

1 Manuscript Title: Role of synaptic inhibition in the coupling of the respiratory rhythms that
2 underlie eupnea and sigh behaviors

3 Abbreviated Title: Synaptic inhibition and the control of breathing rhythms

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29 **ABSTRACT**

30 The preBötzinger Complex (preBötC) gives rise to two types of breathing behavior: eupnea and
31 sighing. Here, we examine the neural mechanisms that couple their underlying rhythms by
32 recording from the preBötC in neonatal mouse brainstem slice preparations. It has been
33 proposed that chloride-mediated synaptic inhibition couples inspiratory (eupnea-related) bursts
34 and sigh bursts, but we find no evidence to support that notion. First, we characterize a
35 fluctuating temporal relationship between sigh bursts and their preceding inspiratory bursts; their
36 coupling is far weaker than previously described. Surprisingly, selective blockade of inhibitory
37 synapses strengthened (rather than weakened) that phasic inspiratory-sigh burst relationship.
38 Furthermore, pharmacological disinhibition did not alter the duration of the prolonged interval
39 that follows a sigh burst prior to resumption of the inspiratory rhythm. These results demonstrate
40 that coupling between inspiratory and sigh rhythms does not depend on synaptic inhibition.

41

42 **SIGNIFICANCE STATEMENT**

43 Breathing consists of eupnea and sigh breaths, which differ in their magnitude and frequency.
44 Both breath types emerge from a brainstem microcircuit that coordinates their timing. Here, we
45 advance understanding of these rhythms by assessing the nature and strength of their
46 coordination, and by showing that synaptic inhibition does not enforce their temporal coupling in
47 contrast to conventional understanding. This study provides insights into the basic neural
48 mechanisms that link oscillations of different amplitude and frequency in a core oscillator.
49

50 INTRODUCTION

51 Breathing behavior consists of two interleaving rhythms and motor patterns: eupnea and
52 sighing. Eupnea is the normal unlabored breathing that underlies periodic lung ventilation and
53 drives alveolar gas exchange. Eupnea occurs at approximately 1-4 Hz in rodents (0.2-0.3 Hz in
54 humans); each breath ventilates a small fraction of lung capacity. Sighs are also inspiratory
55 breaths, but the volume of inhaled air during a sigh is two to five times that of a normal breath,
56 and sigh frequency is an order of magnitude lower than the eupnea rhythm (Li and Yackle,
57 2017). Sighs reinflate collapsed (or collapsing) alveoli and are essential for optimal pulmonary
58 function. Typically, sighs appear to ride atop ongoing eupneic breaths (Cherniack et al., 1981;
59 Orem and Trotter, 1993), which suggests that periodically, but at a much lower frequency, the
60 eupnea cycle triggers the sigh. After a sigh, the next eupneic breath is delayed for a duration
61 roughly equivalent to one additional eupneic cycle (Cherniack et al., 1981; Orem and Trotter,
62 1993). This delay, which we refer to as the post-sigh apnea, suggests that sighs inhibit eupnea
63 at least transiently. Eupnea and sigh breathing rhythms thus appear to be coupled, most likely
64 via neural microcircuits of the brainstem that generate and control breathing movements.

65 In mammals, eupnea and sigh rhythms emanate from the preBötzinger Complex (preBötC) of
66 the lower brainstem (Del Negro et al., 2018; Smith et al., 1991). Both rhythms are maintained in
67 reduced slice preparations that isolate the preBötC as well as inspiratory premotor and motor
68 neurons, and thus encapsulate a minimal breathing-related model system (Lieske et al., 2000;
69 Ruangkittisakul et al., 2008; Chapuis et al., 2014). Because eupnea refers to behavior in living
70 animals, *inspiratory* is the appropriate nomenclature for eupnea-related activity in slice
71 preparations. Inspiratory rhythm depends on network properties, in which recurrent excitation
72 among glutamatergic interneurons is rhythmogenic (Funk et al., 1993; Rekling et al., 2000; Del
73 Negro et al., 2002; Wallen-Mackenzie et al., 2006; Feldman and Kam, 2015). The rhythmogenic
74 mechanism of sighs is unknown, but it depends on neuropeptides released by parafacial
75 respiratory interneurons (Li et al., 2016) as well as excitatory ionotropic and metabotropic
76 receptor-mediated synaptic transmission (Lieske and Ramirez, 2006a, 2006b).

77 Inspiratory bursts appear to trigger sigh-related bursts, and in turn, sigh-related bursts delay the
78 next inspiratory burst by almost an entire cycle (Lieske et al., 2000; Tryba et al., 2008). These
79 observations *in vitro* mirror the *in vivo* coupling behavior described above, which suggests the
80 mechanisms that couple inspiratory and sigh rhythms are contained within the preBötC and can
81 be examined at the cellular and synaptic level *in vitro*.

82 What mechanisms couple these two rhythms? The only existing data suggest that glycinergic
83 synaptic inhibition links the sigh-related burst to its preceding inspiratory burst, thus giving rise
84 to the biphasic shape in which the inspiratory burst appears to trigger the sigh (Lieske et al.,
85 2000). A recent mathematical model (Toporikova et al., 2015) posits two discrete systems for
86 generating eupnea and sigh oscillations. The model inspiratory system acts on the sigh system
87 via synaptic inhibition such that sigh bursts emerge via an escape-like process triggered by
88 disinhibition at the tail end of inspiratory bursts. The model also suggests that the sigh system
89 projects to the inspiratory system via fast excitatory synapses, and the strength of its excitation
90 leads to a transient state of refractoriness, i.e., the post-sigh apnea, in the coupled system.
91 However, the post-sigh apnea might also be attributable to synaptic inhibition from the sigh
92 system onto the inspiratory rhythm generator.

93 Here, we test the role of synaptic inhibition in coupling eupnea and sigh rhythms *in vitro*. First,
94 we elucidate the chloride reversal potential (E_{Cl}) in order to verify that glycine and GABA_A
95 synapses are inhibitory, and not functionally excitatory, as they are during embryonic
96 development (Delpy et al., 2008; Ren and Greer, 2006). Next, we block glycinergic transmission
97 and show that disinhibiting the preBötC *in vitro* does not uncouple the eupnea- and sigh-related
98 rhythms, but in fact appears to couple them more strongly, given our observation of decreased
99 latency between a sigh and its preceding inspiratory burst. We obtain similar results when we
100 simultaneously block GABAergic and glycinergic transmission. We also show the duration of the
101 post-sigh apnea does not depend on glycinergic or GABAergic transmission, which instead
102 suggests that the post-sigh apnea reflects a refractory state attributable to post-synaptic
103 membrane properties evoked by the sigh burst. These findings indicate that the eupnea and
104 sigh rhythms are coupled predominantly by excitatory (rather than inhibitory) synaptic
105 interactions.

106

107 **MATERIALS AND METHODS**

108 ***Ethical approval and animal use***

109 The Institutional Animal Care and Use Committee at our institution approved these protocols,
110 which conform to the policies of the Office of Laboratory Animal Welfare (National Institutes of
111 Health, Bethesda, MD, USA) and the guidelines of the National Research Council (National
112 Research Council (U.S.) et al., 2011). CD-1 mice (Charles River, Wilmington, MA) were

113 maintained on a 14-hour light/10-hour dark cycle at 23° C and were fed *ad libitum* with free
114 access to water.

115 Mouse pups of both sexes were anesthetized by hypothermia and then killed by thoracic
116 transection at postnatal day 0 to 4. The neuraxis was removed in less than two minutes and
117 further dissected in artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl, 3 KCl, 1.5
118 CaCl₂, 1 MgSO₄, 25 NaHCO₃, 0.5 NaH₂PO₄, and 30 dextrose equilibrated with 95% O₂-5% CO₂,
119 pH 7.4.

120 ***Breathing-related measurements in vitro***

121 Isolated neuraxes were glued to an agar block and then cut in the transverse plane to obtain a
122 single 550- μ m-thick slice that exposed the preBötC at its rostral face (Ruangkittisakul et al.,
123 2011, 2014). Slices were then perfused with aCSF at 28° C in a recording chamber mounted
124 below a stereomicroscope that enabled us to position suction electrodes under visual control.
125 Extracellular K⁺ concentration ([K⁺]_o) was increased to 9 mM to elevate preBötC excitability
126 (Funk and Greer, 2013).

127 Inspiratory-related motor output was recorded from the hypoglossal (XII) nerve rootlets, which
128 are captured in transverse slices along with the XII motoneurons and their axon projections to
129 the nerve rootlets, using suction electrodes and a differential amplifier. Also, we simultaneously
130 recorded field potentials from the preBötC by forming a seal over it with a suction electrode at
131 the slice surface. Amplifier gain was set at 2000 and the band-pass filter was set at 300-1000
132 Hz. XII and preBötC bursts were full-wave rectified and smoothed for display and quantitative
133 analyses of burst events. We acquired and digitized the signals at 4 kHz with a low-pass filter
134 set to 1 kHz using a 16-bit analog-to-digital converter (ADInstruments, Colorado Springs, CO).

135 The glycine receptor antagonist strychnine hydrochloride (CAS number 1421-86-9, product
136 S8753, Millipore Sigma, St. Louis, MO) and the GABA_A receptor antagonist picrotoxin (CAS
137 number 124-87-8, product P1675, Millipore Sigma) were bath-applied at 5 μ M while monitoring
138 field potentials in the preBötC and XII motor output.

139 ***Gramicidin patch recordings and glycine and muscimol application***

140 We obtained whole-cell patch-clamp recordings under visual control from slices perfused in a
141 recording chamber on a fixed-stage microscope (Zeiss AxioExaminer, Thornwood, NY). All
142 recordings employed a HEKA EPC 10 patch-clamp amplifier (Holliston, MA). Patch pipettes

143 were fabricated from borosilicate glass (OD: 1.5 mm, ID: 0.86 mm, 4-6 M Ω in bath) and filled
144 with solution containing 150 mM KCl and 10 mM HEPES). We added gramicidin (CAS number
145 1405-97-6, product G0550000 from Millipore Sigma) acutely at the start of the experiment from
146 stock solution (2 mg gramicidin per 1 ml dimethyl sulfoxide) such that the final concentration
147 was 20 μ g/ml. Patch pipettes were back-filled first with gramicidin-free patch solution in order to
148 ensure a proper seal to the membrane. All recordings were corrected offline for a liquid junction
149 of 3.74 mV (Barry and Lynch, 1991; Neher, 1992).

150 The experimental protocol began no earlier than 30 minutes after achieving a seal on the
151 plasma membrane exceeding 1 G Ω (i.e., gigaohm seal), which was sufficient for gramicidin to
152 form ionophores and thus allow intracellular access and current-clamp recording. We also
153 fabricated pipettes (as described above) from which to eject glycine and muscimol (dubbed
154 'puffer' pipettes). Puffer pipettes were filled with 150 μ M glycine and 30 μ M muscimol diluted
155 into ACSF (containing 9 mM [K⁺]_o, as described above). 30 minutes after forming a gigaohm
156 seal on the plasma membrane, the puffer pipette was positioned to within ~50 μ m from the
157 neuron being recorded. 30 μ M muscimol (CAS number 2763-96-4, product M1523, Millipore
158 Sigma) and 150 μ M glycine (CAS number 56-40-6, product 50046, Millipore Sigma) were
159 ejected using 7-9 psi pressure pulses lasting 25-200 ms, which we triggered by TTL commands
160 from the EPC-10 amplifier.

161 Cells were identified as neurons by their ability to discharge action potentials, recognizable ~30
162 min after forming a gigaohm seal. Subsequently, we added 1 μ M tetrodotoxin (TTX) to the bath
163 to prevent spike-mediated chemical synaptic transmission. Neurons were held at a desired
164 membrane potential using bias current. We measured transient changes in membrane potential
165 in response to puffed glycine and muscimol, in which the previous 2 seconds of membrane
166 voltage were used as baseline.

167 ***Identification and categorization of sigh bursts***

168 We distinguished a sigh burst from an inspiratory burst in field recordings based on burst
169 magnitude, period regularity, and the presence of a post-sigh apnea. First, the area of sigh
170 bursts in a given slice exceed the area of inspiratory bursts by more than one standard deviation
171 away from the average area of all inspiratory bursts recorded in that slice preparation (because
172 the frequency of sigh bursts is much lower than the frequency of inspiratory bursts, the average
173 area of all bursts effectively returns the mean inspiratory burst area). Second, the cycle period

174 for sigh bursts measures 1 to 4 minutes, and not outside this range (Lieske et al., 2000;
175 Ruangkittisakul et al., 2008). Third, sigh bursts are followed by a prolonged inter-event interval
176 (post-sigh apnea) greater than 1.3X the average inspiratory cycle time for six consecutive cycles
177 preceding a putative sigh burst (Fig. 1). Burst events meeting two of these three conditions were
178 considered sigh bursts (note that >90% of sigh bursts satisfied all three conditions).

179 We used an algorithmic rule for categorizing sigh bursts into the five characteristic types: *long*
180 *interval*, *doublet*, *classic*, *S to I*, and *conjoint*. Figure 1 provides an accompanying schematic.
181 Epsilon (ϵ) indicates eupnea-related inspiratory cycles. Sigma (σ) indicates sigh-related cycles.
182 Delta (Δ) indicates time or amplitude differences. For each sigh burst, we computed six
183 representative metrics. The average inspiratory cycle time (\bar{T}) is the average inspiratory cycle
184 time, computed from six inspiratory cycles preceding the sigh ($T_{\epsilon_1} \dots T_{\epsilon_6}$). T_{Δ} is the inspiratory-
185 sigh interval computed as the peak time of the sigh burst minus the peak time of the inspiratory
186 burst. T_{σ} is the duration of the post-sigh apnea. For each sigh burst, the duration of the
187 associated inspiratory burst is denoted D_{ϵ} . The peak amplitude of that inspiratory burst is A_{ϵ} ,
188 and the amplitude of the voltage drop from the peak of the preceding inspiratory burst to the
189 trough of the inspiratory-sigh interval is ΔA . Long interval sigh bursts are defined by $D_{\epsilon} < T_{\Delta} <$
190 $\frac{3}{4}\bar{T}$; doublet sighs are defined by $D_{\epsilon} > T_{\Delta}$ and $\Delta A > \frac{1}{2}A_{\epsilon}$; classic sighs are defined by $\Delta A < \frac{1}{2}A_{\epsilon}$;
191 S-to-I sigh bursts are defined by $T_{\Delta} < 0$ (because the inspiratory burst follows the sigh burst);
192 and conjoint sighs are defined by $T_{\Delta} > \frac{3}{4}\bar{T}$.

193 **Measurements and statistics**

194 We measured the amplitude (V) and area (V-s) of eupnea-related inspiratory bursts (frequency
195 range 0.1–0.2 Hz) and lower-frequency sighs (<1 min⁻¹). Burst amplitude was measured from
196 baseline (defined as the average field potential 500 ms prior to the burst) to the burst peak. In all
197 cases inspiratory and sigh burst peaks are distinct and distinguishable. Nevertheless, for
198 classic, S-to-I, conjoint bursts, and some doublet bursts, the amplitude of the trailing burst is
199 enhanced by temporal summation after the preceding burst.

200 Burst area was computed as the area under the curve of the trajectory of the field potential
201 recording. For classic, S-to-I, conjoint bursts, and some doublet bursts, the burst area
202 measurement conflates the two partially coincident events. In such cases, the aggregate area is
203 classified sigh burst area. For long interval sighs – to be consistent with the other categories

204 described above – the area of the sigh burst was always summed with the area of the preceding
205 inspiratory burst regardless of the inspiratory-sigh interval duration.

206 We analyzed data and computed statistics using LabChart 7 (ADInstruments), MATLAB 2018b
207 (Mathworks, Natick MA), and Igor Pro 8 (Wavemetrics, Oswego, OR). We describe the
208 statistical hypothesis tests used as they appear in the main text.

209

210 **RESULTS**

211 ***Chloride-mediated fast synaptic drive in the preBötC of neonatal mice is inhibitory***

212 We recorded preBötC neurons through gramicidin-perforated patches selectively permeable to
213 monovalent cations. The internal chloride concentration remains unchanged so E_{Cl} can be
214 determined (Kyzozis and Reichling, 1995). In the presence of TTX, pressure pulse ejections of
215 muscimol and glycine transiently perturbed the membrane potential, which reversed at or below
216 -45 mV (Fig. 2A and B). E_{Cl} measured -49 ± 8 mV (mean \pm SD, 7 preBötC neurons recorded in
217 6 slices). We observed no relationship between E_{Cl} and age (Fig. 2C), which indicates that E_{Cl} is
218 consistent during early postnatal development.

219 ***Sigh bursts have a variable inspiratory-sigh interval***

220 We measured 343 sigh bursts in 13 slice preparations (26 ± 7 sigh bursts per slice). The classic
221 sigh pattern, which is often described as the sigh burst building off the crest of an inspiratory
222 burst, does not accurately describe the temporal relationship between most sigh bursts and their
223 preceding inspiratory bursts. To illustrate the variability of the inspiratory-sigh intervals we
224 identified five discrete categories (Fig. 3A; the algorithm we used to assign sigh bursts to one of
225 these five categories is explained in Materials and Methods). The classic biphasic sigh burst
226 was only recorded 72 times ($\sim 21\%$). Sigh bursts with long intervals and doublets were observed
227 97 times ($\sim 28\%$) and 56 times ($\sim 16\%$), respectively.

228 We also observed 40 sigh bursts immediately followed by an inspiratory burst, which results in a
229 negative inspiratory-sigh interval ($\sim 12\%$), dubbed S-to-I sigh bursts (Fig. 3A). To our knowledge,
230 the S-to-I phenomenon has not previously been experimentally documented, and it suggests
231 that a sigh burst does not absolutely require a preceding inspiratory burst to trigger it.

232 Further, we recorded 78 conjoint sigh bursts (~23%) in which the inspiratory and sigh bursts
233 appear to occur simultaneously. This interpretation cannot be confirmed because it is possible
234 that the sigh burst we label as conjoint actually occurs with a delay equivalent to an entire
235 inspiratory cycle time. In the other cases shown in Fig. 3A, the delay from the inspiratory to the
236 sigh burst is less than the average inspiratory cycle time so one can perceive their linkage.
237 However, if the conjoint sigh bursts were actually isolated sighs – independent of and separated
238 from preceding inspiratory bursts by an entire cycle – then their amplitude and area
239 measurements should be demonstrably smaller than the amplitude and area of all other
240 categories of sigh bursts. However, the mean burst amplitude of putative conjoint sigh bursts
241 (34 ± 12 mV) was commensurate with the mean amplitude of all other sigh burst types ($36 \pm$
242 13 mV) (Wilcoxon rank sum test, $p = 0.73$, $n = 9$ slices). Similarly, the mean burst area of
243 putative conjoint sigh bursts (18 ± 10 mV-s) and all other sigh burst types (20 ± 11 mV-s) were
244 also commensurate (Wilcoxon rank sum test, $p = 0.26$, $n = 9$ slices). This suggests that the
245 events in question consist of synchronized inspiratory and sigh bursts (i.e., conjoint bursts).

246 Blocking glycinergic synapses with strychnine did not alter the relative prevalence of inspiratory-
247 sigh interval categories (Fig. 3B). However, after blocking all chloride-mediated ionotropic
248 synaptic receptors with strychnine and picrotoxin, it appeared that doublet and classic sighs
249 increased by approximately two-fold or more (13% to 39% and 26% to 38%, respectively), even
250 though we continued to observe all of the inspiratory-sigh interval categories (Fig. 3C). These
251 data show that the temporal coupling between inspiratory and sigh bursts is more variable than
252 previously reported. Furthermore, removal of chloride-mediated inhibition may favor sigh bursts
253 in which temporal coupling is the tightest, i.e., the doublet and classic types of sigh bursts.

254 ***Inhibitory synapses do not couple inspiratory and sigh bursts***

255 In order to quantitatively compare inspiratory-sigh intervals, we calculated their probability
256 distributions (Fig. 3D-F) and cumulative distribution functions (Fig. 4). In control conditions, a
257 sigh burst was most likely to manifest at relatively short intervals following an inspiratory burst.
258 This is illustrated by the peak probability of a sigh burst at the earliest part of the normalized
259 inspiratory cycle; the probability then tapers off at later stages of the normalized cycle. Indeed,
260 24% of sigh bursts occur within the first tenth of the normalized cycle time and the majority
261 (64%) occur within the first half of the normalized cycle (Fig. 3D).

262 Using strychnine to block glycinergic synaptic transmission, the probability distribution of
263 inspiratory-sigh intervals remained weighted towards the first half of the normalized cycle (Fig.
264 3E), suggesting that inspiratory-sigh coupling remained intact. In contrast, previous studies
265 suggested that blocking glycinergic synapses removed any temporal relationship between the
266 sigh burst and its preceding inspiratory burst (Lieske et al., 2000; Chapuis et al., 2014), in which
267 case the probability distribution in Fig. 3E would be uniformly distributed between 0 and 1. Here,
268 the probability of short inspiratory-sigh intervals (T_{Δ}/\bar{T}) increased such that the average
269 normalized inspiratory-sigh interval decreased from 0.31 ± 0.34 in control to 0.25 ± 0.30 in the
270 presence of strychnine. This trend is reflected in the significant leftward shift of the cumulative
271 distribution function from control to treatment with strychnine for cycle time > 0 (Fig. 4,
272 Kolmogorov-Smirnov, test statistic = 0.13, $p = 0.011$, $n = 7$ slices).

273 Similarly, when we simultaneously blocked glycinergic and GABA_A receptors with a strychnine
274 and picrotoxin cocktail, the sigh burst coupled more tightly with the preceding inspiratory burst
275 than in control conditions. The probability of short inspiratory-sigh intervals (T_{Δ}/\bar{T}) increased
276 from control to the strychnine and picrotoxin cocktail such that the average normalized
277 inspiratory-sigh interval decreased from 0.31 ± 0.34 in control to 0.21 ± 0.32 in strychnine and
278 picrotoxin. The significant leftward shift of the cumulative distribution function for cycle time > 0
279 further demonstrates that when both glycinergic and GABA_A receptors were blocked, the sigh
280 burst was more likely to occur earlier with respect to the preceding inspiratory burst than during
281 control (Fig. 4, Kolmogorov-Smirnov, test statistic = 0.31, $p = 4.0E-12$, $n = 6$ slices). It is worth
282 noting that the large standard deviation of the average normalized inspiratory-sigh interval,
283 relative to the mean interval in all conditions, reflects the variability in the timing of a sigh
284 discussed earlier and illustrated in Figure 3A-C.

285 Our analyses (Figs. 3 and 4) show the removal of chloride-mediated synaptic inhibition does not
286 uncouple the sigh from its preceding inspiratory burst, rather it strengthened the temporal
287 coupling of inspiratory and sigh bursts.

288 ***Inhibitory synapses do not influence post-sigh apnea***

289 We calculated the relative post-sigh apnea as the duration of the post-sigh interval (T_{σ}) divided
290 by the average inspiratory cycle time (\bar{T}), thus normalizing it and expressing it as a unitless
291 ratio. Then, we compared the relative post-sigh apnea in control and after blocking either
292 glycinergic transmission, or both glycinergic and ionotropic GABAergic transmission (Fig. 5).

293 The relative post-sigh apnea measured 1.65 ± 0.20 in control, 1.72 ± 0.25 in strychnine, and 1.5
294 ± 0.09 in the strychnine-picrotoxin cocktail. These measurements are statistically
295 indistinguishable (one-way ANOVA, test statistic = 2.12, $p = 0.14$, $n = 13$ slices).

296

297 **DISCUSSION**

298 Inspiratory and sigh-related rhythms both emerge from the preBötC. Their interactions and
299 influence on one another can be studied *in vitro*. The conventional understanding is that sigh
300 bursts build off the crest of inspiratory bursts, which implies strong coupling wherein inspiratory
301 bursts trigger sigh bursts. However, that conceptual framework oversimplifies how sigh bursts
302 actually emerge from preBötC activity. There is previously unrecognized variability in the timing
303 between inspiratory and sigh bursts, which suggests that their coupling is weaker, relatively
304 speaking, than previously appreciated. The existence of S-to-I and conjoint sigh bursts further
305 reinforces this notion of flexibility in the relationship between a sigh burst and an associated
306 inspiratory burst by showing that an inspiratory burst is not strictly necessary to trigger a sigh
307 burst. Nevertheless, more often than not an inspiratory burst does trigger a sigh burst with an
308 interval whose duration is on the order of one-tenth of the average inspiratory cycle time.

309 Here, in early postnatal mouse development (P0-4) with elevated (9 mM) $[K^+]_o$ ACSF to boost
310 slice excitability, E_{Cl} measured -49 mV. This Nernst equilibrium potential is below spike
311 threshold and approximates the level of baseline membrane potential during the interburst
312 interval. Therefore, $GABA_A$ and glycinergic inputs either shunt the membrane – rendering it less
313 responsive to excitatory (depolarizing) drive – or hyperpolarize it directly during the
314 preinspiratory phase or during the inspiratory burst itself when the membrane potential trajectory
315 exceeds -49 mV.

316 We show that chloride-mediated synaptic inhibition is not responsible for the temporal coupling
317 between the inspiratory and sigh bursts. Rather, disinhibition strengthened their coupling.
318 Therefore, our primary conclusion is that excitatory (not inhibitory) synaptic transmission links
319 the inspiratory and sigh rhythms of the preBötC.

320 This conclusion contradicts prior studies showing that blockade of glycinergic transmission
321 decoupled sighs from their preceding inspiratory bursts and created free-running sigh burst
322 rhythms that appeared to be independent from ongoing inspiratory rhythms (Lieske et al., 2000;
323 Chapuis et al., 2014; Toporikova et al., 2015).

324 The discrepancy between those prior results and our present findings are probably attributable
325 to the late embryonic reversal of the chloride electrochemical gradient. Before embryonic day
326 15.5 (E15.5) in mice, the dominant expression of cotransporter NKCC1 in brainstem and spinal
327 cord neurons elevates intracellular chloride concentration (Delpy et al., 2008; Ren and Greer,
328 2006; Viemari et al., 2011) such that chloride-mediated synaptic currents are inward (i.e.,
329 excitatory) at the baseline membrane potential of rhythmically active preBötC interneurons.
330 Perinatally NKCC1 expression decreases in parallel with increasing expression of the chloride
331 symporter, KCC2, which lowers intracellular chloride concentration. In the mature state,
332 dominant KCC2 expression ensures that the chloride equilibrium potential is more
333 hyperpolarized than spike threshold as well as baseline membrane potential during rhythmic
334 activity. The mature gradients ensure that chloride-mediated synaptic currents are outward and
335 inhibitory.

336 Whereas we studied postnatal (P0-4) mice exclusively, Chapuis et al. (2014) and Toporikova et
337 al. (2015) studied embryonic mice (E15.5-18.5). At these ages, with elevated (8 mM) $[K^+]_o$,
338 ACSF, E_{Cl} is above spike threshold and consequently glycinergic synapses serve to depolarize
339 and evoke action potentials in preBötC neurons (Ren and Greer, 2006; Delpy et al., 2008).
340 Under these conditions, we infer that glutamatergic, glycinergic and GABA_Aergic synapses are
341 effectively excitatory and link sigh bursts to their preceding inspiratory bursts. When net
342 excitatory drive is perturbed (such as by blocking chloride-mediated synaptic excitation) then
343 inspiratory-sigh coupling weakens, and the sigh rhythm appears to 'free run' in a manner that is
344 uncoupled from inspiratory rhythm.

345 Development and chloride gradients might also explain the discrepancies between our results
346 and Lieske et al. (2000), who also concluded that glycinergic synapses couple inspiratory and
347 sigh rhythms. Those authors reported using mice aged 0-2 weeks, a postnatal window that
348 overlaps and extends beyond ours. We surmise that their sigh burst experiments were
349 performed exclusively or predominantly using preparations from P0 mice with immature chloride
350 gradients. Then the same explanation holds; chloride gradients favoring inward currents (with
351 suprathreshold reversal potential) render glycine synapses ostensibly excitatory.

352 Chloride-mediated synaptic inhibition does not contribute to the post-sigh apnea. Instead, the
353 post-sigh apnea is caused by activation of the intrinsic cellular mechanisms that help terminate
354 inspiratory bursts, which are recruited to a greater degree during sigh events (compared to
355 typical inspiratory cycles). These burst-terminating mechanisms include activity-dependent

356 outward currents such as the electrogenic Na/K ATPase pump current, Na⁺-dependent K⁺
357 current, and ATP-dependent K⁺ current (Del Negro et al., 2009; Krey et al., 2010), as well as
358 excitatory synaptic depression (Kottick and Del Negro, 2015; Guerrier et al., 2015). It was
359 recently shown that the magnitude of inspiratory burst-related depolarization directly evokes
360 corresponding levels of post-burst hyperpolarization in preBötC neurons, from which the
361 neurons must recover prior to generating the next inspiratory burst (Baertsch et al., 2018). The
362 sigh burst in this context is an extreme version of that same mechanism: the increased
363 magnitude and duration of the sigh event correspondingly evokes larger-than-average activity-
364 dependent refractory (outward) currents and depresses excitatory synapses to a greater extent
365 than during typical inspiratory bursts of lower magnitude and duration. The larger-than-average
366 hyperpolarization (and depressed synapses) extends the duration of the interburst interval, thus
367 creating the post-sigh apnea.

368 Here we demonstrate that chloride-mediated synaptic communication is inhibitory in the
369 preBötC of neonatal mice. Although sigh bursts are often closely preceded by inspiratory bursts,
370 their temporal coordination is more variable than previously documented. Chloride-mediated
371 synaptic inhibition plays no obligatory role in coupling the inspiratory and sigh rhythms in
372 postnatal mice and we speculate that this principle may hold for juvenile and adult stages of
373 development because E_{Cl} is expected to remain below spike threshold and inhibitory; in fact we
374 expect it to descend lower than -49 mV during further maturation. A model of the preBötC core
375 that incorporates inspiratory and sigh oscillators need not include synaptic inhibition to generate
376 or couple the discrete rhythms. This emphasis on the primacy of excitatory synaptic interactions
377 probably extends to embryonic development when chloride-mediated synapses are functionally
378 excitatory.
379

380 Figure Captions

381 Figure 1. Integrated field recording of preBötC activity, which includes inspiratory and sigh
382 bursts, in slices. Inset (upper left) shows normalized inspiratory cycle (\bar{T}) calculated from the
383 average cycle period of the previous six inspiratory bursts ($T_{\epsilon_1} \dots T_{\epsilon_6}$). T_{ϵ} represents period of a
384 single inspiratory cycle; A_{ϵ} represents amplitude of sigh-associated inspiratory burst; ΔA reflects
385 voltage drop from peak of an inspiratory burst to the nadir during the inspiratory-sigh interval; D_{ϵ}
386 reflects the duration of sigh-associated inspiratory burst; T_{Δ} represents the inspiratory-sigh
387 interval; and T_{σ} reflects the duration of the post-sigh apnea.

388 Figure 2. Determining the chloride reversal potential (E_{Cl}) in preBötC neurons in neonatal
389 brainstems. A: Representative traces of membrane voltage before and after puffer application of
390 150 μ M glycine and 30 μ M muscimol. Mean membrane potential before puffer application was
391 calculated from the previous 2 seconds (orange box) of recording. Change in membrane voltage
392 (Δ mV) was calculated as the voltage change from mean membrane potential to peak
393 membrane response. B: Membrane potential changes in response to glycine and muscimol
394 puffs plotted versus holding potential from a single representative experiment (same cell as A).
395 E_{Cl} (Δ mV = 0) is calculated using a linear regression (purple line). C: E_{Cl} from 7 cells (N = 6
396 mice) plotted as a function of postnatal mouse age. Dashed line indicates the average E_{Cl} for
397 the sample.

398 Figure 3. The variability in timing of sigh bursts. A-C: Integrated field recordings of preBötC
399 activity showing the five variations of the inspiratory-to-sigh burst coupling during control (A),
400 and after treatment with 5 μ M strychnine (B), as well as treatment with 5 μ M strychnine and 5
401 μ M picrotoxin (C). Percentages next to sigh categories display the fraction a particular sigh
402 class was observed out of all sighs recorded in that condition (343 control sighs, 292 strychnine
403 sighs, 219 strychnine & picrotoxin sighs). Grey arrows identify the sigh-associated inspiratory
404 burst. D-F: Histograms of the normalized inspiratory-sigh interval (T_{Δ}/\bar{T}) under all three
405 conditions. The width of each bar is $0.1 T_{\Delta}/\bar{T}$.

406

407 Figure 4. Cumulative density distribution of the normalized inspiratory-sigh interval (T_{Δ}/\bar{T}) under
408 control conditions (blue), treatment with 5 μ M strychnine (orange), and treatment with both 5 μ M
409 strychnine and 5 μ M picrotoxin (red).

410

411 Figure 5. Average post-sigh apnea duration normalized by the average inspiratory cycle time (\bar{T})
412 for control conditions (blue), treatment with 5 μ M strychnine (orange), and treatment with both 5
413 μ M strychnine and 5 μ M picrotoxin (red). Each dot represents the average normalized post-sigh
414 apnea for an experiment. Horizontal bars represent the average of all experiments.

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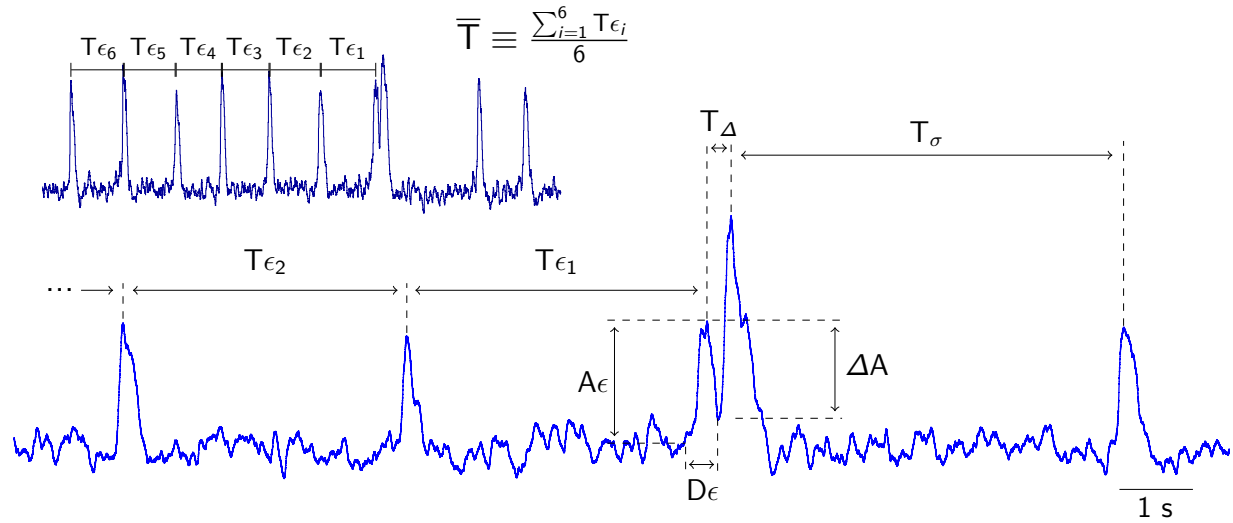
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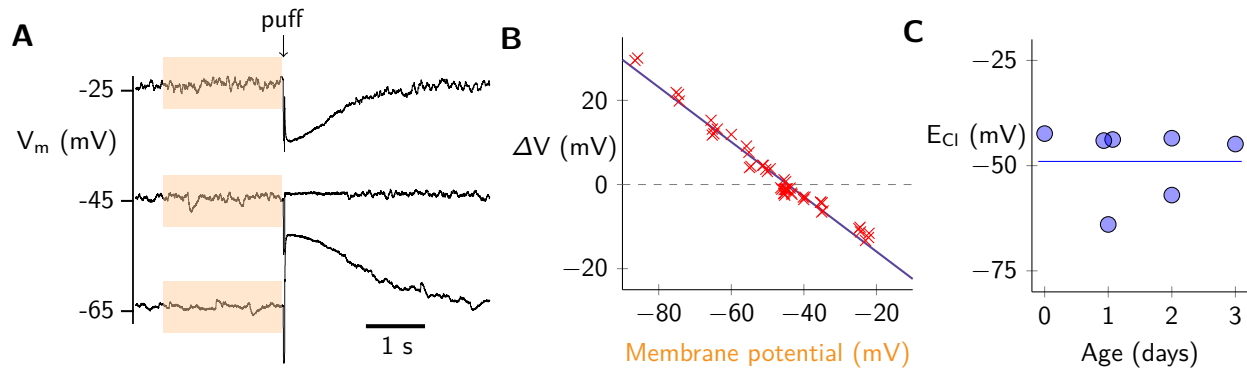
503 **Figures**

504 Figure 1:



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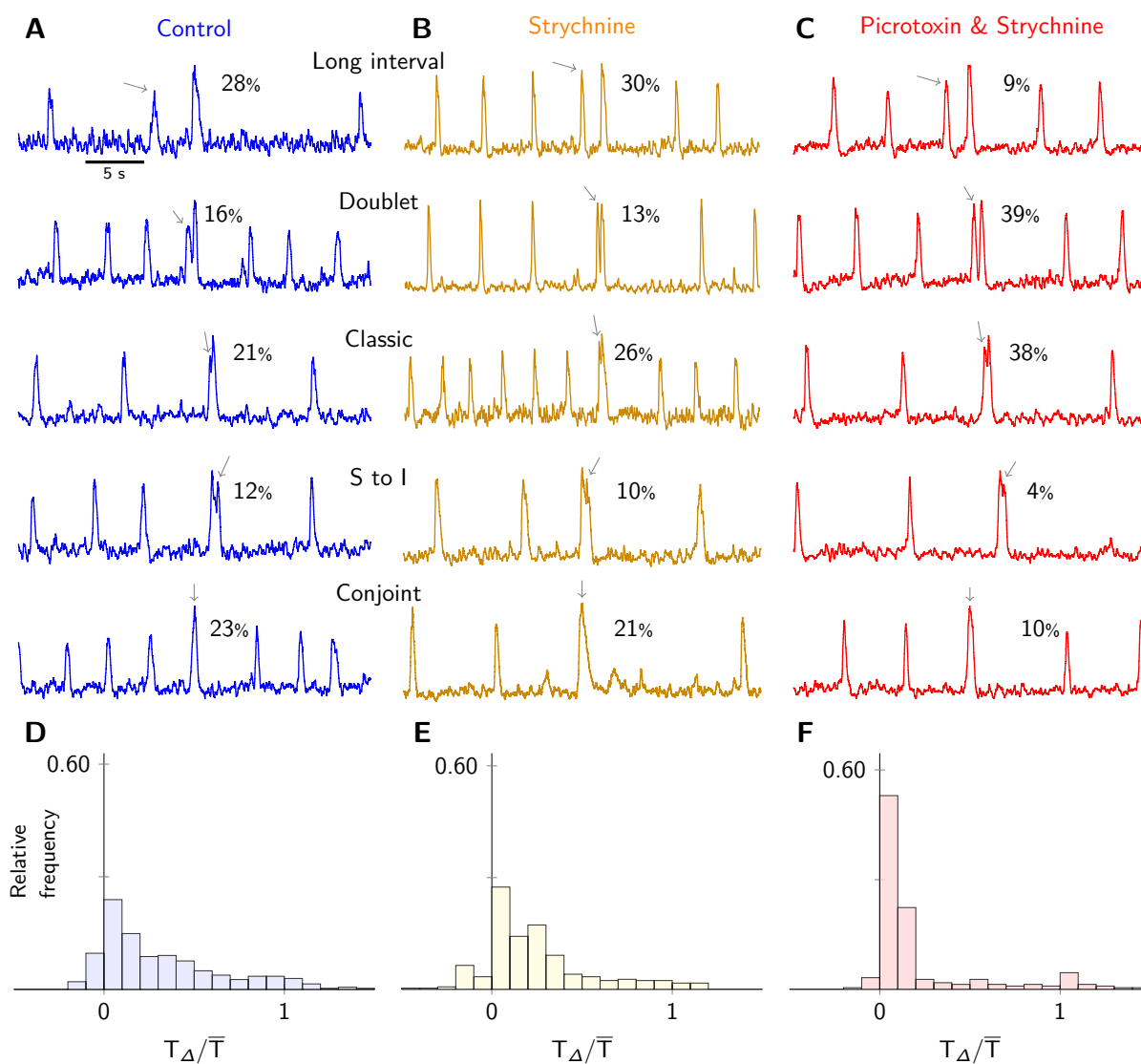
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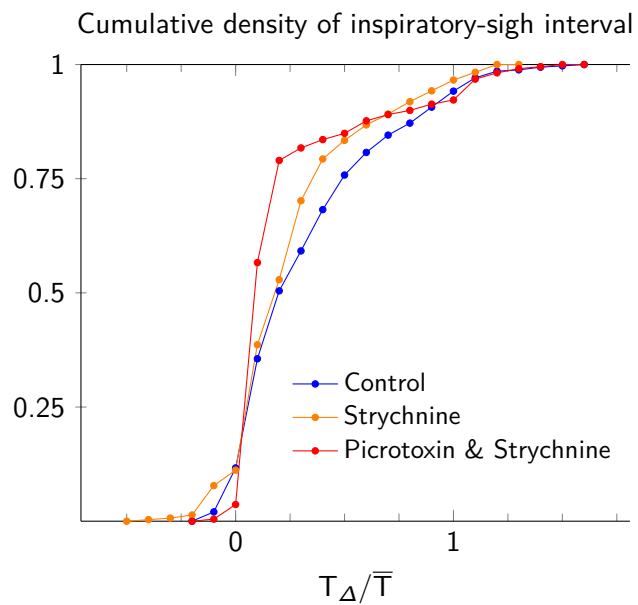


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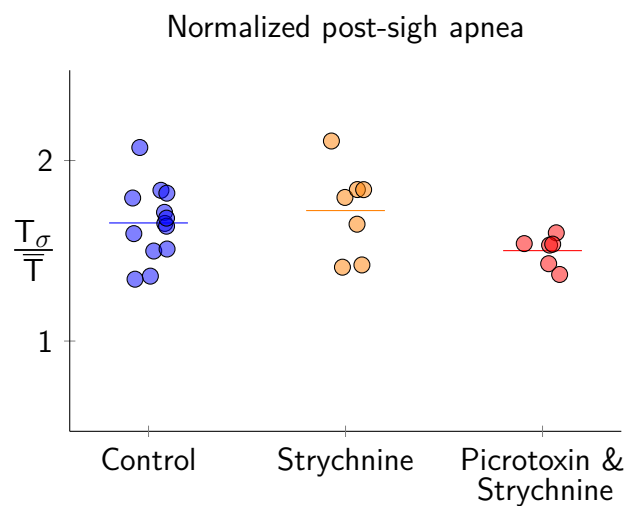
513 Figure 4:



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516 Figure 5:



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