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**The Kölliker-Fuse orchestrates the timing of expiratory abdominal nerve bursting**

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**Running head:** Pontine control of active expiration

**Call for Manuscripts:** Central Pattern Generators

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32 **New & Noteworthy**

33

- 34 • The pons is essential for the formation of the three-phase respiratory pattern, controlling  
35 the inspiratory-expiratory phase transition;
- 36 • We provide functional evidence of a novel role for the KF controlling the emergence of  
37 abdominal expiratory bursts during active expiration;
- 38 • A computational model of the respiratory central pattern generator predicts a possible  
39 mechanism by which the KF interacts indirectly with the parafacial respiratory group  
40 (pFRG) and exerts an inhibitory effect on the expiratory conditional oscillator.

41

42 **Keywords:** abdominal expiratory activity, pons, ventral respiratory column, respiratory pattern,  
43 active expiration.

44

45

46 **Glossary:** AbN, abdominal nerve; aug-E, augmenting expiratory; BötC, Bötzinger complex;  
47 CPG, central pattern generator; cVN, vagus nerve; early-I, early-inspiratory; KF Kölliker-Fuse;  
48 late-E, late-expiratory; pFRG, parafacial respiratory group; post-I, post-inspiratory; pre-BötC,  
49 pre-Bötzinger complex; pre-I/I, pre-inspiratory/inspiratory; ramp-I, ramping inspiratory; RTN,  
50 retrotrapezoid nucleus;

51

52

53 **ABSTRACT**

54

55         Coordination of respiratory pump and valve muscle activity is essential for normal  
56 breathing. A hallmark respiratory responses to hypercapnia and hypoxia is the emergence of  
57 active exhalation, characterized by abdominal muscle pumping during the late one-third of  
58 expiration (late-E phase). Late-E abdominal activity during hypercapnia has been attributed to  
59 the activation of expiratory neurons located within the parafacial respiratory group (pFRG).  
60 However, the mechanisms that control emergence of active exhalation, and its silencing in restful  
61 breathing, are not completely understood. We hypothesized that inputs from the Kölliker-Fuse  
62 nucleus (KF) control the emergence of late-E activity during hypercapnia. Previously, we  
63 reported that reversible inhibition of the KF reduced post-inspiratory (post-I) motor output to  
64 laryngeal adductor muscles and brought forward the onset of hypercapnia-induced late-E  
65 abdominal activity. Herein, we explored the contribution of the KF for late-E abdominal  
66 recruitment during hypercapnia by pharmacologically disinhibiting the KF in *in situ* decerebrate  
67 arterially-perfused rat preparations. These data were combined with previous results and  
68 incorporated into a computational model of the respiratory central pattern generator.  
69 Disinhibition of the KF through local parenchymal microinjections of gabazine (GABA<sub>A</sub>  
70 receptor antagonist) prolonged vagal post-I activity and inhibited late-E abdominal output during  
71 hypercapnia. *In silico*, we reproduced this behavior and predicted a mechanism where the KF  
72 provides excitatory drive to post-I inhibitory neurons, which, in turn, inhibit late-E neurons of  
73 the pFRG. Although the exact mechanism proposed by the model requires testing, our data  
74 confirm that the KF modulates the formation of late-E abdominal activity during hypercapnia.

75

76

## 77 INTRODUCTION

78 Resting respiratory rhythmogenesis in mammals is suggested to emerge primarily from  
79 an inspiratory oscillator located in the medullary respiratory group, which contains neurons that  
80 are sufficient to produce rhythmic inspiratory activity *in vitro* (Anderson et al. 2016; Smith et al.  
81 1991). However, generation of the functionally relevant respiratory rhythm and pattern seen *in*  
82 *vivo* requires interactions between the inspiratory oscillator with other respiratory regions (Smith  
83 et al. 2007). Accumulating evidence supports the notion that descending inputs from the pontine  
84 respiratory group, historically identified as the pneumotaxic center of the brain (Lumsden 1923),  
85 are important for generation of eupneic breathing. Within this region, the Kölliker-fuse nucleus  
86 (KF) has been identified as an essential region to gate the post-inspiratory (post-I) phase and  
87 control I-to-E phase transitions (Dutschmann and Dick 2012; Molkov et al. 2013; Morschel and  
88 Dutschmann 2009). The KF contains neurons that provide mainly excitatory synaptic inputs to  
89 medullary respiratory regions (Ezure and Tanaka 2006; Geerling et al. 2017; Rosin et al. 2006).  
90 Ablation of KF inputs prolongs inspiratory duration and produces apneusis (Bautista and  
91 Dutschmann 2014; Harris and Milsom 2003; Molkov et al. 2013; Morrison et al. 1994; St-John  
92 and Paton 2004), indicating a significant role of the KF neurons in breathing rhythm generation.  
93 Experimental evidence also highlights the role of the KF in the formation of the cranial  
94 respiratory motor activity that controls tongue and laryngeal musculature. Microinjection of  
95 excitatory amino acid agonists into the KF parenchyma promote tonic excitation of post-I motor  
96 activity in the vagus/laryngeal recurrent nerve, increase laryngeal constrictor activity, prolong  
97 expiratory phase duration and promote swallowing-related resetting of the respiratory cycle  
98 (Abdala et al. 2016; Bonis et al. 2013; Dutschmann and Herbert 2006). In contrast,  
99 pharmacological inhibition of the KF eliminates the post-I component of vagus nerve activity,  
100 diminishes hypoglossal nerve and genioglossal activities and reduces upper airway resistance  
101 (Dutschmann and Herbert 2006; Levitt et al. 2015; Silva et al. 2016a). These studies suggest that  
102 KF neurons also interact with the medullary pre-motor respiratory neurons that control upper  
103 airway patency during eupnea or oropharyngeal reflexes.

104 Under conditions of elevated metabolic demand, such as elevated partial pressure of CO<sub>2</sub>  
105 (hypercapnia) or low O<sub>2</sub> (hypoxia) in the blood, an active expiratory pattern emerges to support  
106 pulmonary hyperventilation (Jenkin and Milsom 2014; Lemes and Zoccal 2014). A distinct  
107 feature of active expiration is the generation of rhythmical bursts of abdominal expiratory

108 activity during the late  $\frac{1}{3}$  of expiration (aka late-E or E2-phase) (Moraes et al. 2014; Pagliardini  
109 et al. 2011). In juvenile/adult animals, the generation of abdominal activity relies on the  
110 activation of neurons located in the parafacial respiratory group (pFRG), which are suggested to  
111 comprise a conditional expiratory oscillator (Abdala et al. 2009; Janczewski and Feldman 2006;  
112 Marina et al. 2010; Molkov et al. 2014). Under resting conditions (normoxia and eucapnia), it is  
113 suggested that the expiratory pFRG oscillator is synaptically suppressed (de Britto and Moraes  
114 2017; Molkov et al. 2010; Pagliardini et al. 2011; Rubin et al. 2011). During hypercapnia or  
115 hypoxia, activation of central and peripheral chemoreceptors brings about rhythmic incrementing  
116 bursts in a subpopulation of pFRG neurons, which occur during the late third of the expiratory  
117 period (late-E) and correlate with contractions in abdominal expiratory muscles and active  
118 exhalation (Abbott et al. 2011; Abdala et al. 2009; Barnett et al. 2017; Marina et al. 2010;  
119 Molkov et al. 2011b; Moraes et al. 2012; Silva et al. 2016b). Interestingly, peripheral and central  
120 chemoreceptors are suggested to provide tonic excitatory inputs to pontine-medullary respiratory  
121 neurons (de Britto and Moraes 2017; Mifflin 1990; Moreira et al. 2007), indicating that synaptic  
122 excitatory and inhibitory interactions between the pFRG expiratory oscillator and the respiratory  
123 central pattern generator (CPG) are necessary for abdominal pattern formation.

124 Experiments showing that ponto-medullary transection prevented the emergence of  
125 expiratory bursts in abdominal motor nerves during hypercapnia (Abdala et al. 2009) provide  
126 evidence for the involvement of the pons in the control of active expiratory motor activity. We  
127 recently verified that microinjections of a GABA<sub>A</sub> receptor agonist into the KF parenchyma  
128 caused an earlier onset of hypercapnia-induced late-E abdominal activity (Jenkin et al. 2017).  
129 These data suggest an inhibitory influence for the KF neurons on the pFRG oscillator, regulating  
130 the timing of evoked expiratory bursts in abdominal activity in conditions of metabolic  
131 challenge. Although reciprocal connections have been found between the KF and the pFRG  
132 regions (Rosin et al. 2006; Silva et al. 2016a), it has been reported that the KF projections to  
133 ventromedullary neurons, including those activated during hypercapnia, are predominantly  
134 excitatory (Geerling et al. 2017; Yokota et al. 2015) and that local inhibitory neurons are sparse  
135 (Abdala et al. 2016; Guthmann et al. 1998). These experimental observations suggest that the  
136 inhibitory effect of KF neurons on the pFRG oscillator are indirect.

137 In light of these observations, in the present study we aimed to further investigate the  
138 inhibitory role of KF neurons in the generation of active abdominal expiratory activity.

139 Specifically, we tested the prediction that artificially increased activity of KF neurons during  
140 hypercapnia would cause a suppression of evoked late-E abdominal motor output. The data  
141 obtained in this study were combined with our previous experimental findings (Jenkin et al.  
142 2017) were used to extend a previous computational model of the respiratory central pattern  
143 generator (CPG) (Molkov et al. 2010; Rybak et al. 2007), then generating a hypothetical model  
144 that could provide mechanist insight of how the KF may interact with the respiratory circuitry in  
145 the medulla to control late-expiratory abdominal activity during hypercapnia.

146

## 147 **METHODS**

148

### 149 *Experimental data*

150 Part of the data used here, which relates to pharmacological inhibition of the KF, was  
151 extracted from our recently published study (Jenkin et al. 2017) and was re-analyzed in the  
152 present paper to be included in the model. The new experimental data presented here were  
153 collected as described below.

154

### 155 **Animals**

156 The experimental procedures comply with the guidelines of the National Institutes of  
157 Health (NIH, publication no. 85-23, 1996) and of the Brazilian National Council for Animal  
158 Experimentation Control (CONCEA); and were performed according to the UK Home Office's  
159 Animals (Scientific Procedures) Act (1986) and approved by the University of Bristol Animal  
160 Welfare and Ethical Review Body. Juvenile male Wistar rats (n=6; P21-25, 50-60 g) were  
161 housed with free access to rat chow and water, under controlled conditions of temperature (22 ±1  
162 °C), humidity (50-60%) and light/dark cycle (12:12 lights on at 07:00 am).

163

### 164 *In situ* decerebrate arterially perfused rats

165 *In situ* decerebrate arterially perfused rats (Paton, 1996) were surgically prepared as  
166 previously described (Zoccal et al., 2008). Briefly, rats were heparinized (1000 UI.P.) and  
167 subsequently anaesthetized deeply with halothane until the paw and tail pinch reflexes were  
168 abolished, transected below the diaphragm and submerged in a cold Ringer solution (in mm:  
169 NaCl, 125; NaHCO<sub>3</sub>, 24; KCl, 3.75; CaCl<sub>2</sub>, 2.5; MgSO<sub>4</sub>, 1.25; KH<sub>2</sub>PO<sub>4</sub>, 1.25; dextrose, 10).

170 They were decerebrated (precollicularly) and the cerebellum was removed to expose the IV  
171 ventricle and inferior colliculus. To measure inspiratory motor output, the lungs were removed  
172 the left phrenic nerve was cut distally, and recorded using a bipolar suction electrode. To  
173 measure motor output to laryngeal abductor and adductor muscles, the left vagus nerve (cVN)  
174 was isolated and cut at the cervical level (below the bifurcation of the common carotid artery).  
175 To measure output to abdominal muscles, nerves from the right lumbar plexus at thoracic–  
176 lumbar level (T12-L1) were dissected and cut distally, and are referred to as abdominal nerve  
177 (AbN). Preparations were then transferred to a recording chamber; the descending aorta was  
178 cannulated and perfused retrogradely (21-24 mL.min<sup>-1</sup>; Watson-Marlow 502s, Falmouth,  
179 Cornwall, UK), via a double-lumen cannula, with Ringer solution containing 1.25%  
180 Polyethylene glycol (an oncotic agent, Sigma, St Louis, USA) and vecuronium bromide (a  
181 neuromuscular blocker, 3-4 µg.mL<sup>-1</sup>). The perfusion pressure was held within 55–75 mmHg by  
182 adding vasopressin (0.5 nM, Sigma, St. Louis, MO, USA) to the perfusate. The perfusate was  
183 continuously gassed with 5% CO<sub>2</sub> and 95% O<sub>2</sub> (pH 7.4), warmed to 31–32°C and filtered using a  
184 nylon mesh (25 µm). Arterial perfusion pressure was recorded using a Gould transducer and  
185 amplifier (Series 6600). Bioelectric signals were amplified (×10,000), band-pass filtered (0.3–5  
186 kHz) (AC Amplifier Model 1700, A-M Systems, Sequim, WA, USA) and recorded using an  
187 ADC signal conditioner (10 kHz; Micro1401, Cambridge Electronic Design, Cambridge, UK).

188

### 189 **Brainstem microinjections**

190 Microinjections were performed using custom-made, three-barrel glass micropipettes  
191 (borosilicate, OD 1.5 mm, ID 0.86 mm, Harvard Apparatus, UK) filled with L-glutamate (10  
192 mM, Sigma-Aldrich, UK), gabazine (a GABA<sub>A</sub> receptor antagonist, 0.1-1 mM, Sigma-Aldrich)  
193 and 2% Evans blue dye (Sigma-Aldrich, UK). All drugs were dissolved in artificial cerebrospinal  
194 fluid (aCSF) and adjusted to pH 7.4 when needed. The micropipette tips were positioned 0.3–0.5  
195 caudal to the inferior colliculus, 1.9–2.1 mm from the midline and 1–1.5 mm of the dorsal  
196 surface, as previously described (Abdala et al. 2016). The location of the microinjections was  
197 aided with the use of a surgical binocular microscope and the injection volumes (60 nl)  
198 controlled using a pre-calibrated eyepiece reticule. The right and left KF were functionally  
199 identified with unilateral glutamate microinjections, which evoked PN burst inhibition and  
200 prolonged cVN post-I activity (Dutschmann and Herbert 2006). The left and right-side



201 identifications were performed in random order, and a time interval of 5 min was allowed  
202 between consecutive glutamate microinjections. After a recovery period of at least 10 min, the  
203 KF was pharmacologically disinhibited bilaterally through microinjections of gabazine (Mandel  
204 and Schreihofer 2009). The contralateral injection of gabazine was always performed within 1-2  
205 min of the first injection. After the experiments, microinjection sites were marked with Evans  
206 blue dye and verified histologically *post hoc*. Brainstems were removed and fixed in 4%  
207 paraformaldehyde, cryoprotected in 30% sucrose overnight and sectioned in a freezing  
208 microtome (40  $\mu$ m). Sections were Nissl counterstained (2% Neutral Red) and mounted with  
209 DPX (Sigma-Aldrich, UK). Microinjection sites were photographed and documented on  
210 schematic outlines of the dorsolateral pons (Paxinos and Watson 2007).

211

### 212 **Hypercapnic stimulus**

213 After stabilization and initial baseline recordings, *in situ* preparations were exposed to  
214 hypercapnia by raising the fractional concentration of CO<sub>2</sub> in the perfusate from 5 to 8-10%  
215 (balanced with O<sub>2</sub>) for 5 min to generate active expiration (Abdala et al. 2009; Molkov et al.  
216 2011b). The effects peaked and reached steady-state after 3 min of exposure. The stimulus was  
217 applied before and after bilateral microinjections of gabazine into the KF.

218

### 219 **Data analyses**

220 Analyses were performed on rectified and smoothed signals (time constant of 50 ms)  
221 using custom written subroutines in Spike 7.10 software (Cambridge Electronic Design). PN  
222 burst frequency was calculated in cycles per minute (cpm). The coefficient of variation (CoV) of  
223 PN burst frequency was calculated as an indicator of respiratory cycle variability. The duration  
224 of the cVN post-inspiratory component (decrementing activity during the expiratory phase) was  
225 expressed as a percentage of total expiratory time (i.e. inter PN burst interval). To quantify AbN  
226 activity during eucapnia, each individual expiratory cycle was divided into its initial  $\frac{2}{3}$ , which  
227 was dominated by the post-I phase (also known as E1), and the final  $\frac{1}{3}$  which predominantly  
228 corresponded to the late-E phase (also known as E2). The maximum amplitudes above baseline  
229 of AbN output occurring during the first  $\frac{2}{3}$  and late  $\frac{1}{3}$  of each individual expiratory cycle were  
230 measured and averaged over 15-25 cycles (1 min epoch), and expressed in  $\mu$ V. As described  
231 previously by Abdala et al. (2009), high amplitude incrementing AbN bursts during the final  $\frac{1}{3}$

232 of expiration, referred to as AbN late-E bursts, only emerge in conditions of hypercapnia and/or  
233 hypoxia, and displayed quantal skyping in relation to PN cycles. To quantify these, a threshold  
234 crossing was defined, which was typically set at 75% of the maximum amplitude of AbN activity  
235 during the final  $\frac{1}{3}$  of expiration under a hypercapnia challenge. As previously described (Abdala  
236 et al. 2009), in order to accommodate it, the occurrence of a high amplitude AbN late-E burst in  
237 a given cycle during hypercapnia coincides with a truncated cVN post-I cycle. To quantify this  
238 phenomenon during hypercapnia, we subtracted the length of post-I cycles without AbN late-E  
239 bursts (non-truncated) from cycles with late-E bursts (truncated), and this is referred to as post-I  
240 truncation expressed as  $\Delta PIt$  (ms).

241 All results are reported as mean  $\pm$  standard errors of the means. The normal distribution  
242 of the data was verified using a D'Agostino & Pearson normality test and comparisons were  
243 performed using paired nonparametric Wilcoxon t-test. GraphPad Prism (version 6) was used for  
244 statistical analyses and differences were considered significant when  $P < 0.05$ .

245

## 246 **Computational Methods**

247 The model presented here is an extension of a previous model, which described  
248 sensitization of central chemoreceptors (Molkov et al. 2011b). The Molkov et al. (2011b) work  
249 drew from Molkov et al. (2010), which investigated the recruitment of the late expiratory  
250 oscillator in the pFRG for active expiration. Both models incorporate the network architecture of  
251 previous models (Rybak et al. 2007; Smith et al. 2007) inspired by ultra-precise pontine and  
252 medullary transection studies. As in our previous models, the respiratory neurons were classified  
253 according to their firing pattern (augmenting or decrementing) and phase (inspiratory or  
254 expiratory) relative to the phrenic cycle, as recorded experimentally (Abdala et al. 2009; Orem  
255 and Trotter 1992; Paton 1996; Rybak et al. 1997): early-inspiratory (early-I); ramping inspiratory  
256 (ramp-I); pre-inspiratory (pre-I); post-inspiratory (post-I), augmenting expiratory (aug- E) and  
257 late-expiratory (late-E) neurons. Neuronal populations were composed of either 20 or 50 single  
258 compartment neuron models described in the Hodgkin-Huxley formalism. Synaptic projections  
259 from one population to another were all-to-all, and excitatory projections from drive elements  
260 synapsed onto every postsynaptic neuron. The motoneuron output (phrenic nerve, vagus nerve,  
261 and abdominal nerve) was computed by integrating the excitatory inputs. The connectivity of this  
262 model is detailed in Table 1.

263 *Distinction from previous models*

264 In contrast to previous models, a hyperpolarization-activated cationic current ( $I_h$ ) was  
265 included in the post-I (e) (BötC) population to facilitate adaptation in the post-I component of the  
266 cVN motoneuron output. This current followed the Hodgkin-Huxley formalism; its contribution  
267 to the current-balance equation was  $I_h = \bar{g}_h m_h (V - E_h)$ , where  $V$  was the membrane potential,  
268  $m_h$  was the activation variable, and the expression  $\bar{g}_h m_h$  determined the instantaneous  
269 conductance of the current. The values of its biophysical parameters were adapted from the  
270 description in McCormick and Pape (1990). The maximal conductance ( $\bar{g}_h$ ) was 2 nS and the  
271 reversal potential ( $E_h$ ) was -43 mV. The activation of  $I_h$  was determined by the equation:

272 
$$\frac{dm_h}{dt} = \frac{f(V) - m_h}{\tau(V)}$$

273 where the functions for steady state activation  $f(V)$  and time constant  $\tau(V)$  take the following  
274 form:

275 
$$f(V) = (1 + \exp([V + 60]/5.5))^{-1} \text{ and}$$

276 
$$\tau(V) = 1500/\cosh([V + 80]/13) \text{ ms.}$$

277

278 Numerical simulations were performed using NSM 3.0 software, which was developed  
279 by S. Markin, I. Rybak, and N. Shevtsova at Drexel University (Rybak et al. 2007; Rybak et al.  
280 2004). It was extended to use OpenMPI for high-performance computing clusters by Y. Molkov  
281 (Molkov et al. 2011b). Solutions to ordinary differential equations were computed using the  
282 exponential Euler method for integration with a time step of 0.1 ms.

283

284 *Model adjustments*

285 The response to hypercapnia in the model is mediated by an increase in a tonic drive  
286 attributed to chemosensitive neurons in the RTN. The targets of this drive are the pFRG late-E  
287 population and the pre-I/I population of the pre-BötC. The latter population, in turn, could also  
288 excite late-E populations in the pFRG and contribute to the generation of late-E activity (Barnett  
289 et al. 2017). The gain of this drive with changing CO<sub>2</sub> was set to support late expiratory activity  
290 in the late-E population of the pFRG. We also considered that, for pattern formation, pFRG late-  
291 E neurons receive phasic inhibition during early-inspiration and post-inspiration from the

292 respiratory CPG (Abdala et al. 2009; Molkov et al. 2010; Rubin et al. 2011), which in turn is  
293 excited by a tonic drive from the pons (Rybak et al. 2007; Smith et al. 2007).

294 Anatomical (Gang et al. 1995) and electrophysiological studies (Ezure and Tanaka 2006)  
295 indicate the existence of synaptic projections from the KF to the BötC region. Based on these  
296 observations, and on studies showing that the majority of projections from the KF to lower  
297 brainstem respiratory groups are glutamatergic (Geerling et al. 2017), we proposed that an  
298 inhibitory post-I population of BötC neurons are under direct control by a KF population, which  
299 we model as a tonic excitatory drive (highlighted in Figure 5). Under eupneic conditions, the KF-  
300 driven post-I population of the BötC inhibits both the RTN/pFRG expiratory population and the  
301 pre-I/I population. This inhibition suppresses late expiratory activity in the RTN/pFRG and  
302 contributes to the timing of the onset of inspiration (Figure 5). Our data (this study and Jenkin et  
303 al., 2017) demonstrate that mild inhibition and disinhibition of the KF nucleus modulates the  
304 emergence of late-E activity during hypercapnia, without significantly disrupting eucapnic  
305 respiration. Thus, we reasoned that drive from the KF should also be distributed among both  
306 inspiratory and expiratory populations (Figure 5), and inhibition or disinhibition of the KF  
307 nucleus should result in relatively balanced changes in drive to inspiratory and expiratory  
308 populations of the respiratory central pattern generator. In previous iterations of the model  
309 (Barnett et al. 2017; Molkov et al. 2010), a component of the tonic drive to the early-I population  
310 of the pre-BötC was attributed to the pontine nuclei. Therefore, in the model of the present study,  
311 we attributed a portion of the pontine drive received by the early-I population of the pre-BötC to  
312 the KF (highlighted in Figure 5). For the purposes of simulation, inhibition of the KF was  
313 interpreted as a decrease in the excitatory drive by 35%, and disinhibition of the KF was  
314 interpreted as an increase in the excitatory drive by 30%. These percentage changes in excitatory  
315 conductance of the neurons receiving the KF drive were the values that best reproduced our  
316 experimental data. The remaining pontine drive was separate and independent from the KF drive  
317 and was invariant during increases or decreases to the KF drive.

318 We also adjusted network topology to promote pre-inspiratory and late-E activity that is  
319 distinct in time from inspiratory activity. In Molkov et al. (2010) and Molkov et al. (2011b), the  
320 late-E pFRG population stimulated the pre-I/I population. This projection promoted entrainment  
321 of pre-I/I activity to the pFRG expiratory oscillator. In Barnett et al. (2017), an excitatory  
322 projection from the pre-I/I population to the late-E (pFRG) population was included so that an

323 increased excitability of the pre-I/I population could lower the threshold for emergence of late-  
324 expiratory activity. Here, we removed the excitatory projection from the late-E (pFRG)  
325 population to the pre-I/I population. Excitation from the pre-I/I to the early-I population of the  
326 pre-BötC was diminished, and a component of the phasic excitation to the early-I population was  
327 replaced by a tonic drive, which is sufficient to support an inspiratory burst at the termination of  
328 expiration. We also incorporated an excitatory projection from the late-E pFRG expiratory  
329 population into the respiratory CPG that excited the augmenting expiratory (aug-E) population of  
330 the BötC. In the event of a burst in the RTN/pFRG expiratory oscillator, increased input to aug-E  
331 serves to prevent inspiratory burst initiation during the late-E burst in the pFRG, in agreement  
332 with previous experimental observations (Abdala et al. 2009).

333

## 334 **RESULTS**

### 335 **Anatomical and functional identification of microinjections sites in the KF**

336 Unilateral microinjections of glutamate in the KF increased post-I activity in the cVN,  
337 prolonged expiratory time and reduced PN burst frequency, as illustrated in Figure 1A.  
338 Stimulation of the KF with glutamate did not alter the magnitude of resting AbN activity (Figure  
339 1A). *Post-hoc* histological analyses confirmed that the microinjection sites were in the  
340 boundaries of the KF (Figure 1B), between -8.64 and -8.88 mm in relation to Bregma (Figure  
341 1C) (Paxinos and Watson 2007).

342

### 343 **Baseline respiratory changes after disinhibition of the KF**

344 Under resting conditions (eucapnia), the PN burst showed a ramping pattern of discharge,  
345 the cVN presented an inspiratory component (coincident with the PN burst) followed by post-I  
346 decremting activity, and the AbN exhibited low-amplitude activity (relative to hypercapnia)  
347 during the expiratory phase, which was typically decremting during the first  $\frac{2}{3}$  of expiration.  
348 One preparation also had low amplitude incremting AbN activity during the final  $\frac{1}{3}$  of  
349 expiration. Bilateral microinjections of low doses of gabazine in the KF caused a small but  
350 significant increase in the duration of post-I activity in the cVN ( $68\pm 7$  vs  $59\pm 6$  % of expiratory  
351 time,  $P<0.05$ , Figure 2A, B). This effect was accompanied by an increase in PN burst frequency  
352 variability (CoV:  $0.36\pm 0.10$  vs  $0.15\pm 0.04$ ,  $P<0.05$ , Figure 2C), with no changes in: PN burst  
353 frequency ( $23\pm 4$  vs  $21\pm 4$  cpm, Figure 2D), time of inspiration ( $0.662\pm 0.05$  vs  $0.757\pm 0.085$  s)

354 and time of expiration ( $2.72 \pm 0.41$  vs  $2.96 \pm 1.02$  s). Baseline mean AbN activity ( $3.81 \pm 0.72$  vs  
355  $3.96 \pm 0.71$   $\mu$ V, Figure 2E) did not change after gabazine microinjections in the KF.

356

### 357 **Effect of disinhibition of the KF on the generation of late-E bursts during hypercapnia**

358 With the increase in the fractional concentration of CO<sub>2</sub> in the perfusate (8-10%), late-E  
359 bursts emerged in AbN activity (Figure 3) at a relative frequency ratio of 1:2 with the PN burst  
360 (Figure 3A-C) and 1:1 ratio with the PN frequency at 10% CO<sub>2</sub> (Figure 3D-E). Concomitantly,  
361 post-I duration in the cVN was reduced significantly (Figure 4A and 4D). No significant changes  
362 were observed in PN frequency during steady-state hypercapnia. After disinhibition of the KF,  
363 the magnitude of the AbN late-E response to hypercapnia was markedly suppressed (Figure 3F-  
364 H, Figure 4B) ( $\Delta$ :  $8.2 \pm 0.7$  vs  $1.8 \pm 0.9$   $\mu$ V, respectively before and after gabazine,  $P < 0.05$ ; Figure  
365 4C). This lower amplitude of AbN late-E bursts after gabazine microinjections was associated  
366 with a reduced cVN post-I truncation ( $\Delta$ PIt:  $-0.276 \pm 0.070$  vs  $-0.137 \pm 0.069$  s, respectively before  
367 and after gabazine microinjections,  $P < 0.05$ , Figure 4D). No significant changes were noted in the  
368 PN frequency during steady-state hypercapnia before versus after gabazine microinjections in the  
369 KF ( $\Delta$ :  $2 \pm 1$  vs  $4 \pm 2$  cpm, respectively). Sixty minutes after gabazine microinjections, the  
370 magnitude of late-E bursts in AbN ( $\Delta$ :  $5.5 \pm 0.6$   $\mu$ V) and the cVN post-I truncation ( $\Delta$ PIt: -  
371  $0.551 \pm 0.147$  s) evoked by hypercapnia were equivalent to pre-treatment values.

372

### 373 **Proposed mechanisms through model adjustments**

374 We performed a series of computational experiments aimed at proposing a model that  
375 explains the changes to late-E activity in the AbN pattern during active expiration obtained in  
376 both this study as well as in our previous related study (Jenkin et al. 2017). Based on the sum of  
377 the data, we identified the following key features of the motoneuron output during hypercapnia  
378 after pharmacological manipulation of the KF: (1) microinjections of isoguvacine (GABA<sub>A</sub>  
379 receptor agonist) to inhibit the KF during hypercapnia suppressed the post-I component of the  
380 cVN and advanced the onset of late-E activity in the AbN, increasing its burst duration (Jenkin et  
381 al. 2017); (2) disinhibition of the KF during hypercapnia attenuated the shortening in post-I  
382 activity of cVN and reduced AbN late-E activity (present study). Our model was adjusted to  
383 reproduce these findings, as described in the Methods section (*Model adjustments*).

384

385 **Model validation**

386 *Transient activation of KF drive in the model induces apnea.*

387 Application of glutamate in the KF evokes respiratory apnea during which there is  
388 increased post-I activity in the vagus nerve (Figure 2). We reproduced these results by transiently  
389 increasing the KF drive by a factor of 4 (Figure 6). Post-I (BötC) activity increased in amplitude  
390 and became tonic for the duration of the stimulation period; this population strongly inhibited  
391 aug-E (BötC) neuron activity. The excitatory pre-motor post-I (e) (BötC) population is  
392 responsible for the post-inspiratory component of cVN, and it receives strong inhibition from  
393 aug-E (BötC) neurons. During the epoch of increased drive from the KF, post-I (e) (BötC)  
394 activity was disinhibited since aug-E (BötC) neurons did not fire, hence the activity in the cVN  
395 persisted for the duration of the excess KF input.

396

397 *Suppression of KF drive enhances inspiratory phase duration.*

398 Previous studies have reported that inhibition of KF neurons reduces post-inspiratory  
399 vagal activity and enhances inspiratory phase duration, but does not modify baseline abdominal  
400 activity (Bautista and Dutschmann 2014; 2016; Jenkin et al. 2017). Our model simulations,  
401 which compared the activity of pre-BötC, BötC and pFRG populations in eucapnia before  
402 (Figure 7A) and after KF inhibition (Figure 7B), agree with these previous experimental  
403 observations. KF inhibition in eucapnia reduced the overall firing rate of the post-I (BötC)  
404 populations (inhibitory and excitatory) and increased the phase duration and firing frequency of  
405 the pre-I/I (pre-BötC) population (Figure 7B) compared to the control simulation (Figure 7A).  
406 Also, KF inhibition did not modify the activity of the late-E (pFRG) population.

407

408 *The role of KF drive and pre-I excitation in active expiration*

409 In our previous models, the emergence of active expiration during hypercapnia was  
410 dependent on an increase in the chemosensory drive from the RTN (Molkov et al. 2010; Molkov  
411 et al. 2011b). The same mechanism was implemented in this model. However, the late-E (pFRG)  
412 population also received increased excitatory input from the pre-I/I (pre-BötC) population during  
413 the E2 phase. To investigate the role of pre-I/I (pre-BötC) activity in the emergence and  
414 modulation of active expiration, we performed simulations in eucapnia (control simulation;  
415 Figure 7A) and eucapnia with KF inhibition (Figure 7B). KF inhibition in eucapnia reduced the

416 overall firing rate of the post-I (BötC) population and increased the phase duration and firing  
417 frequency of the pre-I/I (pre-BötC) population (Figure 7B) compared to the control simulation  
418 (Figure 7A). Pharmacological disinhibition of the RTN/pFRG was shown to be sufficient to  
419 evoke late-E activity (Molkov et al. 2010; Pagliardini et al. 2011). However, in Jenkin et al.  
420 (2017), application of isoguvacine to the KF during eucapnia failed to evoke active expiration.  
421 Consistently, in these modeling results, reduction of the KF drive to post-I (BötC) populations  
422 created a combination of weak expiratory disinhibition and weak expiratory excitation, which  
423 was not sufficient to evoke late-E activity in accordance with the isoguvacine experiments during  
424 eucapnia.

425

## 426 **Model performance**

### 427 *Suppression of the KF drive increases AbN late-E burst duration by reducing post-I inhibition*

428 We implemented a pharmacological inhibition of the KF with isoguvacine in our  
429 extended model to explain the change in motoneuron output observed in our previous  
430 experimental results (Jenkin et al. 2017). This was simulated by a decrease in the tonic KF drive  
431 to the post-I (BötC) and early-I (pre-BötC) populations, and we refer to this manipulation in  
432 simulation as inhibition of the KF. Our analysis also included a simulation of hypercapnia with  
433 reduced KF drive (motoneuron output in Figure 8A; central pattern generator activity in Figure  
434 9A) and a simulation of the control condition – hypercapnia only (motoneuron output in Figure  
435 8B; central pattern generator activity in Figure 9B). Similar to the rat experiments with  
436 isoguvacine, a partial decrease in the KF drive during hypercapnia did not disrupt the three-phase  
437 respiratory rhythm in the model. Also, the model reproduced the advanced onset and increased  
438 phase duration of the AbN late-E burst in hypercapnia with reduced KF drive (Figure 8A) as  
439 compared to hypercapnia with no additional manipulation to the KF (Figure 8B).

440 In this model, the post-I (BötC) population fires in a decrementing fashion through the  
441 duration of expiration (Figure 9) and is a main source of expiratory inhibition in the respiratory  
442 cycle. The late-E (pFRG) population receives both expiratory and inspiratory inhibition in  
443 eucapnia (Figure 5). During hypercapnia, the increase in tonic chemosensitive drive from the  
444 RTN lowers its threshold for activation. As the firing rate of the post-I (BötC) population  
445 decrements to a critical value, inhibition of late-E (pFRG) neurons decreases such that the  
446 population fires at the end of expiration (Figure 9B). In the simulation including both reduced



447 KF drive and hypercapnia, the average firing rate of post-I (BötC) neurons is reduced. As such,  
448 late-E (pFRG) neurons reach their threshold for activation earlier in the expiratory phase (Figure  
449 9A) and the duration of late-E bursts extends over a longer interval at the end of expiration.

450 In Jenkin et al. (2017), inhibition of the KF during hypercapnia decreased the amplitude  
451 of post-I activity in the cVN. In the current model, the excitatory post-I (e) (BötC) population  
452 shapes the post-inspiratory component of the cVN activity (Figure 5). This population receives  
453 strong inhibition from aug-E neurons (BötC); this inhibition determines the phase duration of  
454 post-I (e) (BötC) activity and, hence, the phase duration of the post-inspiratory component of  
455 cVN activity. The onset time of aug-E activity (BötC) in the respiratory cycle is determined by  
456 the inhibition that it receives from the inhibitory post-I neurons (BötC). This inhibition is  
457 gradually removed as post-I activity decrements and aug-E activity is eventually released. In the  
458 simulation of hypercapnia and KF inhibition, the average firing rate of post-I neurons (BötC) is  
459 decreased and aug-E (BötC) activity is recruited earlier in the expiratory phase (Figure 9A;  
460 Figure 10) compared to the hypercapnia simulation (Figure 9B; Figure 10). In this way, the  
461 phase duration of post-I (e) (BötC) and the post-inspiratory component of the cVN activity is  
462 reduced through disinhibition of aug-E neurons.

463

464 *Amplification of post-I inhibition abolishes AbN late-E activity.*

465 The effect of microinjections of gabazine in the KF was reproduced in the current  
466 extended model by increasing the KF drive to post-I (BötC) and early-I (BötC) neurons, and we  
467 refer to this manipulation as disinhibition of the KF. It is important to note that our model  
468 simulations reproduced the effects caused by the doses of gabazine used in the present  
469 experimental study, which did not disrupt the three-phase breathing pattern and differ from  
470 results of another study that used higher concentrations of GABA<sub>A</sub> receptor antagonists (Abdala  
471 et al. 2016). The simulation of hypercapnia and KF disinhibition (Figure 8C; Figure 9C)  
472 compliments the simulation of hypercapnia and KF inhibition (Figure 8A; Figure 9A) and the  
473 simulation of hypercapnia alone (control condition; Figure 8B; Figure 9B). In our simulations,  
474 the model reproduced the suppression in the AbN motoneuron output after KF disinhibition  
475 (Figure 8C). We attributed the suppression of active expiration to the increase in the tonic KF  
476 drive to the inhibitory post-I neurons (BötC). The average firing rate of the post-I (BötC)  
477 population increased during simulation of hypercapnia and disinhibition of the KF (Figure 9C)

478 compared to the simulation of hypercapnia without perturbation to the KF drive (Figure 9B). The  
479 increase in firing rate of the post-I population (BötC) lead to an overall increase in inhibition of  
480 late-E neurons (pFRG) during expiration such that late-E activity (pFRG) could not be activated  
481 despite increased chemosensitive drive from the RTN.

482 The post-I (e) (BötC) population increased its firing duration (Figure 9C). An increase in  
483 average firing rate of the post-I (BötC) population distributed more inhibition to its post-synaptic  
484 targets. This increase in inhibition is offset in the early-I population by the increase in tonic KF  
485 drive, but there is no mechanism to compensate for the increase in inhibition to aug-E (BötC)  
486 neurons. The onset time of the E2 burst of aug-E (BötC) neurons was delayed, and, thus, the  
487 duration of post-I (e) (BötC) activity increased (Figure 9C; Figure 10).

488

## 489 **DISCUSSION**

490 Active expiration emerges as a reflex mechanism to increase minute ventilation and  
491 support blood gas homeostasis in states of high metabolic demand (Jenkin and Milsom 2014;  
492 Lemes and Zoccal 2014). An important motor component of the active expiratory pattern is the  
493 development of high-amplitude bursts in abdominal expiratory motor activity, not seen in  
494 eupnea. Under hypercapnic conditions, these abdominal bursts are present during the late-E  
495 phase of the respiratory cycle, in association with reduced upper airway resistance and increased  
496 sympathetic outflow (Abdala et al. 2009; de Britto and Moraes 2017; Molkov et al. 2011a). This  
497 evoked late-E discharge in AbN activity reported in the decerebrate *in situ* preparation resembles  
498 the pattern recorded in *in vivo* (more intact) preparations (Iizuka and Fregosi 2007; Moraes et al.  
499 2013; Pagliardini et al. 2011), indicating that this pattern formation depends primarily on neural  
500 mechanisms within the brainstem. In the present study, we provide novel insights supporting a  
501 conditional inhibitory role of the KF neurons regulating the timing of abdominal late-E bursts in  
502 conditions of high chemosensory drive. Based on experimental data, we present modeling  
503 simulations elucidating possible interactions between the KF and the pFRG, through the BötC,  
504 which might help to explain, at least in part, the inhibitory effect of KF inputs on the threshold  
505 for activation of the expiratory oscillator.

506 The generation of active expiration has been attributed to a conditional expiratory  
507 oscillator located in the pFRG (Janczewski and Feldman 2006), whose activity is suggested to be  
508 defined by a balance between excitatory and inhibitory inputs (Marina et al. 2010; Molkov et al.

509 2010; Moraes et al. 2012; Pagliardini et al. 2011; Rubin et al. 2011). Under normoxic/eucapnic  
510 conditions, the expiratory neurons of the pFRG are tonically suppressed by inhibitory synapses  
511 (Pagliardini et al. 2011). During hypercapnia or hypoxia, this oscillator is activated due to  
512 increased excitation from central and peripheral chemoreceptors (Marina et al. 2010; Moraes et  
513 al. 2012) and reduction in the inhibitory drive to the pFRG (Abdala et al. 2009; de Britto and  
514 Moraes 2017). Under eucapnic normoxic conditions, activation and disinhibition of the KF with  
515 microinjections of glutamate and gabazine, respectively, did not modify abdominal activity. This  
516 finding agrees with previous studies demonstrating that baseline abdominal activity did not  
517 change after inhibition of the KF, despite significant modifications in phrenic and vagal motor  
518 outputs (Bautista and Dutschmann 2016; Jenkin et al. 2017). These data suggest that, under  
519 resting conditions, the KF inputs to the respiratory CPG are important for three-phase respiratory  
520 pattern formation, contributing to the inspiratory off-switch and the control of inspiratory-  
521 expiratory phase transition (Dutschmann et al. 2014), but do not influence the generation of  
522 abdominal activity and the activity of pFRG expiratory neurons.

523 On the other hand, pharmacological manipulations of the KF during hypercapnia revealed  
524 that changes in KF drive to the respiratory CPG affect the emergence and timing of the AbN  
525 late-E activity in conditions of elevated chemosensitive drive. We previously demonstrated in  
526 rats that inhibition of the KF with isoguvacine reduced baseline post-I vagal activity and  
527 advanced the onset of late-E abdominal bursts in the expiratory phase (Jenkin et al. 2017).  
528 Herein, we report that KF disinhibition with low doses of gabazine (100  $\mu$ M) during hypercapnia  
529 generate a modest increase in baseline post-I activity in the vagus nerve, but markedly attenuate  
530 the emergence of late-E bursts in the abdominal motor output. In these experiments, we sought to  
531 promote a moderate disinhibition of the KF by using lower doses of a GABA<sub>A</sub> receptor  
532 antagonist [100  $\mu$ M of gabazine versus 5 mM of bicuculline in Abdala et al. (2016)] to avoid  
533 excessive KF overactivity and the generation of exaggerated post-I bursts in vagus nerve activity  
534 associated with periods of apnea, as described previously (Abdala et al. 2016). These findings  
535 reveal a conditional inhibitory role of the KF that effectively controls the timing and generation  
536 of abdominal bursts during hypercapnia. Teppema et al. (1997) reported an increase in fos  
537 expression at the region of the KF after hypercapnia exposure. Interestingly, most of  
538 hypercapnia-activated KF neurons that send projections to respiratory neurons in the lower  
539 brainstem and spinal cord are glutamatergic (Yokota et al. 2015). Based on this, we hypothesize

540 that the KF impedes the emergence of late expiratory activity through an indirect inhibitory  
541 pathway that controls the pFRG neuronal activity. The recruitment of this KF-driven pathway  
542 during hypercapnia might be important for promoting phasic inhibition of expiratory neurons in  
543 the pFRG, then contributing to late-expiratory pattern formation. Interestingly, modifications in  
544 the strength of this pathway may modulate the active expiratory pattern, as seen during sleep-  
545 wake cycles (Andrews and Pagliardini 2015; Leirao et al. 2017).

546 We consider the possibility that the KF controls the threshold for activation of the late-E  
547 (pFRG) neuronal population and the emergence of late-E activity in the AbN through inhibition  
548 from post-I (BötC) neurons. This assumption was based on the following experimental  
549 observations: i) projections from the KF to the other pontine and medullary regions that control  
550 respiratory function are predominantly excitatory (glutamatergic) (Geerling et al. (2017); ii) we  
551 found an inverse relationship between post-I and late-E motor activities (present study and  
552 Jenkin et al., 2017); iii) post-I neurons of the BötC are suggested as an important source of  
553 inhibition to the pFRG (Molkov et al. 2010); iv) the activity of post-I neurons in the BötC  
554 requires pontine drive (Smith et al., 2007); and v) studies using retrograde labeling (Gang et al.  
555 1995) and antidromic stimulation (Ezure and Tanaka 2006) suggest the existence of projections  
556 from the KF to the BötC region. This hypothesis was incorporated and tested in our model of  
557 respiratory CPG. In this model, the KF provided an excitatory drive to inhibitory post-I neurons  
558 in the BötC, which, in turn, inhibited late-E neurons in the pFRG. This KF-driven post-I  
559 population in the BötC also established inhibitory connections with inspiratory population in the  
560 pre-BötC. In model simulations, mild KF inhibition during hypercapnia led to suppression of  
561 post-I (BötC) activity which reduced the threshold necessary for the activation of late-E (pFRG)  
562 neurons, thus advancing the breakthrough of late-E activity and prolonging its burst duration. In  
563 the simulation of mild KF disinhibition during hypercapnia, the enhanced post-I (BötC) activity  
564 did not decrement enough to release late-E (pFRG) activity. Hence, late-E bursts did not appear  
565 in the AbN. Therefore, our modeling simulations suggest a potential mechanism by which KF  
566 inputs may determine the intensity of post-I (BötC) discharge, which, in turn, finely controls the  
567 emergence of AbN late-E activity and its duration during hypercapnia. This theoretical  
568 mechanism still awaits experimental confirmation but parallels previous studies showing that  
569 more extreme reductions in the pontine drive to post-I (BötC) neurons, as seen in conditions of

570 ischemia, generate late-E/post-I biphasic abdominal bursts (Abdala et al. 2009; Molkov et al.  
571 2010; Rubin et al. 2011).

572 Previously, we investigated the role of phasic ponto-medullary interactions in models that  
573 included pulmonary feedback (Molkov et al. 2013; Rybak et al. 2004). Based on these  
574 interactions, it is safe to assume that, qualitatively, the variation of excitability of pontine  
575 neurons would give rise to variation in the tonic drive to the populations they project to.  
576 Therefore, in the present study, for simplicity, we implemented the contributions of the pontine  
577 nuclei as a variable tonic excitatory drive. This allowed us to make some generic assumptions  
578 about the effect of the pharmacological manipulations delivered by isoguvacine and gabazine  
579 without addressing specific cell-types in the pons. Those assumptions were: (1) there is a tonic  
580 pontine drive that is invariant in the face of these manipulations: and (2) there is a tonic pontine  
581 drive that we attribute to the KF whose amplitude is subject to our manipulations. This  
582 simplification also allowed us to formulate a specific hypothesis for the role of KF input to the  
583 CPG in the regulation of the emergence of active expiration.

584 Previous studies demonstrated that the antagonism of GABA<sub>A</sub> and glycinergic receptors  
585 in the pFRG generates abdominal late-E activity under normoxic and eucapnic conditions (de  
586 Britto and Moraes 2017; Molkov et al. 2010; Pagliardini et al. 2011). Our current model supports  
587 the hypothesis that late-E activity is markedly controlled by inhibitory inputs from the  
588 respiratory CPG. In our *in situ* experiments, we observed that KF inhibition did not evoke active  
589 expiration at rest (Jenkin et al. 2017). Although our microinjections may have not inhibited all  
590 neurons in the KF, these data suggest that other sources of inhibitory input to the pFRG may also  
591 contribute to tonic inhibition of the expiratory oscillator. These sources may include inhibitory  
592 projections from the nucleus of the Solitary Tract (NTS) (Takakura et al. 2007).

593 In conclusion, our experimental data indicate that KF-driven inputs to the respiratory  
594 CPG control the presence and onset timing of AbN late-E bursts during exposure to high CO<sub>2</sub>.  
595 Our model simulations propose that this inhibitory effect of the KF on hypercapnia-induced AbN  
596 activity is indirect and involves connections with post-I neurons of the BötC, which, in turn,  
597 inhibit expiratory neurons in the pFRG. Although the proposed model requires further anatomic  
598 and functional validation, our findings are relevant for exploring the mechanisms underpinning  
599 the generation of active expiration in conditions of chronic intermittent (Zoccal et al. 2009;  
600 Zoccal et al. 2008) or sustained hypoxia (Moraes et al. 2014). In these experimental conditions,

601 the emergence of late-E activity at rest is suggested to be associated with depressed post-I BötC  
602 activity (Moraes et al. 2014; Zoccal et al. 2008) and sensitization of chemosensitive neurons in  
603 the RTN (Molkov et al. 2011a). Therefore, the hypoxia-induced reduction of the pontine drive to  
604 the medullary respiratory circuits, causing a reduction in excitability of the post-I (BötC)  
605 population, may promote active expiration in eucapnia by reducing the baseline expiratory  
606 inhibition in late-E. Moreover, hyperactivity of the KF neurons has been suggested as a  
607 mechanism that generates respiratory instabilities in the mouse model of Rett syndrome (Abdala  
608 et al. 2016). During central apneas in *Mecp2*-deficient mice, sustained expiratory activities are  
609 observed in the hypoglossal, vagus and abdominal nerves (Abdala et al. 2010). This mouse  
610 model also exhibits reduced ventilatory responses to hypercapnia (Toward et al. 2013). Although  
611 our current model does not explain the generation of the respiratory phenotype seen in Rett  
612 Syndrome, it certainly forms a platform from which to conceptualize the mechanisms  
613 responsible for the generation of central apneas and respiratory cycle irregularity in multiple  
614 disorders.

615

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620

## 621 **DISCLOSURES**

622 No conflicts of interest are declared by the authors.

623

## 624 **AUTHOR CONTRIBUTION**

625 APA and DBZ conceived and designed the experiments. DBZ performed all the *in situ*  
626 experiments. WHB and YIM designed and performed modeling simulations. APA, DBZ, WHB  
627 and YIM assembled, analyzed and interpreted the data. DBZ, WHB and YIM drafted the article.  
628 APA, DBZ, JFRP, SEMJ, WHB, WKM and YIM revised and edited the article critically for  
629 important intellectual content. All authors have approved the final version of the manuscript.

630

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794 **TABLES**

795

796 **Table 1.** Detailed connectivity of the computational model.

Target Population	Excitatory drive [weight of synaptic input] or presynaptic source population [weight of synaptic input from single neuron]
aug-E (BotC)	Drive (PONS) [0.42] <sup>a</sup> , Drive (RTN) [2.3] <sup>b</sup> , early-I (1) (pre-BötC) [-0.135], late-E (pFRG) [0.03] <sup>b</sup> , post-I (BötC) [-0.3]
early-I (1) (pre-BotC)	Drive (RTN) [2] <sup>a</sup> , Drive (KF) [0.6] <sup>bd</sup> , aug-E (BötC) [-0.265], post-I (BötC) [-0.45], pre-I (pre-BötC) [0.05] <sup>a</sup>
early-I (2) (rVRG)	Drive (PONS) [2.5], aug-E (BötC) [-0.25], post-I (BötC) [-0.5]
late-E (pFRG)	Drive (RTN) [0.225, 0.325] <sup>ac</sup> , early-I (1) (pre-BötC) [-0.05] <sup>a</sup> , late-E (pFRG) [0.024] <sup>a</sup> , post-I (BötC) [-0.0275] <sup>a</sup> , pre-I (pre-BötC) [0.013] <sup>b</sup>
post-I (BotC)	Drive (PONS) [1] <sup>a</sup> , Drive (KF) [0.65] <sup>bd</sup> , early-I (1) (pre-BötC) [-0.025]
post-I (e) (BotC)	Drive (PONS) [0.8] <sup>a</sup> , aug-E (BötC) [-0.3] <sup>a</sup> , early-I (1) (pre-BötC) [-0.2] <sup>a</sup>
pre-I (pre-BotC)	Drive (RTN) [0.198, 0.286] <sup>c</sup> , Drive (PONS) [0.65], Drive (Raphe) [0.15] <sup>a</sup> , aug-E (BötC) [-0.01] <sup>a</sup> , post-I (BötC) [-0.19] <sup>a</sup> , pre-I (pre-BötC) [0.02] <sup>a</sup>
ramp-I (rVRG)	Drive (PONS) [2], aug-E (BötC) [-0.1], early-I (2) (rVRG) [-0.3], post-I (BötC) [-2], pre-I (pre-BötC) [0.12]
	<sup>a</sup> Different value from Molkov et al. (2011b) <sup>b</sup> Not Present in Molkov et al. (2011b) <sup>c</sup> Drive (RTN) [Eucapnia, Hypercapnia] <sup>d</sup> Isoguvacine and Gabazine

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800 **FIGURE LEGENDS**

801

802 **Figure 1. Functional and histological identification of the Kölliker-Fuse.** **A:** Integrated  
803 recordings of abdominal (AbN), phrenic (PN) and cervical vagus (cVN) nerve activities from an  
804 *in situ* rat preparation, representative of the group, illustrating the respiratory responses to  
805 microinjections of glutamate (arrow) in the left (top) and right sides (bottom) of Kölliker-Fuse  
806 (KF). \* represents an artifact generated during the removal of the injection micropipette. **B:**  
807 Photomicrography of coronal section from the brainstem of a representative *in situ* rat  
808 preparation, illustrating the site of microinjection in the KF (arrow). **C:** Schematic  
809 representations of all microinjection sites (black circles) into the KF (n = 6 each side).  
810 Abbreviation: DLL – dorsal nucleus of the lateral lemniscus; scp: superior cerebellar peduncle;  
811 s5 – sensory root of trigeminal nerve; 4V – fourth ventricle.

812

813 **Figure 2. Changes in baseline activities after microinjections of gabazine in the Kölliker-**  
814 **Fuse.** **A:** Raw and integrated recordings of cervical vagus (cVN), phrenic (PN) and abdominal  
815 nerve activities (AbN) from a representative *in situ* rat preparation, illustrating the respiratory  
816 pattern before and after gabazine microinjections in the Kölliker-Fuse (KF). **B-E:** Average  
817 values of cVN post-I duration (normalized by expiratory time), coefficient of variation of PN  
818 burst frequency, mean PN burst frequency and mean abdominal activity, respectively, before and  
819 after gabazine microinjections in the KF. \* - different from baseline, P<0.05, n=6.

820

821 **Figure 3. Disinhibition of the Kölliker-Fuse suppresses the generation of abdominal late-E**  
822 **activity during hypercapnia.** Recordings, from representative *in situ* rat preparations, depict the  
823 activity of central vagus nerve (cVN), phrenic nerve (PN), and abdominal nerve (AbN) in  
824 eucapnia and hypercapnia, pre-treatment (**A-E**) and after gabazine microinjections (**F-J**). All  
825 recordings performed after gabazine microinjections are indicated in the light grey box. Filled  
826 arrows and unfilled arrows in panels A and F indicate the beginning and end of the change in  
827 perfusate CO<sub>2</sub> fractional concentration from 5% to 8%, respectively. Zoomed in traces, **B, D, G**  
828 **and I** depict respective preceding eucapnia epochs; **C and H** depict 8% hypercapnia; and **E and**  
829 **J** depict 10% hypercapnia. Traces **A-C** and **F-H** are from a preparation challenged with 8% CO<sub>2</sub>.  
830 Traces **D, E, I** and **J** come from a separate preparation which was challenged with 10% CO<sub>2</sub>.

831  
832 **Figure 4. Disinhibition of the Kölliker-Fuse prevented the reduction in vagal post-**  
833 **inspiratory activity and restrained the generation of late-E abdominal activity during**  
834 **hypercapnia. A-B:** Superimposed traces of integrated cervical vagus (cVN, gray) and  
835 abdominal nerve activities (AbN, black) during hypercapnia, from a representative *in situ* rat  
836 preparation, before and after gabazine microinjections in the Kölliker-Fuse (KF). The gray box  
837 indicates the inspiratory phase (coincident with phrenic burst) and the arrows indicate the end of  
838 post-inspiratory (post-I) activity in cVN. Note that before gabazine microinjections, the onset of  
839 the late-E burst in AbN during hypercapnia is associated with a clear truncation in post-I activity.  
840 After gabazine microinjections, the amplitude of AbN late-E burst reduced and post-I cVN  
841 activity presented minor changes. **C-D:** Average values of AbN late-E burst amplitude and  
842 variation of cVN post-I duration during hypercapnia, before and after gabazine microinjections  
843 in the KF. \* statistically significantly different from baseline,  $P < 0.05$ ,  $n = 6$ .

844  
845 **Figure 5. Schematic of the brainstem respiratory network model** including the inhibitory  
846 post-inspiratory (post-I), excitatory post-inspiratory (post-I(e)), and augmenting expiratory (aug-  
847 E) populations of the Bötzinger complex (BötC); the pre-inspiratory/inspiratory (pre-I/I) and  
848 early-inspiratory (early-I (1)) populations of the pre-Bötzinger complex (pre-BötC); the ramping  
849 inspiratory (ramp-I) and early-inspiratory [early-I (2)] populations of the rostral ventral  
850 respiratory group (rVRG); and the late-expiratory (late-E) population of the parafacial  
851 respiratory group (pFRG). This model includes excitatory drive elements, which are not modeled  
852 as populations and provide constant excitation to post-synaptic populations: drive from the  
853 pontine nuclei (Drive Pons), drive specifically from the Kölliker-Fuse (Drive KF), and drive  
854 from the retrotrapezoid nucleus (Drive RTN). Excitatory and inhibitory populations of 20 to 50  
855 neurons are respectively depicted as orange and blue elements. Similarly, projections from  
856 excitatory and inhibitory populations are respectively colored orange and blue. Drive elements  
857 and projections associated with these elements are represented as green triangles or green arrows.  
858 The new additions to this model—Drive KF and its projections—are emphasized with black  
859 outline.

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863 **Figure 6. Simulation of the glutamate microinjection into the Kölliker-Fuse** by transient  
864 increase in drive from the Kölliker-Fuse to the respiratory CPG (arrow). The figure shows the  
865 activity of the phrenic nerve (PN) and the central vagus nerve (cVN) as well as the inhibitory  
866 post-inspiratory (post-I), excitatory post-inspiratory (post-I (e)), and augmenting-expiratory (aug-  
867 E) populations of the Bötzing complex (BötC).

868

869 **Figure 7. Simulation of the Kölliker-Fuse inhibition in eucapnia.** Model activity of central  
870 pattern generator populations under (A) eucapnia and (B) KF inhibition. The figure shows the  
871 activities of the pre-inspiratory/inspiratory (pre-I/I) population of the pre-Bötzing complex  
872 (pre-BötC); the inhibitory post-inspiratory (post-I), augmenting expiratory (aug-E), and  
873 excitatory post-inspiratory (post-I (e)) populations of the Bötzing complex (BötC); and the  
874 late-expiratory (late-E) population of the pFRG. The horizontal black line emphasizes the change  
875 in amplitude of the inhibitory post-I population between (A) and (B).

876

877 **Figure 8. Simulation of the Kölliker-Fuse inhibition and disinhibition in hypercapnia.**  
878 Motoneuron output in the model under (A) hypercapnia plus KF inhibition, (B) hypercapnia, and  
879 (C) hypercapnia plus KF excitation each depicting activity of the phrenic nerve (PN), the central  
880 vagus nerve (cVN), and the abdominal nerve (AbN).

881

882 **Figure 9. Simulation of the Kölliker-Fuse inhibition and disinhibition in hypercapnia.**  
883 Model activity of central pattern generator populations under (A) hypercapnia plus KF  
884 inhibition, (B) hypercapnia, and (C) hypercapnia plus KF excitation. The post-I (BötC) traces are  
885 shaded grey for emphasis. The horizontal dashed black line in the post-I (BötC) traces indicate  
886 the approximate threshold for activation of the late-E (pFRG). The vertical dashed grey lines  
887 indicate the phase of activation of late-E (pFRG). The included populations are the pre-  
888 inspiratory/inspiratory (pre-I/I) population of the pre-Bötzing complex (pre-BötC); the  
889 inhibitory post-inspiratory (post-I), augmenting expiratory (aug-E), and excitatory post-  
890 inspiratory (post-I (e)) populations of the Bötzing complex (BötC); and the late-expiratory  
891 (late-E) population of the pFRG.

892

893 **Figure 10. Comparison of post-inspiratory activity among three hypercapnia simulations.**  
894 Activity of the post-I (e) (BötC) determines the post-inspiratory component of the cVN burst.  
895 The vertical grey line in each trace indicates the end of the post-inspiratory burst.

896 **FIGURES**

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898 **Figure 1**

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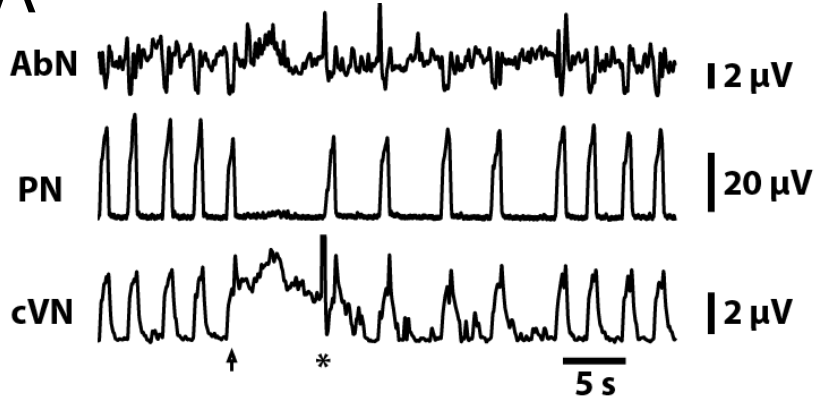
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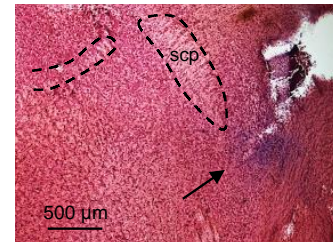
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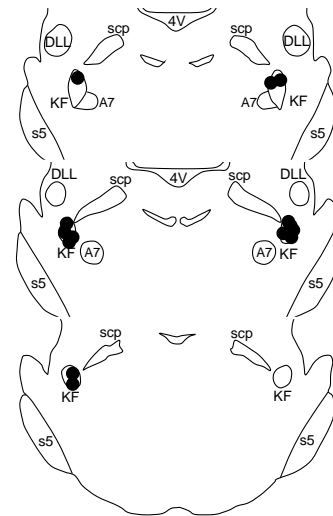
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931 **Figure 2**

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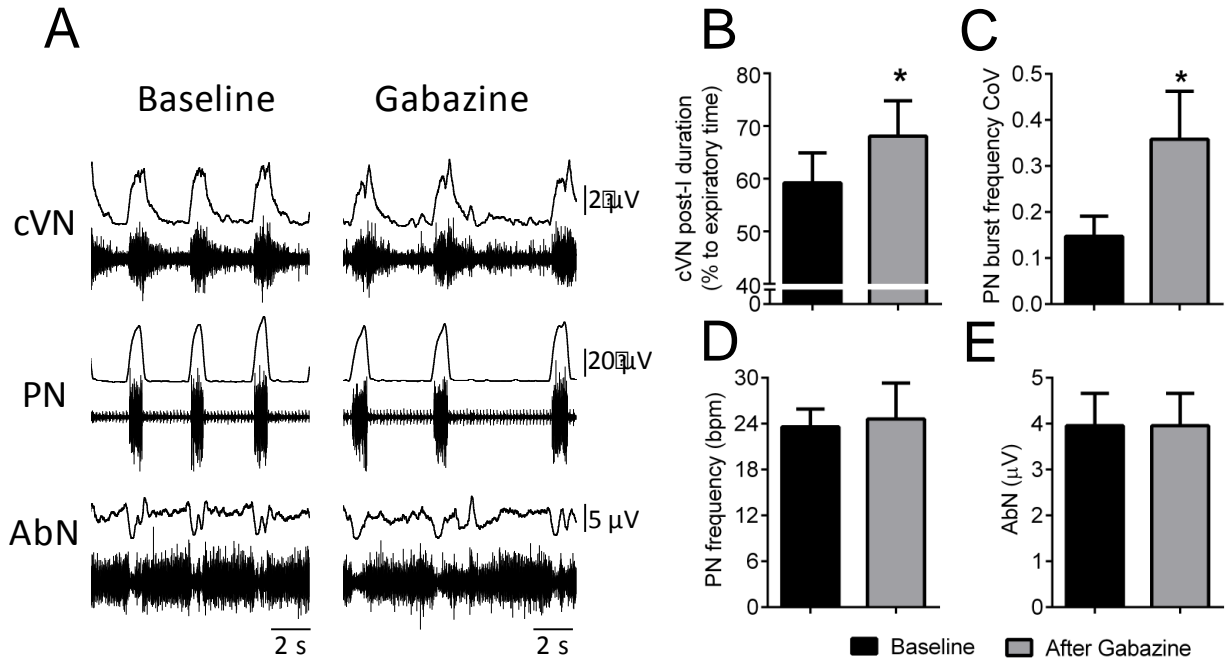
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968 **Figure 3**

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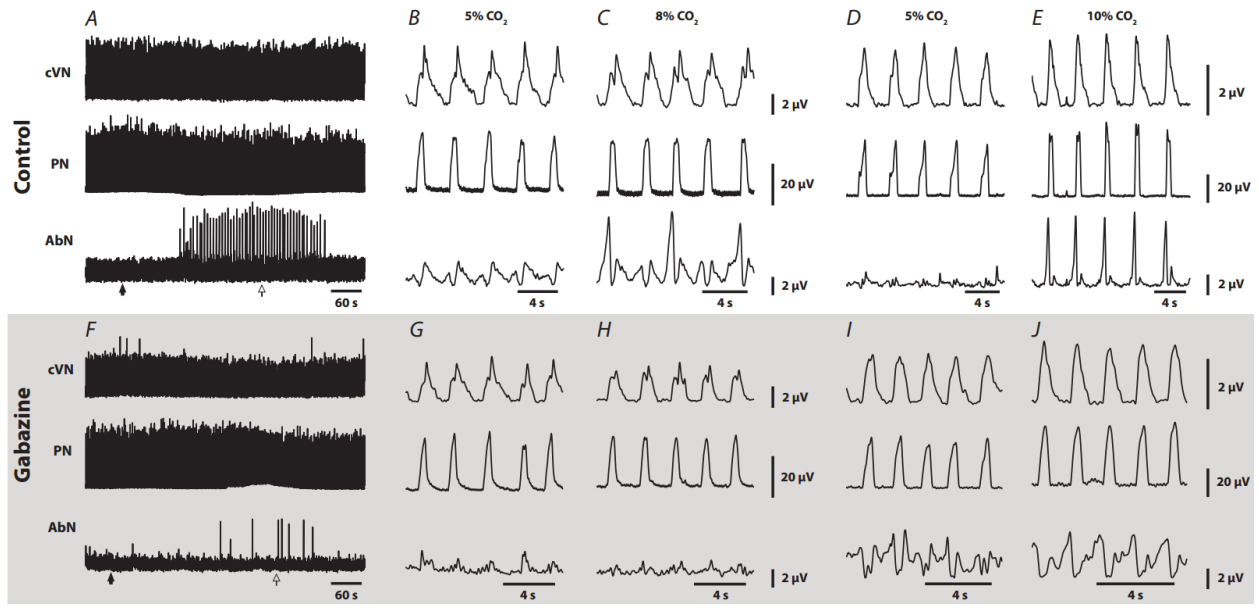
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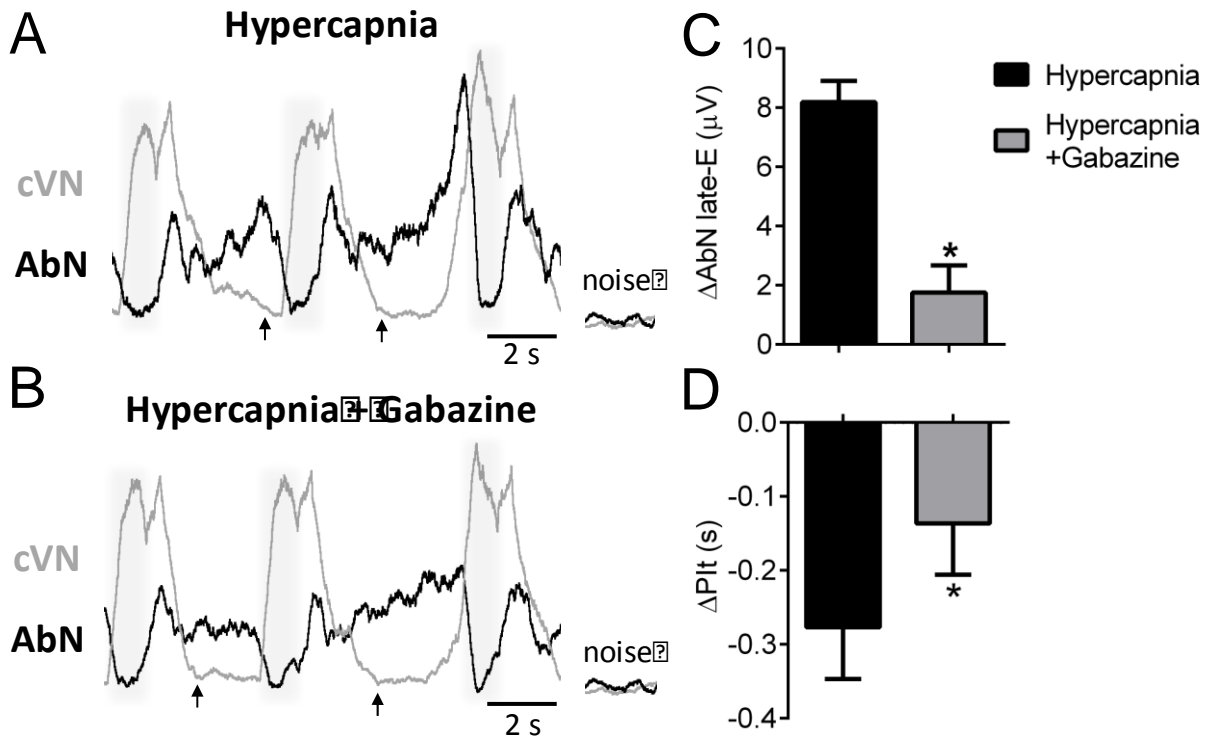
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1006 **Figure 4**

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1038 **Figure 5**

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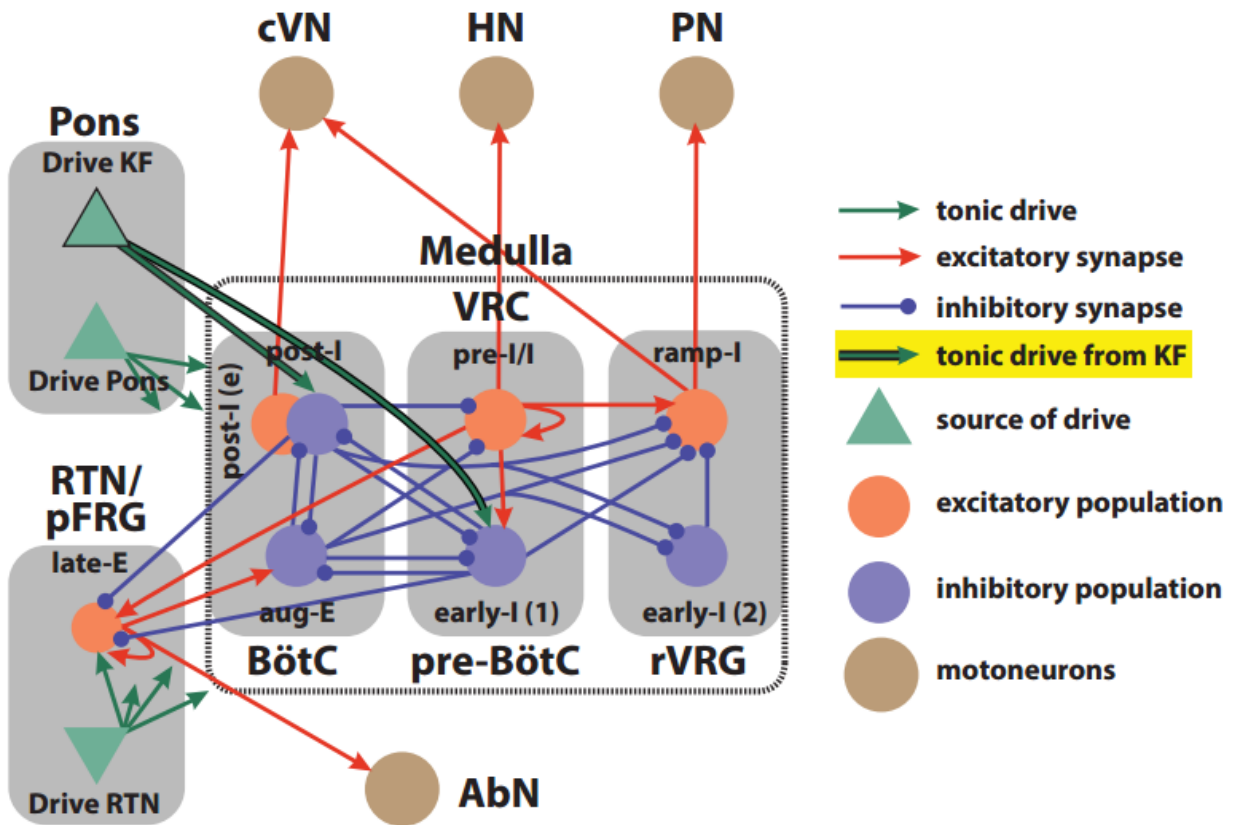
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1079 **Figure 6**

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**PN**

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**cVN**

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**post-I  
(BötC)**

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**aug-E  
(BötC)**

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**post-I (e)  
(BötC)**

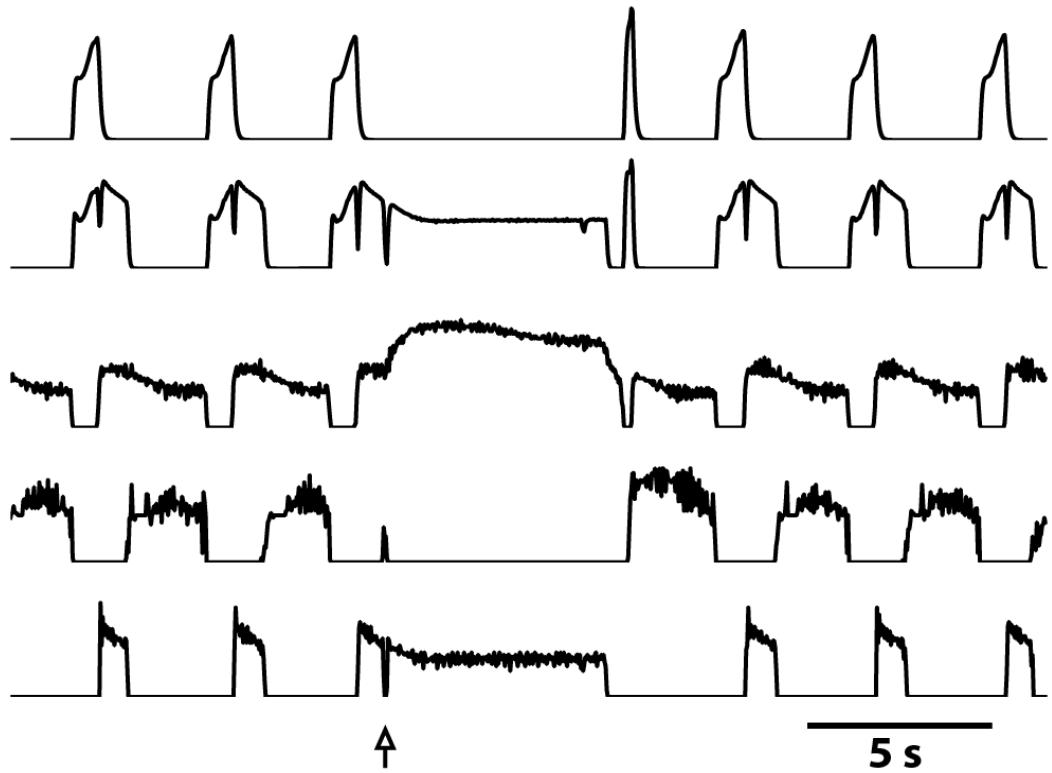
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1097 **Figure 7**

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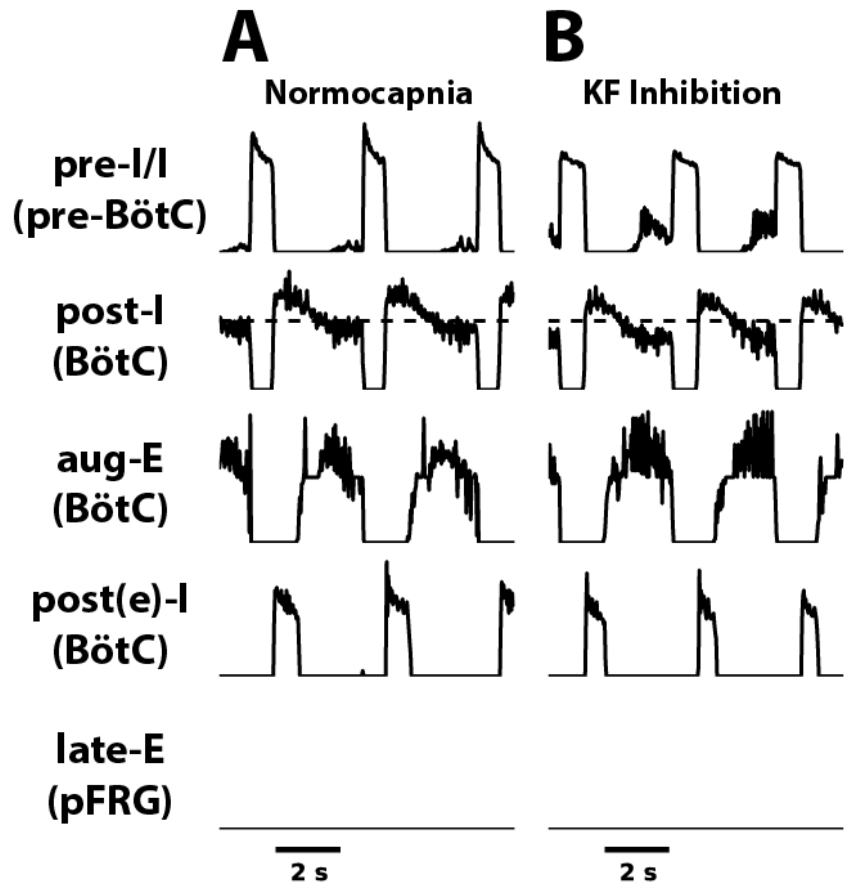
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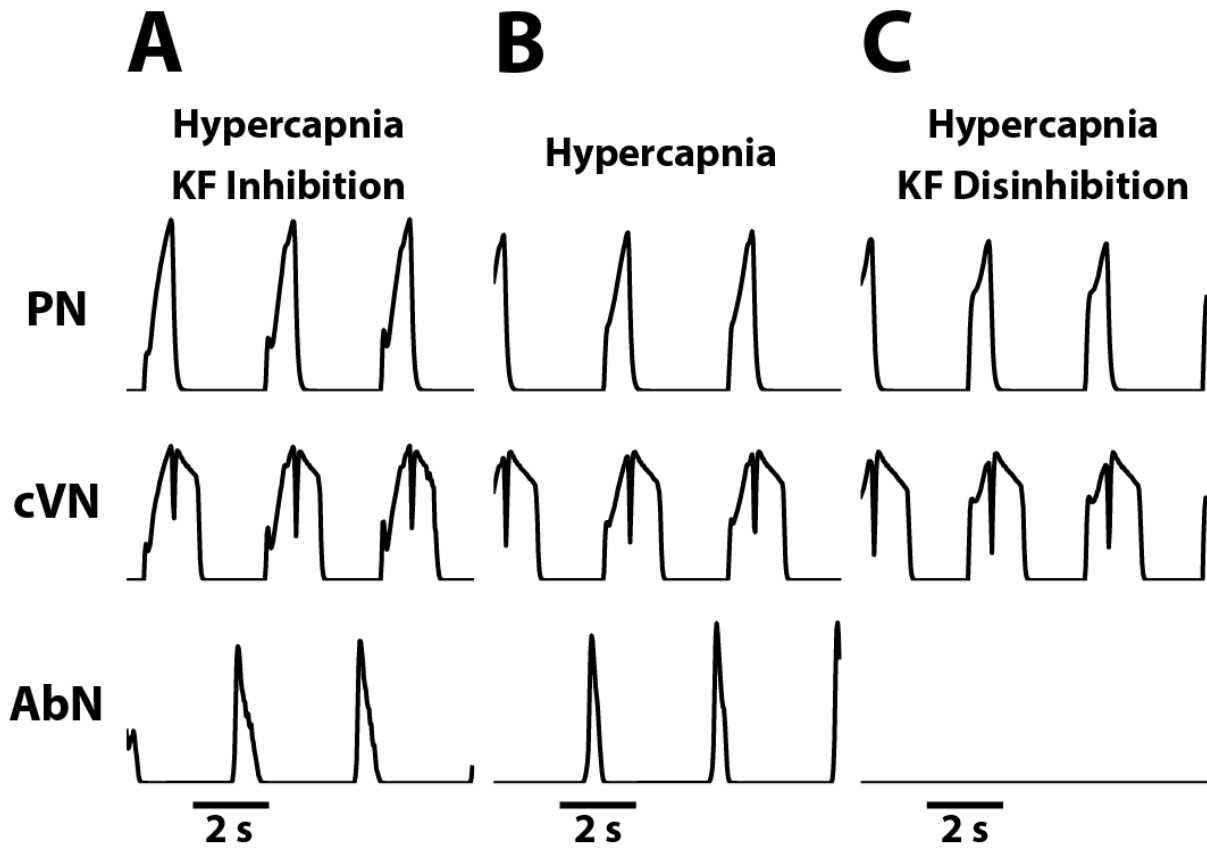
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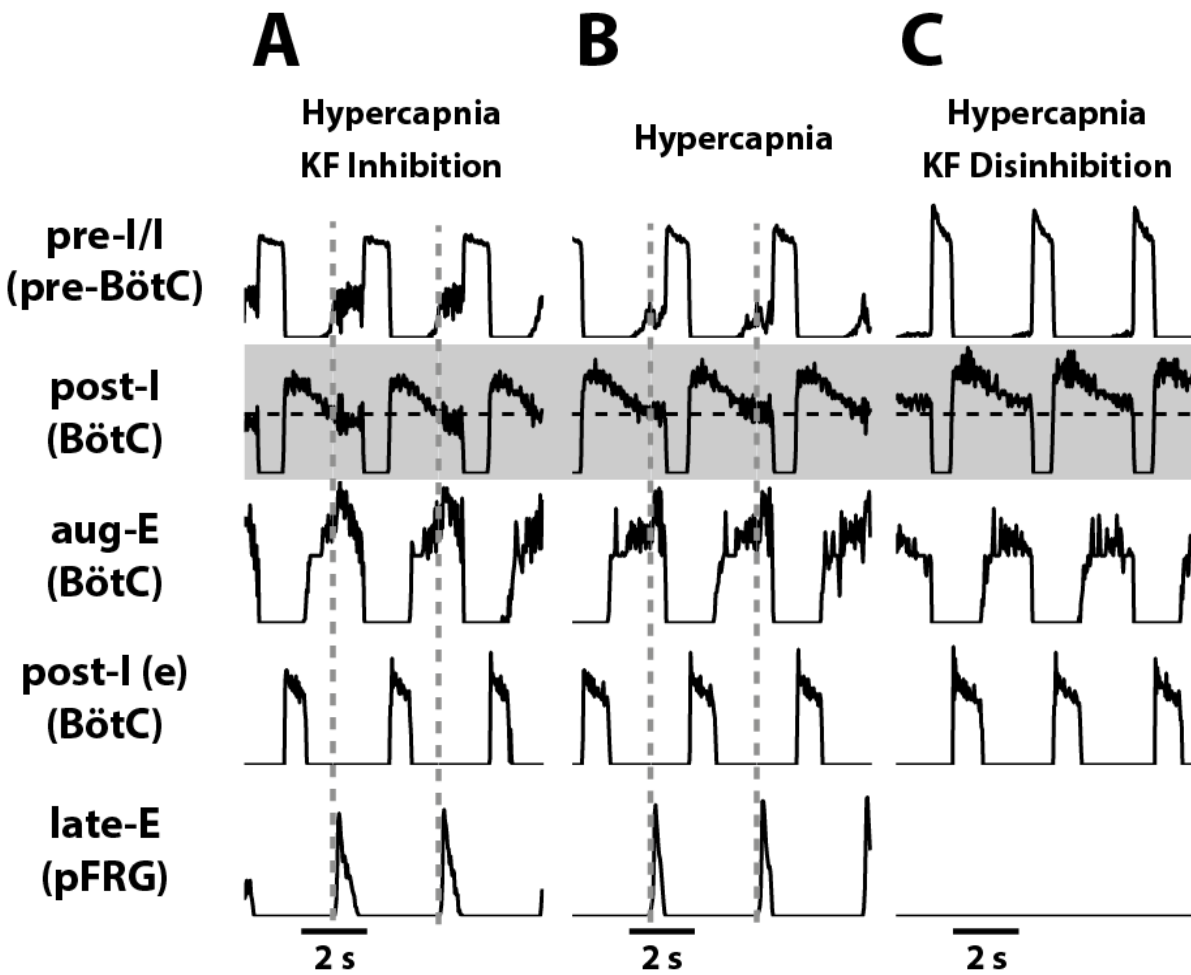
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Figure 8



1158 **Figure 9**

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Figure 10

