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Glycinergic pacemaker neurons in preBötzinger Complex of neonatal mouse

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Abstract

The preBötzinger Complex (preBötC) is essential for normal respiratory rhythm generation in rodents, for which the underlying mechanisms remain unknown. Excitatory preBötC pacemaker neurons are proposed to be necessary for rhythm generation. Here we report the presence of a population of preBötC glycinergic pacemaker neurons. We used rhythmic *in vitro* transverse slice preparations from transgenic mice where neurons expressing the glycine transporter 2 (GlyT2) gene co-express enhanced green fluorescent protein (EGFP). We combined epifluorescence and whole-cell patch-clamp recording to study preBötC EGFP-labeled, i.e., glycinergic, inspiratory-modulated neurons with pacemaker properties. We defined glycinergic pacemaker neurons as those preBötC EGFP neurons that exhibited: 1) ectopic bursting in rhythmic slices when depolarized during their normally silent period, and; 2) bursting when depolarized in non-rhythmic slices (following AMPA receptor blockade). 42% 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 preBötC inspiratory-modulated glycinergic, presumably inhibitory, pacemaker neurons that constitute a substantial fraction of all preBötC pacemaker neurons. These findings challenge contemporary models for respiratory rhythmogenesis that assume the excitatory nature of preBötC pacemaker neurons. Testable and non-trivial predictions of the functional role of excitatory and inhibitory pacemaker neurons need to be proposed and the necessary experiments performed.

Keywords

respiratory rhythm generation; breathing; GlyT2-EGFP mice; glycine; inhibition; Pacemaker

INTRODUCTION

The preBötzinger Complex (preBötC) 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); preBötC neurons with pacemaker properties dependent on persistent sodium current (I_{NaP}) or Ca^{2+}-activated non-specific cationic current (I_{CAN}) of

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undetermined neurotransmitter phenotype were subsequently identified (Johnson et al., 1994; Thoby-Brisson and Ramirez, 2001; Del Negro et al., 2002; Pena 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; Rybak et al., 2008). Pharmacological studies suggest that pacemaker neurons are not obligatory for rhythmogenesis (Del Negro et al., 2002; Del Negro et al., 2005; Feldman and Del Negro, 2006). Regardless, they could play a role in modulating/stabilizing the rhythm (Purvis et al., 2007). Recently, preBötC 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 preBötC contains excitatory and inhibitory neurons (Winter et al., 2009; Stornetta et al., 2003). How pacemakers are distributed between these two populations is unknown. PreBötC 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 preBötC 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; Busselberg 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 preBötC. 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: i) ectopic bursting induced by depolarization during their silent period in rhythmic slices, and; ii) 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 preBötC pacemaker neurons are excitatory is incorrect. Establishing the neurotransmitter(s) used by preBötC 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 preBötC 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, decerebrated and the neuroaxis was isolated. The brainstem was serially-sectioned (Vibratome™, St Louis, MO) in the transverse plane until the nucleus ambiguus and inferior olive, were visible. A slice (450–500 μm) containing the preBötC was cut (Del Negro et al., 2002; Ruangkittisakul et al., 2006). The dissection was performed in artificial cerebrospinal fluid (ACSF) containing (in mM) 128 NaCl, 3 KCl, 1.5 $CaCl_2$, 1 $MgSO_4$, 23.5 $NaHCO_3$, 0.5 NaH_2PO_4 and 30 glucose, bubbled with 95% O_2/5% CO_2 at 27°C. The slice was perfused with ACSF (6 ml/min) in a 1 ml recording chamber.

Electrophysiological recording

Respiratory-related motor output was recorded from hypoglossal nerves (XIIn) 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. XIIn activity was amplified, bandpass filtered (0.3–1 kHz), rectified and integrated (τ=20 ms; $\int$XIIn). Whole-cell patch-clamp recordings were performed using an Axopatch 200A amplifier (Molecular Devices, Sunnyvale, CA) in current-clamp mode. preBötC inspiratory neurons were visualized using infrared-enhanced differential interference contrast (IR-DIC) video microscopy. Electrodes were pulled from borosilicate glass (O.D., 1.5 mm; I.D., 0.86 mm) and filled with solution containing (in mM): 130 K-gluconate, 10 NaCl, 10 HEPES, 0.1 $CaCl_2$, 1.1 EGTA, 2 Mg-ATP, 0.3 mM 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 AD/DA board (Molecular Devices, Sunnyvale, CA).

Neuron visualization

We detected EGFP-labeled neurons using an upright microscope (DMLFS, Leica, Wetzlar, Germany) equipped for epifluorescence, a 63X objective (HCX/APO 0.90NA, Leica), and dichroics (I3 and N2.1, Leica). In some experiments 15–30 images were acquired with a CCD camera (Watec, Japan), digitized (Scion LG-3), and averaged (Scion Image, Scion Corporation, Frederick, MD). Image processing was performed in ImageJ (NIH, Bethesda, MD).

Drugs

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

Immunohistochemistry

Mice (n=6, P4–P21 from 3 litters) were anaesthetized and transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline (PFA/PBS). The brainstems were dissected and placed in 4% PFA/PBS overnight, cryoprotected in 30% sucrose/PBS, embedded in OCT medium and cut in 40 μm transverse sections with a freezing microtome. Freely floating sections were processed for neurokinin-1 receptor (NK1R) immunolabeling (Gray et al., 1999). The preBötC was identified in transverse sections as the NK1R-immunoreactive (-ir) zone ventral to the subcompact region of the nucleus ambiguus (Gray et al., 2001),

Imaging

Confocal image stacks were acquired with a Zeiss LSM 510 microscope and software. Lasers (488 nm 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 multi-track mode or ensured that the NK1R-ir signal was unaltered after modifications of the power of the 488 nm laser. We used 40X and 63X 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 roughly 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 ± S.E.M. 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 V_m at ~−60 mV during the period between XIIn bursts. 58% (n=67/115) of these neurons were either silent or had an irregular firing pattern, i.e., were non-respiratory-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 XIIn inspiratory bursts (Figure 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 (Figure 1A and B).

To determine the presence of I_{NaP} or I_{CAN}, we bath-applied the I_{CAN} blocker flufenamic acid (FFA) or the I_{NaP} blocker riluzole. EGFP-labeled preBötC 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 preBötC 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 (Pena et al., 2004; Del Negro et al., 2005). Our small data set suggests a developmental dependence of glycinergic pacemakers. We infrequently found EGFP-labeled inspiratory preBötC 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).

Neurokinin 1 receptor (NK1R) expression in EGFP-labeled inspiratory neurons

The preBötC 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 preBötC EGFP-labeled neurons were NK1R-ir (n=140, Figure 2B).

We measured soma size of preBötC NK1R-ir (n=24) and EGFP-labeled (n=45) neurons from 3 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 preBötC of neonatal rodents there is a population of pacemaker neurons that are glycinergic and inspiratory-modulated.

Are the GlyT2-EGFP neurons exclusively glycinergic?

Studies of the anatomy of GlyT2-EGFP mice using immunohistochemistry against glycine or GlyT2 showed that >90% of EGFP fluorescent neurons are glycine-ir (in soma and dendrites) and GlyT2-ir (in axon terminals) (Zeilhofer et al., 2005).

Precursors of GABAergic interneurons in the molecular layer of the cerebellum transiently express GlyT2 during development (Simat et al., 2007). This suggests that, at least in cerebellum, GlyT2 is a marker of immature GABAergic neurons. While Cl^- currents are outward in some neurons at early stages of development, activation of glycinergic and $GABA_{A/B}$ receptors in the mouse respiratory network *in vitro* in all stages of postnatal development hyperpolarizes inspiratory neurons (Ramirez et al., 1996; Zhang et al., 1999), confirming the inhibitory nature of glycinergic and GABAergic neurons. Moreover, glycine elicits IPSPs in preBötC neurons (Shao and Feldman, 1997). We are unaware of experimental evidence suggesting a dual excitatory/inhibitory neuronal phenotype, such as the glutamatergic/GABAergic phenotype expressed by developing hippocampal granule cells (Gutierrez and Heinemann, 2006). While glycine modulates excitability by acting as co-agonist of NMDA receptor, NMDA receptors are not required for generation of respiratory rhythm or motor output (Morgado-Valle and Feldman, 2007) in standard *in vitro* conditions in the medullary slice.

Here we established the presence of pacemaker properties in EGFP-labeled inspiratory preBötC neurons in GlyT2-EGFP mice. Given the lack of studies of preBötC neurons in GlyT2-EGFP mice that suggest otherwise, we refer to EGFP-labeled neurons in these mice as "glycinergic" with presumptive inhibitory function.

The presence of inspiratory-modulated glycinergic, presumably inhibitory, pacemaker neurons in the preBötC constitutes a novel subpopulation of preBötC neurons that challenges the current models of respiratory rhythm generation where only excitatory pacemakers are stipulated, e.g., (Butera et al., 1999a, b; Smith et al., 2000; Del Negro et al., 2001; Rybak et al., 2003; Rybak et al., 2008). This stipulation is based on previous recordings of pacemaker neurons in the preBötC where the nature of neurotransmission (excitatory or inhibitory) was assumed but not determined. The glycinergic pacemakers we identified cannot directly generate inspiratory rhythm in these models because they would inhibit activity during the inspiratory phase. Recently identified excitatory pacemakers in the preBötC also appear inconsistent with these models insofar as these neurons may not possess pacemaker properties under normal conditions (St-John et al., 2009).

Glycinergic neurons are not NK1R-ir

In the preBötC, glycinergic and NK1R-ir neurons appear to be two distinct, non-overlapping, populations, as we found colocalization of NK1R-ir 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% (Pena 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 (Pena 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 (P6–8) rats identified 4 preBötC intrinsic pacemaker neurons (St-John et al., 2009); 3 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 Cl^--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 Ramirez, 2001; Del Negro et al., 2002; Pena 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; Rybak et al., 2003; Rybak et al., 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 rhythmogenesis under normal conditions in more intact preparations, even *in vivo*, and certainly could affect patterning of respiratory output, even in slices. While inhibitory pacemaker neurons are obligatory for rhythmogenesis 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 preBötC 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 preBötC pacemaker neurons can have a sampling bias towards neurons with larger somas with strong inspiratory drive. In the present sample, size was not an explicit or implicit criteria. There also is sampling bias in studies using blind patch recording (Johnson et al., 1994; Thoby-Brisson and Ramirez, 2001; Pena 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 preBötC 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 preBötC glycinergic pacemaker neurons suggests that this remains to be done.

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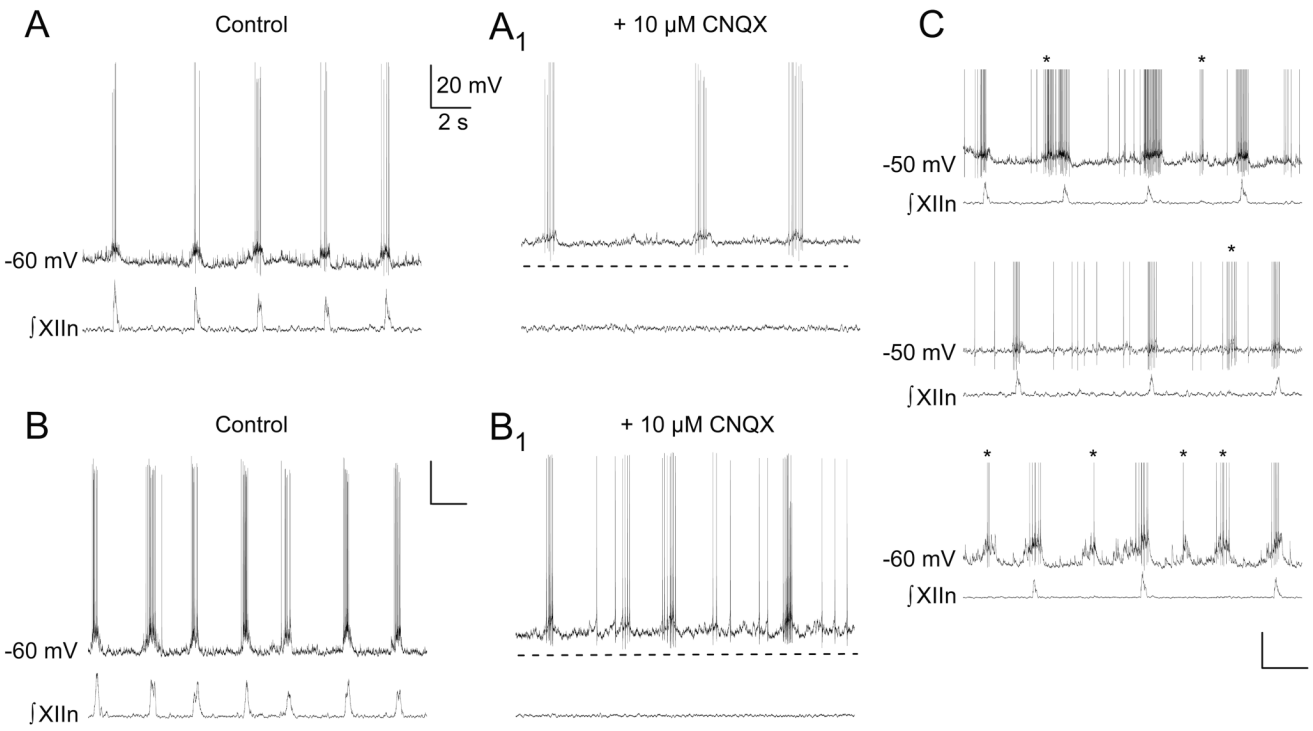

Figure 1. Examples of preBötC GlyT2-EGFP pacemaker neurons

A. Respiratory-modulated discharge in a GlyT2-EGFP pacemaker neuron current-clamped at interburst $V_m \approx -60$ mV. **A1**. Bursting activity in 10 μM CNQX bath-applied. Network activity is blocked. **B, B1**. Another example of a respiratory-modulated discharge and bursting in a GlyT2-EGFP neuron. **C**. Examples of respiratory-modulated discharge and "ectopic bursts" (asterisks) after depolarizing V_m. Action potentials have been truncated.

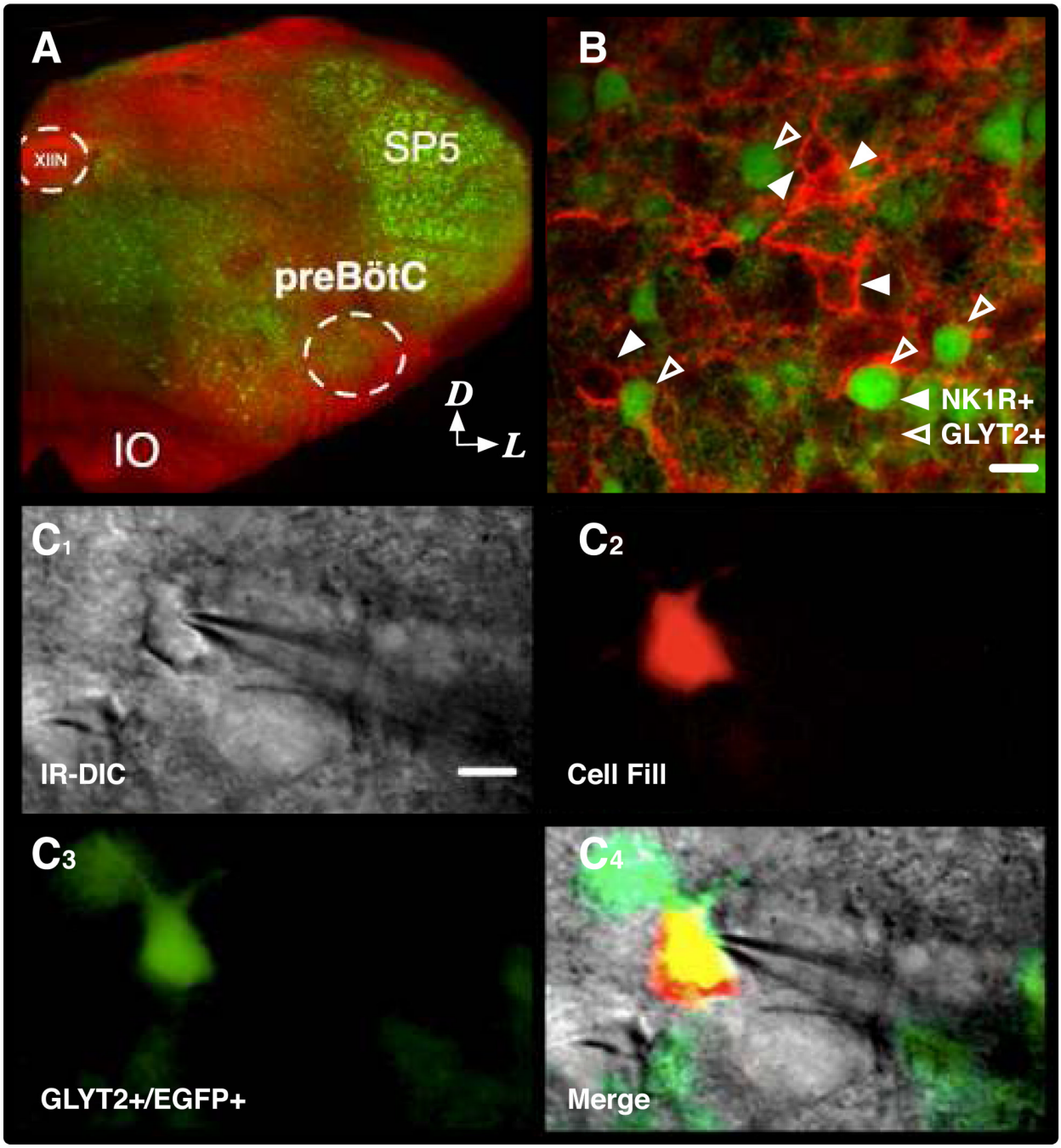

Figure 2. NK1R and GlyT2-EGFP do not colocalize in the preBötC

A. EGFP-labeled neurons in transverse medullary slices of neonatal GlyT2-EGFP mice. NK1R-ir (red) was used to identify the preBötC. **B**. We observed little to no colocalization between NK1R-ir and EGFP signal. Note the absence of yellow in the merged channels. **C1–C4**. Whole-cell patch recordings were made under IR-DIC (**C1**). Patched cells were tested electrophysiologically for pacemaker properties and filled with rhodamine via the patch electrode (**C2**). We confirmed that the neurons are GlyT2-EGFP by merging the images taken with EGFP and rhodamine filters (**C2–C4**).

Scale bar=15 μm. IO=inferior olive; SP5=spinal trigeminal nucleus; XIIN=hypoglossal nucleus. Arrows indicate dorsal and lateral orientation of slice.

Table 1

Estimated numbers of preBötC neurons in various categories. PreBötC, NK1R$^+$ and GlyT2$^+$ estimated from histological counts. Other estimated numbers/ percentages extrapolated from recordings of neurons of various types.

Neurons	***Estimated***		**Recorded**					
	# Total	**% *Total***	**#**	**% of GlyT2**	**#**	**% GlyT2 inspiratory**	**#**	**% Ectopic bursters**
PreBötC	3000	*100%*						
NK1R$^+$	300	*10%*						
GlyT2$^+$	*600*	*20%*	115	100%				
GlyT2$^+$ non-inspiratory	*348*	*12%*	67	58%				
GlyT2$^+$ inspiratory	*252*	*8%*	48	42%	-----	100%		
GlyT2$^+$ inspiratory ectopic burster	*93*	*3%*			18	37%	------	100%
GlyT2$^+$ inspiratory ectopic burster pacemaker	*57*	*2%*					11	61%

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