Glycinergic Pacemaker Neurons in PreBötzinger Complex of Neonatal Mouse

Consuelo Morgado-Valle,1,2 Serapio M. Baca,1 and Jack L. Feldman1
1Systems Neurobiology Laboratory, Department of Neurobiology, David Geffen School of Medicine at UCLA, University of California, Los Angeles, Los Angeles, California 90095-1763, and 2Programa de Neurobiología, Universidad Veracruzana, Xalapa, Veracruz, Mexico, C.P. 91190

The preBötzinger complex (preBöC) is essential for normal respiratory rhythm generation in rodents, for which the underlying mechanisms remain unknown. Excitatory preBöC pacemaker neurons are proposed to be necessary for rhythm generation. Here we report the presence of a population of preBöC glycinergic pacemaker neurons. We used rhythmic in vitro transverse slice preparations from transgenic mice where neurons expressing the glycine transporter 2 (GlyT2) gene coexpress enhanced green fluorescent protein (EGFP). We combined epifluorescence and whole-cell patch-clamp recording to study preBöC EGFP-labeled, i.e., glycinergic, inspiratory-modulated neurons with pacemaker properties. We defined glycinergic pacemaker neurons as those preBöC EGFP neurons that exhibited the following: (1) ectopic bursting in rhythmic slices when depolarized during their normally silent period and (2) bursting when depolarized in nonrhythmic slices (following AMPA receptor blockade). Forty-two percent of EGFP-labeled neurons were inspiratory modulated neurons with pacemaker properties. We defined glycinergic pacemaker neurons as those preBöC EGFP neurons that exhibited the following: (1) ectopic bursting in rhythmic slices when depolarized during their normally silent period and (2) bursting when depolarized in nonrhythmic slices (following AMPA receptor blockade). Forty-two percent of EGFP-labeled neurons were inspiratory (n = 48 of 115), of which 23% (n = 11 of 48 inspiratory; 10% of the total recorded) were pacemakers. We conclude that there is a population of preBöC inspiratory-modulated glycinergic, presumably inhibitory, pacemaker neurons that constitute a substantial fraction of all preBöC pacemaker neurons. These findings challenge contemporary models for respiratory rhythmogenesis that assume the excitatory nature of preBöC pacemaker neurons. Testable and nontrivial predictions of the functional role of excitatory and inhibitory pacemaker neurons need to be proposed and the necessary experiments performed.

Introduction

The preBötzinger complex (preBöC) is essential for normal breathing in rodents (Tan et al., 2008) and is postulated to be an essential site for respiratory rhythm generation (Smith et al., 1991). In brainstem–spinal cord (en bloc) and transverse slice in vitro preparations, respiratory rhythm persists in the absence of postsynaptic inhibition (Feldman and Smith, 1989; Onimaru et al., 1990; Shao and Feldman, 1997; Brockhaus and Ballanyi, 1998). This observation led to the hypothesis that intrinsically rhythmic excitatory pacemaker neurons drive the respiratory rhythm (Smith et al., 1991); preBöC neurons with pacemaker properties depend on persistent sodium current (I\text{\textsubscript{NaP}}) or Ca\textsuperscript{2+}-activated nonspecific cationic current (I\text{\textsubscript{CaL}}) of underdetermined neurotransmitter phenotype were subsequently identified (Johnson et al., 1994; Thoby-Brisson and Ramirez, 2001; Del Negro et al., 2002; Peña et al., 2004). Many models for rhythmogenesis predicate a significant role, often obligatory, for excitatory, presumably glutamatergic, pacemaker neurons (Butera et al., 1999a,b; Smith et al., 2000; Del Negro et al., 2001; Rybak et al., 2003, 2008). Pharmacological studies suggest that pacemaker neurons are not obligatory for rhythmogenesis (Del Negro et al., 2002, 2005; Feldman and Del Negro, 2006). Regardless, they could play a role in modulating/stabilizing the rhythm (Purvis et al., 2007). Recently, preBöC pacemakers expressing the vesicular glutamate transporter 2 (VGluT2) and presumably glutamatergic were identified, but they were few in number and may not possess pacemaking properties under normal conditions (St-John et al., 2009).

The preBöC contains excitatory and inhibitory neurons (Stornetta et al., 2003; Winter et al., 2009). How pacemakers are distributed between these two populations is unknown. PreBöC rhythm is abolished in vitro (Greer et al., 1991) and in vivo (Chitravanshi and Sapru, 1996) after blockade of glutamatergic transmission. Although inhibition is not necessary for rhythmogenesis in the preBöC in in vitro slices (Feldman and Smith, 1989; Del Negro et al., 2009), inhibition is important in respiratory patterning (Feldman and Smith, 1989; Bianchi et al., 1995; Shao and Feldman, 1997; Brockhaus and Ballanyi, 1998; Büsselberg et al., 2001; Richter and Spyer, 2001; Ren and Greer, 2006). In some invertebrates, inhibitory pacemakers play a fundamental role in rhythmic behavior (Cardi and Nagy, 1994; Mamiya and Nadim, 2004). Here, we sought to determine whether there are inhibitory neurons with pacemaker properties within the preBöC. We recorded from inspiratory-modulated glycinergic neurons in an in vitro transverse slice preparation from mice with EGFP expressed in GlyT2-containing neurons (Zeilhofer et al., 2005). We used whole-cell recording and epifluorescence to test for two distinct pacemaker properties: (1) ectopic
bursting induced by depolarization during their silent period in rhythmic slices and (2) bursting induced by depolarization in non-rhythmic slices (following AMPA receptor blockade). Approximately 23% of these inspiratory-modulated glycinergic neurons had both pacemaker properties. We conclude that the presumption that all preBo¨tC pacemaker neurons are excitatory is incorrect. Establishing the neurotransmitter(s) used by preBo¨tC pacemaker neurons is essential for understanding their functional role, if any, in generating or modulating respiratory pattern, and a prerequisite for validating models that stipulate pacemakers as an essential element of the rhythm generating mechanism. The inspiratory glycinergic, presumably inhibitory, pacemaker neurons in preBo¨tC are a novel class of neurons that may modulate the respiratory network.

Materials and Methods

Medullary slice preparation. Experiments were performed on transverse brainstem slices generating respiratory-related motor output (Smith et al., 1991) from GlyT2-EGFP mice (Zeilhofer et al., 2005). The Office for the Protection of Research Subjects, University of California Research Committee approved all protocols. Mice (n = 26, P0 –P7 from 10 litters) were anesthetized with isoflurane and decerebrated, and the neuraxis was isolated. The brainstem was serially sectioned (Vibratome) in the transverse plane until the nucleus ambiguus and inferior olive were visible. A slice (450 –500 μm) containing the preBo¨tC was cut (Del Negro et al., 2002; Ruangkittisakul et al., 2006). The dissection was performed in artificial CSF (ACSF) containing (in mM): 128 NaCl, 3 KCl, 1.5 CaCl2,1 MgSO4,23.5 NaHCO3, 0.5 NaH2PO4, and 30 glucose, bubbled with 95% O2/5% CO2 at 27°C. The slice was perfused with ACSF (6 ml/min) in a 1 mm transverse sections with a freezing microtome. 

Electrophysiological recording. Respiratory-related motor output was recorded from hypoglossal nerves (XIIIn) using suction electrodes. To obtain a robust, stable rhythm, ACSF K+ concentration was elevated to 9 mM. Slices were perfused for 30 min before experimental manipulations. XIIIn activity was amplified, bandpass filtered (0.3–1 kHz), rectified, and integrated (τ = 20 ms; fXIIIn). Whole-cell patch-clamp recordings were performed using an Axopatch 200A amplifier (Molecular Devices) in current-clamp mode. preBo¨tC inspiratory neurons were visualized using infrared-enhanced differential interference contrast (IR-DIC) video microscopy. Electrodes were pulled from borosilicate glass (outer diameter, 1.5 mm; inner diameter, 0.86 mm) and filled with solution containing the following (in m): 130 K-gluconate, 10 NaCl, 10 HEPES, 0.1 CaCl2, 1.1 EGTA, 2 Mg-ATP, and 0.3 GTP-Na, pH 7.3; in some experiments, 0.01% rhodamine was added. Electrophysiological signals were low-pass filtered and digitized at 4 kHz using pCLAMP software and a Digidata 1320 A/D/DA board (Molecular Devices).

Neuron visualization. We detected EGFP-labeled neurons using an upright microscope (DMILFS, Leica) equipped for epifluorescence, a 63× objective (HCX/APO 0.90 numerical aperture, Leica), and dichroics (I3 and N2.1, Leica). In some experiments, 15–30 images were acquired with a CCD camera (Water), digitized (Scion LG-3), and averaged (Scion Image). Image processing was performed in ImageJ (National Institutes of Health).

Drugs. Drugs were bath applied. 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) and flufenamic acid (FFA, 10 –500 μM) were obtained from Sigma Chemical, Riluzole (10–20 μM) was obtained from Tocris Bioscience.

Confocal image stacks were acquired with a Zeiss LSM 510 microscope and software. Lasers (488 and 543 nm) and appropriate filters were used to visualize EGFP and NK1R-ir. To avoid cross talk between channels, and therefore false colocalization of EGFP and NK1R-ir signals, we acquired images in multitrack mode or ensured that the NK1R-ir signal was unaltered after modifications of the power of the 488 nm laser. We used 40× and 63× objectives to estimate soma size and determine colocalization of the EGFP and NK1R-ir signals.
No striking qualitative differences between the shapes of EGFP-labeled and NK1R-ir somas were found. Somas were approximately spherical. Therefore we measured the diameter at the largest cross-sectional region of the neuron using confocal stacks. We did not explore differences in their neuropil nor attempt other measures. Data are expressed as the mean ± SEM. A t test was used to determine statistical differences between mean values.

Results
Glycinergic inspiratory neurons
Under epifluorescence microscopy and regardless of size or shape, EGFP-labeled neurons (n = 115) from P0–P7 GlyT2-EGFP neonatal mice were whole-cell patch clamped at their somas. Using current-clamp mode, we maintained neuronal Vm at approximately −60 mV during the period between X1In bursts. Fifty-eight percent (n = 67/115) of these neurons were either silent or had an irregular firing pattern, i.e., were non-riparatory-modulated, and 42% (n = 48/115) had inspiratory-modulated membrane depolarization and spiking. Of these inspiratory-modulated neurons, 27% (13/48) showed delayed excitation when depolarized by a square pulse from a hyperpolarized (−70 mV) membrane potential, a signature of type 1 neurons (Rekling et al., 1996; Gray et al., 1999), and the remaining 73% (35/48) showed a sag during a hyperpolarizing square pulse and postinhibitory rebound, a signature of type 2 neurons.

Pacemaker properties in EGFP-labeled neurons
In rhythmic slices, when EGFP-labeled inspiratory neurons (n = 48) were depolarized by current injection, 37% (n = 18/48) produced ectopic bursts of action potentials, i.e., burst out of phase with X1In inspiratory bursts (Fig. 1C). For these latter neurons, rhythm in the slice was abolished by bath application of CNQX (10 μM), blocking fast glutamatergic transmission; upon depolarization, 61% (n = 11/18) showed voltage-dependent intrinsic bursting (Fig. 1A,B).

To determine the presence of I_NaP or I_CAN, we bath applied the I_CAN blocker FFA or the I_NaP blocker riluzole. EGFP-labeled preBoC pacemaker neurons were sensitive to 10 μM riluzole, which abolished bursting within 3 min (n = 7/7; included in this group are two neurons that were insensitive to 10 μM FFA, but further application of 10 μM riluzole abolished intrinsic bursting). In a small sample, EGFP-labeled inspiratory preBoC pacemaker neurons were also extremely sensitive to FFA, which rapidly abolished bursting at 500 μM (n = 2/2 tested) or 100 μM (n = 2/2 tested).

There is an early postnatal age dependence of I_CAN-dependent pacemaker activity (Peña et al., 2004; Del Negro et al., 2005). Our small dataset suggests a developmental dependence of glycnergic pacemakers. We infrequently found EGFP-labeled inspiratory preBoC pacemaker neurons in transverse slices from P0–P3 mice (n = 3 pacemaker neurons from 11 slices). The likelihood of finding EGFP-labeled pacemaker neurons tripled in P4–P7 mice (n = 8 neurons from 10 slices).

NK1R expression in EGFP-labeled inspiratory neurons
The preBoC contains a high density of NK1R-ir neurons (Gray et al., 1999; Wang et al., 2001; Pagliardini et al., 2005). Less than 1% (1/140) of preBoC EGFP-labeled neurons were NK1R-ir (n = 140) (Fig. 2B).

We measured soma size of preBoC NK1R-ir (n = 24) and EGFP-labeled (n = 45) neurons from three preparations. Somas of EGFP-labeled neurons were smaller than those of NK1R-ir neurons (soma diameters: EGFP-labeled 13.6 ± 0.5 μm; NK1R-ir 16.3 ± 0.6 μm; p < 0.001).

Discussion
Our principal result is that in the preBoC of neonatal rodents, there is a population of pacemaker neurons that are glycnergic and inspiratory-modulated.

Are the GlyT2-EGFP neurons exclusively glycnergic?
Studies of the anatomy of GlyT2-EGFP mice using immunohistochemistry against glycine or GlyT2 showed that >90% of EGFP

Figure 2. NK1R and GlyT2-EGFP do not colocalize in the preBoC. A, EGFP-labeled neurons in transverse medullary slices of neonatal GlyT2-EGFP mice. NK1R-ir (red) was used to identify the preBoC. Arrows indicate dorsal (D) and lateral (L) orientation of slice. B, We observed little to no colocalization between NK1R and EGFP signal. Note the absence of yellow in the merged images taken with EGFP and rhodamine filters (C2–C4). Scale bar, 15 μm. IO, Inferior olive; SP5, spinal trigeminal nucleus; X1In, hypoglossal nucleus.
two distinct, non-overlapping populations, as we found colocalization of NK1R-ir neurons in ~1% of EGFP-labeled preBötC neurons. This lack of overlap follows from previous work. mRNA encoding GlyT2 is detected only in ~1% NK1R-ir neurons (Wang et al., 2001). Furthermore, in the ventral respiratory group (VRG) at least 77 ± 9% of NK1R-ir neurons are excitatory, as they contain mRNA that encodes the VGluT2, a reliable marker of glutamatergic neurons (Guyenet et al., 2002).

Can we estimate what fraction of preBötC neurons are GlyT2-EGFP pacemakers?
The rat preBötC contains ~300 NK1R-ir neurons that represent ~10% of all preBötC neurons (Gray et al., 1999; Wang et al., 2001). In our experience ~60% of neurons that we record in active slices from neonatal rodents are inspiratory modulated. Based on our cell counts in the mice studied here, we estimate that ~20% of all preBötC neurons were EGFP labeled, making GlyT2-EGFP pacemaker neurons ~2% of all preBötC neurons and ~3% of preBötC inspiratory neurons (see Table 1). The estimate of the prevalence of pacemaker neurons in randomly recorded preBötC inspiratory neurons (with the presumption of no sampling bias, see below) ranges from 5% (Del Negro et al., 2002) to 25% (Peña et al., 2004). Based on our data and estimates, GlyT2-EGFP pacemaker neurons represent ~3% of preBötC inspiratory neurons, making them up to ~50% of preBötC pacemaker neurons in our experimental conditions or as low as ~10% in different conditions (Peña et al., 2004). Conversely, from ~50% up to ~90% of preBötC inspiratory pacemaker neurons may be excitatory.

A recent blind-patch study in perfused in situ preparations of juvenile (P14–P21) and neonatal (P6–P8) rats identified four preBötC intrinsic pacemaker neurons (St-John et al., 2009); three were positive for VGluT2, suggesting that they were excitatory. The remaining VGluT2-positive pacemakers (15/19) were located caudal to the preBötC in a region that does not appear obligatory for rhythmogenesis (Smith et al., 1991). Many of these VGluT2-positive neurons exhibited pacemaker properties only after blocking CI−–mediated inhibition, elevating local K+ or by applying sodium cyanide, so whether they are pacemakers during normal breathing, or contribute to the breathing rhythm, remains unresolved.

From our limited sample, GlyT2-EGFP preBötC pacemaker neurons express both I_{NaP} and I_{CAN}. Whether GlyT2-EGFP preBötC pacemaker neurons have different biophysical, synaptic, or network properties from those of excitatory preBötC neurons also remains unresolved.

Neurotransmitter phenotype of preBötC pacemaker neurons
The hypothesis that pacemaker neurons play an obligatory role in respiratory rhythmogenesis in vitro arose from the observation that inhibition is not essential for generation of inspiratory rhythm (Feldman and Smith, 1989). The presence of inspiratory-modulated preBötC pacemaker neurons is well documented (Smith et al., 1991; Johnson et al., 1994; Thoby-Brisson and

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**Table 1. Estimated numbers of preBötC neurons in various categories**

| Neurons | Estimated | Recorded |
|---------|-----------|----------|
|         | # Total | % Total | # | % of GlyT2 | # | % GlyT2 inspiratory | # | % Ectopic bursters |
| PreBötC | 3000 | 100 |     |     |     |     |     |     |
| NK1R + | 300 | 10 |     |     |     |     |     |     |
| GlyT2 − | 600 | 20 |     |     |     |     |     |     |
| GlyT2 − noninspiratory | 348 | 12 |     |     |     |     |     |     |
| GlyT2 − inspiratory | 252 | 8 |     |     |     |     |     |     |
| GlyT2 − inspiratory ectopic burster | 93 | 3 |     |     |     |     |     |     |
| GlyT2 − inspiratory ectopic burster pacemaker | 57 | 2 |     |     |     |     |     |     |

PreBötC, NK1R +, and GlyT2 − were estimated from histological counts. Other estimated numbers/percentages were extrapolated from recordings of neurons of various types.
Ramirez, 2001; Del Negro et al., 2002; Peña et al., 2004), but in none of these papers (cf. St-John et al., 2009) was the transmitter phenotype determined. In the authors’ interpretations of these various papers, and in models that cite them as evidence of the presence of pacemaker neurons [e.g., Butera et al. (1999a,b), Smith et al. (2000), Del Negro et al. (2001), and Rybak et al. (2003, 2008)], they are universally presumed to be excitatory. However, there is no a priori reason that pacemaker neurons need to be excitatory to either generate or modulate rhythm.

Models of respiratory rhythm in vitro recognize that inhibitory interactions are not obligatory as rhythm persists when synaptic inhibition is blocked. Nonetheless, such interactions could play a role in rhythmonogenesis under normal conditions in more intact preparations, even in vivo, and certainly affect pat terning of respiratory output, even in slices. While inhibitory pacemaker neurons are obligatory for rhythmonogenesis in other systems, such as in the generation of the pyloric rhythm in invertebrates (Cardi and Nagy, 1994; Mamiya and Nadim, 2004), we propose that the principal role of glycinergic preBo¨tC pacemaker neurons is in modulation/stabilization of respiratory rhythm.

Neuron size and sampling bias

By virtue of exploiting visualized recording, we were limited to recording relatively superficial neurons in the slice, up to ~120 μm deep. However, patch-clamping methodologies used in several studies of preBo¨tC pacemaker neurons can have a sampling bias toward neurons with larger somas with strong inspiratory drive. In the present sample, size was not an explicit or implicit criterion. There also is sampling bias in studies using blind patch recording (Johnson et al., 1994; Thoby-Brisson and Ramirez, 2001; Peña et al., 2004; St-John et al., 2009) and extracellular recording (Del Negro et al., 2001), which can record neurons much deeper in the slice but require detecting strong, likely somatic, electrical signals. This increases the probability of recording from larger somas, reducing the probability of recording smaller neurons that, according to our observations, are more likely to be glycinergic.

Establishing the role of any preBo¨tC neuronal class, including pacemakers, in respiratory rhythm generation requires determination of their neurotransmitter phenotype. A basic requirement for validation of models that stipulate that excitatory pacemaker neurons are essential for rhythm generation in vitro is that this is actually the case. The demonstration of preBo¨tC glycinergic pacemaker neurons suggests that this remains to be done.

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