
- Pearlstein E, Ben Mabrouk F, Pflieger JF, Vinay L. Serotonin refines the locomotor-related alternations in the in vitro neonatal rat spinal cord. *European Journal of Neuroscience* 2005;21:1338–1346. [PubMed: 15813943]
- Pena F, Ramirez J-M. Endogenous Activation of Serotonin-2A Receptors Is Required for Respiratory Rhythm Generation In Vitro. *J Neurosci* 2002;22:11055–11064. [PubMed: 12486201]
- Pena F, Parkis MA, Tryba AK, Ramirez J-M. Differential Contribution of Pacemaker Properties to the Generation of Respiratory Rhythms during Normoxia and Hypoxia. *Neuron* 2004a;43:105–117. [PubMed: 15233921]

- Pena F, Parkis MA, Tryba AK, Ramirez JM. Differential contribution of pacemaker properties to the generation of respiratory rhythms during normoxia and hypoxia. *Neuron* 2004b;43:105–117. [PubMed: 15233921]
- Prinz AA, Bucher D, Marder E. Similar network activity from disparate circuit parameters. *Nature Neurosci* 2004;7(12):1345–52. [PubMed: 15558066]
- Ramirez JM, Tryba AK, Pea F. Pacemaker neurons and neuronal networks: an integrative view. *Curr Opin Neurobiol* 2004a;14:665. [PubMed: 15582367]
- Ramirez JM, Tryba AK, Pena F. Pacemaker neurons and neuronal networks: an integrative view. *Curr Opin Neurobiol* 2004b;14:665–674. [PubMed: 15582367]
- Saint DA. The role of the persistent Na⁺ current during cardiac ischemia and hypoxia. *J Cardiovasc Electrophysiol* 2006;17:S96. [PubMed: 16686689]
- Smith JC, Ellenberger HH, Ballanyi K, Richter DW, Feldman JL. Pre-Botzinger Complex: a brainstem region that may generate respiratory rhythm in mammals. *Science* 1991;254:726. [PubMed: 1683005]
- St-John WM, Rudkin AH, Harris MR, Leiter JC, Paton JF. Maintenance of eupnea and gasping following alterations in potassium ion concentration of perfusates of in situ rat preparation. *J Neurosci Methods* 2005;142:125–129. [PubMed: 15652625]
- Stafstrom CE. Persistent Sodium Current and Its Role in Epilepsy. *Epilepsy Currents* 2007;7:15–22. [PubMed: 17304346]
- Steriade M, McCormick DA, Sejnowski TJ. Thalamocortical oscillations in the sleeping and aroused brain. *Science* 1993;262:679–685. [PubMed: 8235588]
- Steriade M, Timofeev I, Grenier F. Natural waking and sleep states: a view from inside neocortical neurons. *J Neurophysiol* 2001;85:1969–1985. [PubMed: 11353014]
- Taddese A, Bean BP. Subthreshold Sodium Current from Rapidly Inactivating Sodium Channels Drives Spontaneous Firing of Tuberomammillary Neurons. *Neuron* 2002;33:587–600. [PubMed: 11856532]
- Telgkamp P, Cao YQ, Basbaum AI, Ramirez JM. Long-term deprivation of substance P in PPT-A mutant mice alters the anoxic response of the isolated respiratory network. *J Neurophysiol* 2002;88:206. [PubMed: 12091546]
- Thoby-Brisson M, Ramirez J-M. Identification of Two Types of Inspiratory Pacemaker Neurons in the Isolated Respiratory Neural Network of Mice. *J Neurophysiol* 2001;86:104–112. [PubMed: 11431492]
- Topolnik L, Steriade M, Timofeev I. Hyperexcitability of intact neurons underlies acute development of trauma-related electrographic seizures in cats in vivo. *Eur J Neurosci* 2003;123:299–336.
- Tryba AK, Ritzmann RE. Multi-joint coordination during walking and foothold searching in the *Blaberus* cockroach. II. Extensor motor neuron pattern. *J Neurophysiol* 2000;83:3337–3350. [PubMed: 10848553]
- Tryba AK, Ramirez JM. Hyperthermia modulates respiratory pacemaker bursting properties. *J Neurophysiol* 2004a;92:2844–2852. [PubMed: 15190095]
- Tryba AK, Ramirez JM. Background sodium current stabilizes bursting in respiratory pacemaker neurons. *J Neurobiol* 2004b;60:481–489. [PubMed: 15307152]
- Tryba AK, Pena F, Ramirez J-M. Stabilization of Bursting in Respiratory Pacemaker Neurons. *J Neurosci* 2003;23:3538–3546. [PubMed: 12716963]
- Tryba AK, Pena F, Ramirez J-M. Gasping Activity In Vitro: A Rhythm Dependent on 5-HT_{2A} Receptors. *J Neurosci* 2006;26:2623–2634. [PubMed: 16525041]
- Tryba AK, Pena F, Lieske SP, Viemari JC, Thoby-Brisson M, Ramirez J-M. Differential modulation of neural network and pacemaker activity underlying eupnea and sigh breathing activities. *J Neurophysiol* 2008;99(5):2114–25. [PubMed: 18287547]
- van Drongelen W, Koch H, Marcuccilli C, Pena F, Ramirez JM. Synchrony levels during evoked seizure-like bursts in mouse neocortical slices. *J Neurophys* 2003;90:1571–1580.
- Viemari J-C, Ramirez J-M. Norepinephrine Differentially Modulates Different Types of Respiratory Pacemaker and Nonpacemaker Neurons. *J Neurophysiol* 2006;95:2070–2082. [PubMed: 16394066]
- Viemari J-C, Roux J-C, Tryba AK, Saywell V, Burnet H, Pena F, Zanella S, Bevengut M, Barthelemy-Requin M, Herzing LBK, Moncla A, Mancini J, Ramirez J-M, Villard L, Hilaire G. Mecp2 Deficiency

Disrupts Norepinephrine and Respiratory Systems in Mice. *J Neurosci* 2005a;25:11521–11530. [PubMed: 16354910]

Viemari JC, Roux JC, Tryba AK, Saywell V, Burnet H, Pena F, Zanella S, Bevengut M, Barthelemy-Requin M, Herzing LB, Moncla A, Mancini J, Ramirez JM, Villard L, Hilaire G. Mecp2 deficiency disrupts norepinephrine and respiratory systems in mice. *J Neurosci* 2005b;25:11521–11530.

Viemari JC, Roux JC, Tryba AK, Saywell V, Burnet H, Pena F, Zanella S, Bevengut M, Barthelemy-Requin M, Herzing LB, Moncla A, Mancini J, Ramirez JM, Villard L, Hilaire G. Mecp2 deficiency disrupts norepinephrine and respiratory systems in mice. *J Neurosci* 2005c;25:11521–11530. [PubMed: 16354910]

Wilders R, Jongsma HJ. Beating irregularity of single pacemaker cells isolated from the rabbit sinoatrial node. *Biophys J* 1993;65:2601–2613. [PubMed: 8312495]

Zhong G, Masino MA, Harris-Warrick RM. Persistent Sodium Currents Participate in Fictive Locomotion Generation in Neonatal Mouse Spinal Cord. *J Neurosci* 2007;27:4507–4518. [PubMed: 17460064]

Fig. 1. Fictive eupneic population activity becomes irregular in low- $[Na^+]_o$ ACSF

a) Diagram of the medullary brain-slice that contains the pre-Bötzinger complex (preBötC) within the ventral respiratory group (VRG). The hypoglossal nuclei (XII) are also within the slice. Extracellular population recordings are made from the slice surface (VRG). The integrated trace ($\int$ VRG) is dominated by inspiratory neuron activity as it is in-phase with inspiratory XII motor neuron activation (Tryba et al., 2006). Overlay of averaged $\int$ VRG bursts ($n=50$ bursts averaged) and $\int$ XII bursts ($n=48$ bursts averaged), indicates the onset of the central respiratory network, VRG bursts, precedes XII motor neuron burst onset. The $\int$ VRG averaged peak time was 51.8ms before the averaged $\int$ XII burst peak time. **b)** In control ACSF, both integrated $\int$ VRG and $\int$ XII population activities are synchronized and bursting occurs at regular

intervals compared to **c**) after washing on ACSF containing 20% lower $[\text{Na}^+]_o$ than control (80% $[\text{Na}^+]_o$ ACSF). Note different time scales are used in 1b and 1c. **d**) Expanded and overlaid averaged [VRG] or [XII] population bursts indicate the burst duration is similar in control ACSF (black line) and following lowering to 80% $[\text{Na}^+]_o$ ACSF (red lines). Note the baselines are offset to make it easier to distinguish data collected during control (black line) versus 80% $[\text{Na}^+]_o$ ACSF (red line).

Fig. 2. a) The regularity of fictive inspiratory eupneic bursts generated by the ventral respiratory group (VRG) is reduced in low-[Na⁺]_o ACSF whereas **b)** the VRG burst amplitude and **c)** burst duration were not altered by reducing the [Na⁺]_o in ACSF to either 87%, 80% or 75% of control ACSF. After application of 80%-[Na⁺]_o ACSF, subsequent application of control (100%-[Na⁺]_o) restored VRG bursting regularity.

Fig. 3. A subset of preBötC inspiratory neurons have endogenous voltage-dependent pacemaker bursting properties

Inspiratory neurons burst in-phase with fictive eupneic $\int$ VRG activities and, as in this case, a subset of inspiratory neurons continue to generate voltage-dependent bursting properties, following blockade of ionotropic receptors with CNQX, CPP, bicuculline and strychnine and (bottom panels) loss of network ($\int$ VRG) rhythmic bursting. As shown by current injection, inspiratory pacemakers retain voltage-dependent bursting properties following synaptic isolation (bottom two cell traces).

Fig. 4. Fictive inspiratory pacemaker activity becomes irregular in low- $[Na^+]_o$ ACSF

a) In control ACSF, fictive inspiratory pacemaker activity occurs at regular intervals compared to **b)** bursting occurring at irregular intervals after washing on ACSF containing, for example, 25% lower $[Na^+]_o$ than control (75% $[Na^+]_o$ ACSF). **c)** Isolated pacemaker bursting irregularity increased after Reducing ACSF $[Na^+]_o$ to either 87% or 75% of control. **d)** In 87% $[Na^+]_o$ ACSF, the Vm, action potential amplitude (A.P. Amp.), burst duration (BD) and burst frequency were unchanged, while the number (#) of action potentials per burst was reduced. **e)** In synaptically isolated cadmium insensitive (CI-) pacemakers, adding cadmium to block VGCC increased their bursting regularity. Subsequent application of ketanserin to block endogenous 5HT_{2a} signaling increased bursting irregularity, consistent with background

sodium currents being critical to burst regularity. F) Elevating ACSF $[K^+]_o$ from 3mM (100%) to 8mM did not alter synaptically isolated pacemaker bursting irregularity score.

Fig. 5. Decreasing ACSF [Na⁺]_o did not alter fictive eupneic nonpacemaker cell properties Compared to **a-i**) control, lowering ACSF [Na⁺]_o to **a-ii**) 75%, decreased VRG and nonpacemaker bursting regularity. **a-iii**) blocking glutamatergic transmission with cnqx and cpp eliminated VRG and nonpacemaker cell bursting. Lowering ACSF [Na⁺]_o to either **b**) 87%; or **c**) 75% of normal, does not significantly alter inspiratory nonpacemaker action potential amplitude (AP Amp.), number of AP per burst (#AP/Brst), burst duration (Brst Dur.), AP frequency (AP Freq.) or Vm.

Fig. 6. Lowering ACSF $[Na^+]_o$ 87% of normal reduced the persistent sodium, but not fast sodium current in medullary neurons

In voltage clamp, we held medullary neurons in the VRG region at $V_{hold} = -60mV$ to record synaptic input. **a)** Four of the $n=6$ medullary neurons tested are likely inspiratory neurons, having synaptic currents synchronous with fictive eupneic population bursts; **b)** an expanded view of synaptic currents synchronized to the $\int VRG$ population (boxed region in (a)). **c)** In VRG neurons receiving rhythmic synaptic input after isolating the peak transient and persistent I_{Na+} , washing on 87% $[Na^+]_o$ did not alter the fast transient sodium current (black trace) but reduced the persistent sodium current (red trace). The peak current was evoked by stepping from $V_{hold} = -80mV$ to $V_{step} = -20mV$ for 200ms. **d)** The mean % change ($\pm SD$) in peak fast

I_{Na^+} ($I_{\text{Na}^+}(\text{f})$) and persistent ($I_{\text{Na}^+}(\text{p})$) currents following reducing the ACSF $[\text{Na}^+]_o$ to 87% of normal.