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1                   **Upregulation of breathing rate during running exercise by**  
2                   **central locomotor circuits**

3                   Coralie Hérent<sup>1,2</sup>, Séverine Diem<sup>1</sup>, Gilles Fortin<sup>3</sup> and Julien Bouvier<sup>1\*</sup>

4                   <sup>1</sup> Université Paris-Saclay, CNRS  
5                   Institut des Neurosciences Paris-Saclay  
6                   91190, Gif-sur-Yvette, France.

7                   <sup>2</sup> Present address:  
8                   Champalimaud Neuroscience Program,  
9                   Champalimaud Center for the Unknown,  
10                  Portugal

11                  <sup>3</sup> Institut de Biologie de l'École Normale Supérieure (IBENS),  
12                  École Normale Supérieure, CNRS, INSERM, PSL Research University,  
13                  75005 Paris, France.

14                  \***Correspondence:**  
15                  Dr. Julien Bouvier  
16                  [Julien.bouvier@cnr.fr](mailto:Julien.bouvier@cnr.fr)  
17                  ORCID ID: [0000-0002-1307-4426](https://orcid.org/0000-0002-1307-4426)

## 18 **Abstract**

19           While respiratory adaptation to exercise is compulsory to cope with the increased metabolic supply  
20 to body tissues and with the necessary clearing of metabolic waste, the neural apparatus at stake remains  
21 poorly identified. Using viral tracing, *ex vivo* and *in vivo* optogenetic and chemogenetic interference  
22 strategies in mice, we unravel interactive locomotor and respiratory networks' nodes that mediate the  
23 respiratory rate increase that accompanies a running exercise. We show that the mesencephalic locomotor  
24 region (MLR) and the lumbar spinal locomotor pattern generator (lumbar CPG), which respectively initiate  
25 and execute the locomotor behavior, access the respiratory network through distinct entry points. The MLR  
26 directly projects onto the inspiratory rhythm generator, the preBötzinger complex (preBötC), and can trigger  
27 a moderate increase of respiratory frequency, prior to, or even in the absence of, locomotion. In contrast,  
28 the lumbar CPG projects onto the retrotrapezoid nucleus (RTN) that in turn contacts the preBötC to enforce,  
29 during effective locomotion, higher respiratory frequencies. These data expand, on the one hand, the  
30 functional implications of the MLR beyond locomotor initiation to a *bona fide* respiratory modulation. On the  
31 other hand, they expand the adaptive respiratory ambitions of the RTN beyond chemoception to “locomotor-  
32 ception”.

## 33 Introduction

34 Breathing is a vital behavior which must combine extreme robustness with continuous adaptability.  
35 One striking example is the abrupt augmentation of ventilation at the transition from rest to running in order  
36 to maintain homeostasis in spite of increased metabolic demand (DiMarco et al., 1983; Duffin and  
37 Bechbache, 1983; Mateika and Duffin, 1995). This “exercise hyperpnoea” is manifested by an increase in both  
38 respiratory frequency and volume. It has long been proposed that its main trigger, at least for acute exercise,  
39 is of neuronal nature, i.e. relies on activatory signals from locomotor effectors or circuits impacting onto the  
40 respiratory generator in the brainstem (Mateika and Duffin, 1995; Gariepy et al., 2010; Duffin, 2014;  
41 Paterson, 2014). However, the underlying cells and circuits are not fully elucidated.

42 We recently uncovered that running hyperpnoea can, at least in mice but very likely in all  
43 quadrupeds, occur without temporal synchronization of breathes to strides (Hérent et al., 2020). This,  
44 together with the ventilatory response seen in men during mental simulation of exercise (Tobin et al., 1986;  
45 Decety et al., 1991; Decety et al., 1993) or in the absence of peripheral signals (Fernandes et al., 1990),  
46 highlights that the main neuronal trigger of running hyperpnoea is of central, rather than peripheral, origin.  
47 Of particular interest are therefore brain regions that command or execute locomotor movements and could  
48 provide a parallel drive to respiratory centers (Eldridge et al., 1981; Eldridge et al., 1985). The mesencephalic  
49 locomotor region (MLR) in the dorsal midbrain is considered the main site of locomotor initiation throughout  
50 the animal kingdom (Shik et al., 1966; Le Ray et al., 2011; Ryczko et al., 2016; Chang et al., 2021) likely  
51 including humans (Jahn et al., 2008; Sébille et al., 2019). Stimulation of the MLR, and particularly its cuneiform  
52 nucleus (CnF) component, engages forward locomotion at a speed that is commensurate to the intensity of  
53 the stimulus (Bachmann et al., 2013; Roseberry et al., 2016; Caggiano et al., 2018; Josset et al., 2018; Dautan  
54 et al., 2020; van der Zouwen et al., 2021), making it a candidate neuronal encoder and driver of running  
55 intensity. The possibility that the MLR may provide a parallel activation of respiratory centers is suggested by  
56 work in the lamprey (Gariepy et al., 2011), an ancestral vertebrate specie, but this has yet not been  
57 investigated in terrestrial mammals. Another central drive to respiratory centers may originate in the circuits  
58 of the spinal cord that elaborate the locomotor rhythm and coordinate the motor output during ongoing  
59 locomotor movements, often referred to as a “Central Pattern Generator” or CPG (Grillner, 2006; Kiehn,  
60 2016; Grillner and El Manira, 2020). Indeed, pharmacological activation of the lumbar enlargement, where  
61 the hindlimb CPG circuit is thought to reside, can upregulate the frequency of respiratory-like activities on *ex*  
62 *vivo* preparations from neonatal rats (Le Gal et al., 2014; Le Gal et al., 2020). While this is suggestive of  
63 ascending projections to respiratory centers, the underlying circuit and its functionality during running has  
64 not been documented.

65 Another gap of knowledge resides in the identification of the respiratory neurons targeted by  
66 descending (e.g., MLR) or ascending (e.g., from the CPG) locomotor drives. In mammals, the respiratory  
67 rhythm is paced by a confined cluster of neurons in the ventromedial medulla, the pre-Böttinger complex

68 (preBötC, Smith et al., 1991; Del Negro et al., 2018). Direct activation or inactivation of preBötC glutamatergic  
69 neurons respectively increases, or reduces and even arrests, respiratory rate (Tan et al., 2008; Alshafi et al.,  
70 2015; Cui et al., 2016; Vann et al., 2018). The preBötC receives inputs from several brain areas including the  
71 midbrain (Yang et al., 2020) and, in the lamprey, MLR neurons were shown to contact a presumed homologue  
72 of the preBötC (Mutolo et al., 2010; Gariepy et al., 2012). This makes the preBötC a candidate for promptly  
73 entraining respiration during exercise in mammals. More rostrally, an area collectively referred to as the  
74 parafacial (pF) respiratory region may be another candidate of respiratory regulation during metabolic  
75 challenges including effort. In particular in this region, non-catecholaminergic *Phox2b*-expressing neurons  
76 (defining the Retrotrapezoid Nucleus, RTN) are well-known for their capacity to rapidly upregulate  
77 respiratory rate in the context of central CO<sub>2</sub> chemoreception (Abbott et al., 2009; Abbott et al., 2011). They  
78 might also support active expiration which is thought to accompany exercise (Ainsworth et al., 1989; Iscoe,  
79 1998; Abraham et al., 2002). *Phox2b*-positive neurons in the pF region have been shown to be activated  
80 during running (Barna et al., 2012, 2014), during locomotor-like activity on *ex vivo* neonatal rat preparations  
81 (Le Gal et al., 2014), and their chemogenetic silencing limits exercise capacity in running rats (Huckstepp et  
82 al., 2015).

83 Here we sought to investigate the central circuits interfacing locomotor and respiratory centers in  
84 the resourceful mouse model. We found the existence of both a descending drive from the MLR, and of an  
85 ascending drive from the locomotor CPG of the lumbar spinal cord. Remarkably, the MLR is capable of  
86 upregulating breathing rate even before the initiation of actual limb movements. We further uncovered that  
87 the two systems both have access to respiratory rhythm generation mechanisms albeit through two different  
88 synaptic schemes. The MLR directly projects to the preBötC, but not to the pF respiratory region, while the  
89 lumbar spinal cord targets the RTN which in turns contacts the preBötC. Our work therefore demonstrates  
90 two locomotor central drives that may underlie breathing adaptability during running and their synaptic  
91 nodes in the respiratory central network.

## 92 **Results**

### 93 **Glutamatergic CnF neurons project to the preBötC.**

94 We first examined whether locomotor-promoting MLR neurons in mice, as is the case with the  
95 lamprey (Gariepy et al., 2012), contact neuronal groups involved in respiratory rhythm generation. The MLR  
96 contains two major subdivisions, the cuneiform nucleus (CnF) containing glutamatergic (Glut+) neurons, and  
97 the pedunculopontine nucleus (PPN) containing both glutamatergic and cholinergic neurons (Roseberry et  
98 al., 2016; Caggiano et al., 2018; Josset et al., 2018). Since locomotor initiation is mostly attributed to the  
99 former, we traced the projections of CnF neurons by unilateral stereotaxic injections of a Cre-dependent  
100 Adeno Associated Virus (AAV) coding the fluorescent protein eYFP in *Vglut2<sup>Cre</sup>* adult mice (Vong et al., 2011)  
101 (Figure 1a, b). Abundant eYFP-positive fibers were detected in the preBötC, defined as located ventrally to

102 the nucleus ambiguus, containing SST-positive neurons and between antero-posterior levels -7.0 and -7.4  
103 from bregma (Stornetta et al., 2003; Tan et al., 2008). These projections were found bilaterally with an  
104 ipsilateral predominance (Figure 1c, d, g). In contrast, projections were very sparse in the pF respiratory area,  
105 defined as located immediately ventral, ventro-median and ventro-lateral to the facial motor nucleus (7N,  
106 between antero-posterior levels -6.5 and -5.7 from Bregma, Figure 1e-g). We also observed CnF projections  
107 to the parabrachial nucleus and to the nucleus of the tractus solitarius (data not shown). To verify that CnF  
108 neurons synaptically target preBötC neurons, we made use of a genetically restricted two-virus approach  
109 (Kim et al., 2016) to reveal preBötC putative inputs anatomically. Functional preBötC neurons can be  
110 efficiently delineated as Glut+ neurons with commissural projections (Koshiya and Smith, 1999; Bouvier et  
111 al., 2010). We therefore drove the expression of the TVA receptor and the rabies protein G using a  
112 retrogradely transported Cre-dependent Herpes Simplex Virus (HSV, Neve et al., 2005; Reinhard et al., 2019)  
113 injected in the preBötC on one side. Seven days later, a G-deleted and EnvA-pseudotyped rabies (Rb) virus  
114 coding mCherry was injected in the preBötC on the other side (Figure 1h). As demonstrated previously, this  
115 leads to the expression of the Rb virus in projection-defined neuronal somata (Usseglio et al., 2020), here  
116 commissural Glut+ neurons of the preBötC (Figure 1i), and, from these starter cells, to its transynaptic spread  
117 to upstream neurons. Transynaptically labelled neurons, i.e., expressing only the Rb-driven fluorophore,  
118 were detected in the CnF and PPN nuclei bilaterally (Figure 1j). Putative inputs to Glut+ preBötC neurons  
119 were also detected in the contralateral preBötC, the periaqueductal grey, the superior colliculus and the NTS,  
120 and only few cells were detected in the pF respiratory region on either side (data not shown, Figure S1).  
121 Altogether, these converging anterograde and retrograde tracings demonstrate that Glut+ CnF neurons make  
122 direct contact with candidate respiratory rhythm generating neurons in the preBötC, but not in the pF  
123 respiratory region.

#### 124 **Glutamatergic CnF neurons modulate inspiratory rhythm generation mechanisms.**

125 We next tested by optogenetics the ability of Glut+ CNF neurons to functionally impact the preBötC.  
126 One hallmark of preBötC responses to phasic incoming inputs is their phase-dependency to the ongoing  
127 rhythm (Cui et al., 2016; Baertsch et al., 2018). To evaluate this for CnF-evoked preBötC responses, we virally  
128 delivered the light-activated excitatory opsin Channelrhodopsin 2 (ChR2) in the CnF on one side (AAV-DIO-  
129 ChR2-eYFP) of *Vglut2<sup>Cre</sup>* adult mice, and implanted an optic fiber over the injection site (Figure 2a). Breathing  
130 cycles were measured in awake animals with whole body plethysmography (WBP, (DeLorme and Moss,  
131 2002), Figure 2b) while short single-pulse (50 ms) photoactivations were delivered randomly during the  
132 respiratory cycle. We calculated the resultant phase shift, expressed as the perturbed cycle duration over the  
133 control cycle duration as done previously (Cui et al., 2016; Baertsch et al., 2018, Figure 2b). A phase shift <1  
134 (perturbed cycle duration lower than the control one) indicates a shortening of the respiratory cycle, a phase  
135 shift >1 (perturbed cycle duration higher than the control one) indicates a lengthening, and a phase shift  
136 equal to 1 (perturbed cycle duration equal to the control one) indicates no effect. We found that unilateral

137 photostimulation of CnF neurons elicited an ectopic inspiratory burst and shortened the respiratory cycle  
138 (Figure 2c) but that this effect was dependent on the timing of light-activation during the respiratory cycle.  
139 Specifically, the most significant cycle shortening was seen when delivering light-pulses during early  
140 expiration (phase: 0.5 - 0.6, phase shift:  $0.80 \pm 0.10$ ,  $p < 0.0001$  Figure 2c). In contrast, the effect was only  
141 minimal when photostimulations were delivered in late expiration. Intriguingly, in addition to the immediate  
142 inspiratory response to photostimulation, we observed a shortening (although less drastic) of the next two  
143 consecutive cycles suggesting that activation of the CnF also determines a longer lasting modulatory action  
144 on preBötC rhythm generation (Figure S2a-d). No alteration of the respiratory cycle was however seen in  
145 mock trials in control mice that do not express Chr2 (Figure S3a, b), ruling out a contribution of light-evoked  
146 temperature changes locally (Stujenske et al., 2015). To ascertain that this modulation of respiratory rhythm  
147 generation owes to direct projections of Glut+ CnF neurons to the preBötC, we next aimed at photo-  
148 activating Chr2-expressing fibers in the preBötC following the delivery of the Chr2 virus in the CnF (Figure  
149 2d). This led to a similar phase-dependent shortening of the respiratory cycle, with again a maximal  
150 shortening observed when light-activations are delivered in early expiration (phase: 0.5 - 0.6; phase shift:  
151  $0.79 \pm 0.15$ ,  $p < 0.0001$ , Figure 2e, f). This effect disappeared after the 2<sup>nd</sup> subsequent respiratory cycle ( $\theta+3$ ,  
152 Figure S2e-h). Light deliveries in the preBötC of control mice that do not express Chr2 did not produce any  
153 noticeable effect (Figure S3d, e). Overall, this indicates that the direct projections of the Glut+ CnF neurons  
154 indeed conform to phase-dependent activation of preBötC neurons, highlighting excitatory modulations of  
155 inspiratory burst generation.

#### 156 **Glutamatergic CnF neurons modulate breathing in synergy with locomotion.**

157 Through their access to rhythm generating neurons in the preBötC, Glut+ CnF neurons might be  
158 capable of upregulating breathing frequency in synergy with locomotor initiation. To access respiratory  
159 parameters during vigorous displacement movements, we made use of our recently-developed method for  
160 chronic electromyographic (EMG) recordings of the diaphragm, the main inspiratory muscle (Hérent et al.,  
161 2020). Animals were thus made to express Chr2 in Glut+ CnF neurons as above, EMG-implanted, and placed  
162 in a linear corridor. Light was delivered in trains of 1 s duration at increasing pulse frequencies when animals  
163 were stationary at one end of the corridor (Figure 3). Animals were filmed from the side and their  
164 displacement speed computed using markerless video-tracking (Mathis et al., 2018) as performed previously  
165 (Hérent et al., 2020). In line with numerous studies (Roseberry et al., 2016; Caggiano et al., 2018; Josset et  
166 al., 2018; Dautan et al., 2020), we found that photoactivation of Glut+ CnF neurons at 15 Hz or more engages  
167 animals in forward locomotion (Figure 3a), and that higher stimulation frequencies impose faster regimes,  
168 shorten the delay between light onset and locomotor initiation and increase the occurrence of left-right  
169 synchronous gaits (Figure S4). Importantly, CnF photostimulations were associated with an increased  
170 respiratory rate (Figure 3b, c), an effect that was not seen in control mice that do not express Chr2 (Figure  
171 S3c). In what we consider a remarkable observation, during CnF photoactivations that effectively engage

172 running, respiratory rate increased in a two-step sequential manner. In a first step, that we term the “pre-  
173 loco” phase, a modest increase was seen immediately at light onset but before the first locomotor  
174 movements (i.e., during the delay between light onset and the initiation of locomotion, Figure 3d). The mean  
175 respiratory rate during this “pre-loco” phase was found significantly higher than baseline but was not  
176 correlated to the photostimulation frequency (Figure 3e). In a second step, when the animals effectively  
177 engage in locomotion (“loco” phase), the respiratory rate was further augmented (Figure 3d). There,  
178 respiratory rate was still not strongly dependent on stimulation frequency, however it was proportional to  
179 the actual displacement speed (Figure 3f). This likely reflects the variability of locomotor velocities at a given  
180 stimulation frequency (Figure S4b). However, we found that during CnF photoactivations, respiratory and  
181 locomotor rhythms were not temporally synchronized (Figure S5), in line with what we recently reported  
182 during spontaneous running in mice (Hérent et al., 2020). These results indicate that respiratory frequency  
183 during CnF-evoked locomotion is upregulated immediately at light onset and before the initiation of  
184 locomotion, and further upregulated during actual locomotion yet without a phasic relation to limb cycles.

185 The former observation above suggests that Glut+ CnF neurons can modulate respiratory activity  
186 independently of their action on limb movements, in line with what was reported in the lamprey (Garipey et  
187 al., 2012). To demonstrate this further, we reduced the intensity of photostimulations below the threshold  
188 for locomotor initiation (5 and 10 Hz, Figure 3g, Figure S4b). We found that, in spite of absent locomotor  
189 movements, the respiratory frequency was indeed significantly increased from baseline (Figure 3h).  
190 Altogether, these analyses demonstrate that i) Glut+ CnF neurons can upregulate breathing before, or even  
191 in the absence of, locomotor movements, ii) during CnF-evoked locomotion, the highest increase in breathing  
192 rate from rest occurs when actual locomotor movements are engaged, iii) respiratory frequency increase  
193 during the “loco” phase are proportional to the displacement speed, and iv) breaths are not phase-locked to  
194 cyclic limb movements.

### 195 **The spinal locomotor circuits project to the pF respiratory region.**

196 From the above observation we reasoned that the engagement in actual locomotor movements may  
197 be associated with a stronger activating drive onto respiratory centers which could originate in executive  
198 lumbar locomotor circuits. We thus examined the ascending projections to the brainstem of glutamatergic  
199 lumbar neurons that are essential in the regulation of locomotor speed (Kjaerulff and Kiehn, 1996; Hagglund  
200 et al., 2010; Hagglund et al., 2013; Talpalar et al., 2013). To do so, we injected a Cre-dependent AAV-eYFP  
201 vector bilaterally in the ventral laminae of the 2<sup>nd</sup> lumbar segment (L2) of adult *Vglut2<sup>Cre</sup>* animals (Figure 4a)  
202 and examined projections in the brainstem reticular formation. In contrast to the anterograde tracings from  
203 the CnF, this revealed very few, if any, eYFP-positive fibers in the preBötC but their abundant presence in the  
204 pF respiratory region (Figure 4b-f). To discriminate passing fibers from putative synaptic contacts, we first  
205 repeated these spinal cord injections with an AAV that drives a presynaptic, synaptophysin-fused, GFP (AAV-  
206 DIO-Syp-GFP) and indeed observed GFP-positive puncta in the pF (Figure 4g). We also performed similar



207 spinal injections this time with a high-titer AAV coding the Cre recombinase (AAV1-Syn-Cre, Zingg et al.,  
208 2017), on wild-type mice. This vector has been shown to be transported anterogradely down the axon and  
209 to enable expression of the Cre-recombinase in postsynaptic target neurons that then are amenable to  
210 visualization through Cre-dependent transgene expression. We therefore injected, one week after the first  
211 viral injection in the spinal cord, a Cre-dependent AAV-eYFP vector in the pF region. This led to numerous  
212 eYFP-expressing cells (Figure 4h). Since this region does not project to the lumbar spinal cord (Figure S6), the  
213 Cre-dependent labelling cannot owe to spurious retrograde transport of the AAV1-Syn-Cre virus. Therefore,  
214 ascending spinal projections synaptically target the pF respiratory region.

#### 215 **Lumbar locomotor circuits upregulate breathing rate through the RTN<sup>Phox2b/Atoh1</sup> *ex vivo*.**

216 To investigate functionally the possibility that lumbar locomotor circuits can upregulate breathing,  
217 we used the *ex vivo* isolated brainstem/spinal cord preparation from neonatal mouse. Although long-used  
218 for monitoring locomotor (Kjaerulff and Kiehn, 1996; Bouvier et al., 2015) or respiratory-like (Bouvier et al.,  
219 2010; Ramanantsoa et al., 2011a) activities, monitoring both simultaneously using pharmacological  
220 activation of the lumbar CPG had only been achieved on neonatal rat preparations (Le Gal et al., 2014; Le Gal  
221 et al., 2020). We adapted the method to the neonatal mouse using a split-bath allowing independent  
222 pharmacological manipulation of the brainstem and spinal cord superfused by dedicated artificial-  
223 cerebrospinal fluids (a-CSFs, see methods). In these conditions, we recorded respiratory-like activity on the  
224 4<sup>th</sup> cervical ventral root and locomotor-like activity on the 2<sup>nd</sup> lumbar ventral root (Figure 5a, b).

225 When both the brainstem and spinal cord were superfused with their respective control a-CSF  
226 solution, the frequency of respiratory-like activities was found to be ranging from 2.5 to 8.3 bursts/min, with  
227 an average of  $4.3 \pm 1.6$ . Bath-application of the neuroactive substances N-methyl-D-aspartate (NMDA) and  
228 serotonin (5-HT) in the spinal cord compartment evoked locomotor-like activities associated with an  
229 increased frequency of the respiratory-like activity (average of  $7.2 \pm 3.6$  bursts/min which represents  $162 \pm$   
230  $30$  % of baseline, Figure 5b, c). Reminiscent of what we observed in freely running mice (Hérent et al., 2020),  
231 we found that respiratory- and locomotor-like activity were not temporally synchronized (data not shown).  
232 To rule out drug leakage from the spinal compartment we first verified that this frequency increase of  
233 respiratory-like activities was abolished following a cervical transection of the medulla (Figure S7a-c). We  
234 also examined respiratory changes following the engagement of locomotor-like activities by targeted  
235 optogenetic activations of lumbar excitatory neurons (*Vglut2<sup>Cre</sup>; ChR2<sup>flox</sup>*), in the absence of NMDA and 5-  
236 HT (Hagglund et al., 2010; Hagglund et al., 2013; Le Gal et al., 2020). In this optogenetic paradigm, the  
237 frequency of inspiratory activity was increased similarly to the pharmacologically-induced locomotion ( $170 \pm$   
238  $38$  % from baseline, Figure S7d-f). Altogether, these experiments indicate that the lumbar spinal segments  
239 containing the locomotor CPG exert an excitatory effect on respiratory activity.

240 Since our anatomical observations place the pF respiratory region as a candidate target of ascending  
241 pathways from the spinal cord (Figure 4), we next addressed the functional contribution of this region. In a  
242 first set of experiments, we eliminated physically the pF by a complete transection below the facial motor  
243 nucleus (Figure 5d). Respiratory-like activities persisted on the 4<sup>th</sup> cervical root, but pharmacological  
244 activation of lumbar circuits was no longer capable of significantly upregulating their frequency (Figure 5e,  
245 f). In a second set of experiments, we examined specifically the contribution of RTN neurons in the pF region  
246 (Abbott et al., 2009; Abbott et al., 2011). For this we made use of the fact that RTN neurons relevant for  
247 modulating respiratory rhythm generation, at least in the context of central chemoception, are i) best  
248 identified by their combined history of expression of the transcription factors *Phox2b* and *Atoh1* during  
249 embryogenesis (thereafter RTN<sup>*Phox2b/Atoh1*</sup> neurons; Ramanantsoa et al., 2011a; Ruffault et al., 2015) and ii)  
250 can be deleted when expressing a mutated allele of PHOX2B (*Phox2b*<sup>27Ala</sup>) in rhombomeres 3 and 5  
251 (*Egr2*<sup>cre</sup>;*Phox2b*<sup>27AlaCKI</sup> mutants; Figure S8; Ramanantsoa et al., 2011a; Ruffault et al., 2015). We thus  
252 generated RTN mutant pups and recorded respiratory- and locomotor-like activities as above. Preparations  
253 showed persistent inspiratory-like activity on the C4 root, but pharmacological activation of lumbar circuits  
254 was no longer capable of significantly upregulating its frequency (Figure 5g-i). These experiments altogether  
255 highlight the capacity of spinal lumbar circuits to upregulate respiratory-like activities through RTN<sup>*Phox2b/Atoh1*</sup>  
256 neurons in the pF respiratory region.

### 257 **Silencing RTN<sup>*Phox2b/Atoh1*</sup> neurons reduces respiratory increase during running exercise *in vivo*.**

258 The importance of RTN<sup>*Phox2b/Atoh1*</sup> neurons revealed above on *ex vivo* neonatal preparations prompted  
259 to address their contribution to respiratory activity during running in behaving adult mice. For this, we used  
260 the *Atoh1*<sup>FRTCre</sup>;*Phox2b*<sup>Flpo</sup> intersectional background, i.e., in which Cre-expression from the *Atoh1* locus is  
261 conditional to Flpo expression driven by the *Phox2b* locus (Ruffault et al., 2015), and injected in the pF region  
262 bilaterally a Cre-dependent AAV coding the inhibitory DREADD receptor hM4Di (Figure 6a, b). Three weeks  
263 later, animals underwent the chronic implantation of diaphragmatic EMG electrodes (Hérent et al., 2020).  
264 Inspiratory frequency was then measured before, and 2-3 h after, the administration of the DREADD ligand  
265 Clozapine-N-Oxide (CNO) while animals were at rest or running at a set frequency on a motorized treadmill.

266 At rest, we found that CNO administration had no significant effect on the mean inspiratory  
267 frequency (Figure 6c, d), supporting previous findings that the RTN only minimally contributes to setting the  
268 baseline breathing rate (Korsak et al., 2018). When animals were made to run for 1.5 min at a velocity of 40  
269 cm/s set by the treadmill, they presented an augmented respiratory frequency (from  $3.9 \pm 0.2$  bursts/s at  
270 rest to  $10.4 \pm 1.1$ , which represents a 271 % increase, Figure 6e, f). We have recently reported that this value  
271 is characteristic of running mice on a treadmill, regardless of the trotting speed (Hérent et al., 2020).  
272 Following CNO administration, while animals were still capable to run at 40 cm/s for 1.5 min, their breathing  
273 rate was significantly reduced when compared to controls ( $8.4 \pm 1.2$  bursts/s, which represents a 227 %  
274 augmentation from rest). Administration of saline in hM4Di-injected mice or of CNO on wild-type mice did

275 not significantly reduce breathing rate during running sessions (Figure S9). These experiments altogether  
276 indicate that the activity of RTN<sup>Phox2b/Atoh1</sup> neurons is required for setting the adapted ventilatory frequency  
277 during running exercise.

#### 278 RTN<sup>Phox2b/Atoh1</sup> neurons project to the preBötC inspiratory generator.

279 The reduced breathing rate following the silencing of RTN<sup>Phox2b/Atoh1</sup> neurons suggests that this  
280 genetically defined neuronal subset may have access to the main inspiratory generator, the preBötC. While  
281 the pF region was previously shown to send axonal projections to the ventral respiratory column and possibly  
282 the preBötC (Smith et al., 1989; Li et al., 2016), this had not been determined for RTN<sup>Phox2b/Atoh1</sup> neurons. For  
283 this, similarly to what we did for CnF neurons (Figures 1, 2), we injected, in the pF region of  
284 *Atoh1<sup>FRTCre</sup>;Phox2b<sup>Flpo</sup>* animals, a Cre-dependent AAV vector coding the ChR2 and a fluorescent protein (AAV-  
285 DIO-ChR2-eYFP; Figure 7a, b) and implanted an optic fiber above the injection site. Anatomically, we  
286 observed abundant eYFP-labelled varicosities of RTN<sup>Phox2b/Atoh1</sup> neurons in the preBötC region with a strong  
287 ipsilateral dominance (Figure 7c, d). To demonstrate functionally the capacity of RTN<sup>Phox2b/Atoh1</sup> neurons to  
288 directly modulate rhythm generation mechanisms in the preBötC, we examined respiratory responses to  
289 short single-pulse (50 ms) photoactivations with the same analytic tools described earlier for CnF activations  
290 (Figure 2). We found that unilateral photostimulation of RTN<sup>Phox2b/Atoh1</sup> neurons could elicit an ectopic  
291 inspiratory burst and shorten the respiratory cycle (Figure 7e). However, when compared to CnF  
292 photostimulation, shortenings of the respiratory peaked earlier during the inspiration phase (phase 0.2-0.3  
293 and 0.3 - 0.4; phase shift:  $0.70 \pm 0.13$  and  $0.69 \pm 0.09$ ;  $p < 0.0001$ ) and, contrary to the CnF, stimulation of the  
294 RTN during late expiration caused a significant lengthening of the respiratory cycle (phases 0.9 - 1; phase  
295 shift:  $1.11 \pm 0.12$ ;  $p = 0.0004$ ; Figure 7f). Therefore here again, stimulation of RTN<sup>Phox2b/Atoh1</sup> neurons led to  
296 phase dependent inspiratory responses in keeping with the preBötC excitability dynamics. Yet unlike the CnF,  
297 no shortening of the subsequent cycles following the stimulus was observed (Figure S10). Moreover, 1 s  
298 pulsed-light stimulations of the RTN<sup>Phox2b/Atoh1</sup> neurons resulted in significant augmentations of respiratory  
299 frequency during the stimulus (Figure 7g-h). The capacity of RTN<sup>Phox2b/Atoh1</sup> to contact the preBötC directly is  
300 further corroborated by the detection of eYFP puncta in the preBötC following the injection of a Cre-  
301 dependent AAV coding a synaptophysin-fused eYFP in the pF area on *Atoh1<sup>FRTCre</sup>;Phox2b<sup>Flpo</sup>* animals (Figure  
302 7i-k). Altogether, these results demonstrate that the genetically defined subset of RTN neurons co-expressing  
303 *Atoh1* and *Phox2b* can upregulate respiratory rate through direct projections to the preBötC. Interestingly,  
304 the phase-dependent shortening (in inspiration) and lengthening (in late expiration) observed when  
305 stimulating RTN<sup>Phox2b/Atoh1</sup> neurons are reminiscent of those reported when photostimulating inhibitory  
306 preBötC neurons (Baertsch et al., 2018). This suggests that RTN<sup>Phox2b/Atoh1</sup> and CnF locomotor-related drives  
307 may also differ by their targeting of a distinct balance of excitatory versus inhibitory preBötC neurons.

## 308 Discussion

309 A neuronal substrate for hyperpnea during running has long been proposed but its underlying cells  
310 and circuits had remained speculative. We uncover here two systems by which the central locomotor  
311 network can enable respiratory rate to be augmented in relation to running activity. On the one hand, we  
312 demonstrate the capacity of the MLR subnucleus CnF, a conserved locomotor controller (Shik et al., 1969;  
313 Dubuc et al., 2008; Le Ray et al., 2011; Bachmann et al., 2013; Roseberry et al., 2016; Caggiano et al., 2018;  
314 Josset et al., 2018; Dautan et al., 2020; Chang et al., 2021; van der Zouwen et al., 2021), to upregulate  
315 breathing. On the other hand, we demonstrate that the lumbar enlargement of the spinal cord, containing  
316 the hindlimb CPG, also acts as a potent upregulator of breathing rate. Using cell-type specific circuit tracing,  
317 we further characterize both locomotor drives by identifying their distinct neuronal targets in the respiratory  
318 network.

### 319 Multiple locomotor drives set respiratory rhythm frequency.

320 One intriguing feature we observed with CnF stimulations is that respiratory rate is upregulated  
321 before the engagement of effective locomotor movements, or for stimulations intensities that are below the  
322 threshold for locomotor initiation (Figure 3). This might be consequent to the direct access of the CnF to the  
323 preBötC (Figures 1, 2), while its access to the limb controller requires the crossing of multiple synapses,  
324 including onto relay reticulospinal neurons (Jordan et al., 2008; Capelli et al., 2017; Caggiano et al., 2018)  
325 that likely accounts for the latencies observed from light onset to locomotor movements (Figure S4c). The  
326 regulation of breathing by the CnF in the absence of locomotor movements, revealing the CnF as a *bona fide*  
327 respiratory modulator structure, may bear physiological relevance as an anticipatory mechanism to the  
328 planned motor action. In the lamprey, spontaneous swimming bouts are preceded by a marked increase in  
329 respiratory frequency, an effect that may incriminate the MLR (Gravel et al., 2007; Gariépy et al., 2012). Men  
330 informed of an upcoming exercise (Decety et al., 1991; Decety et al., 1993; Thornton et al., 2001; Williamson  
331 et al., 2001) or imagining performing an exercise (Krogh and Lindhard, 1913; Tobin et al., 1986; Green et al.,  
332 2007), also show increased ventilation and cardiovascular responses, although less drastic than during actual  
333 movements, reminiscent of the “pre-loco” phase resulting here from CnF stimulations (Figure 3).  
334 Interestingly, the CnF subcomponent of the MLR is associated with escape-like fast regime running, rather  
335 than exploratory locomotion (Caggiano et al., 2018; Josset et al., 2018) and may be part of a larger command  
336 system for defensive behaviors in the broad sense (Mitchell et al., 1988a; Depoortere et al., 1990; Korte et  
337 al., 1992; Ryczko and Dubuc, 2013). It may therefore bear output connectivity allowing to engage a composite  
338 response with both cardiovascular (Mitchell et al., 1988b; Korte et al., 1992), respiratory (this study and  
339 Gariépy et al., 2012), and if needed, locomotor components. A higher threshold and longer latency for the  
340 latter would ensure sufficient priming of relevant autonomic respiratory and cardiovascular controls. Data in  
341 the lamprey indicate that locomotor and respiratory centers are not contacted by the same individual MLR  
342 neurons with branched collaterals but rather by distinct neurons (Gariépy et al., 2012). Pending an equivalent

343 examination in mice, it is possible that the MLR may, similarly to other descending motor pathways  
344 (Sathyamurthy et al., 2020; Usseglio et al., 2020) host projection-defined subsets that each control one trait  
345 of a multi-faceted adaptive response.

346 We also report the existence of a respiratory-modulatory drive from the lumbar spinal segments that  
347 contains the hindlimb locomotor circuits (Grillner, 2006; Kiehn, 2016; Grillner and El Manira, 2020). Such an  
348 ascending drive, previously suggested in rats (Le Gal et al., 2014; Le Gal et al., 2020) is demonstrated here in  
349 mice by local pharmacological (Figure 5) or optogenetic (Figure S7) activations of the lumbar enlargement on  
350 reduced preparations *ex vivo*. Indeed, *ex vivo*, the absence of the MLR and of peripheral structures, as well  
351 as the experimental control of the extracellular solution, allow to isolate the functional contribution of the  
352 spinal ascending drive from descending, peripheral feedbacks and central chemoceptive ones. Since the  
353 activity of the spinal locomotor circuits is proportional to locomotor speed (Talpalar and Kiehn, 2010), it is  
354 possible that this ascending drive continuously informs supra-spinal structures including respiratory centers  
355 on the state of ongoing locomotion. We thus propose that active locomotor executive circuits are, at least  
356 partly, causal to the further increase of respiratory rate seen when animals engage in locomotion (i.e., the  
357 “loco” phase) following CnF stimulations. In support of this, we show *ex vivo* that fictive respiratory rates are  
358 no longer upregulated by engagement of fictive locomotion after elimination of the RTN<sup>Phox2b/Atoh1</sup> (Figure 5).  
359 However, acute silencing of the RTN<sup>Phox2b/Atoh1</sup> neurons *in vivo* reduces, but does not completely prevent, the  
360 capacity to accelerate breathing during treadmill running (Figure 6). This could owe to suboptimal efficiency  
361 of the chemogenetic inhibition that relies on a double Flpo and Cre recombination strategy and/or variable  
362 levels of expression of DREADDS receptors in transfected neurons only partially reducing neuronal activity.  
363 In addition, we cannot rule out a persisting activity of CnF neurons during the running exercise (Caggiano et  
364 al., 2018). This leaves open the possibility that, during ongoing running, the two locomotor drives, from the  
365 CnF and the lumbar spinal cord, may synergize to set the respiratory frequency.

366 While our results localize the locomotor ascending drive to the lumbar spinal cord, the identity of  
367 incriminated neurons will remain to be characterized. Spinal neurons of V2a, V0<sub>v</sub> or V3 genetic identity as  
368 well as Shox2 and Hb9-expressing ones stand as candidates, by virtue of their localization in ventral laminae,  
369 their glutamatergic nature and their direct contribution to locomotor rhythm and pattern (Zhang et al., 2008;  
370 Dougherty and Kiehn, 2010; Dougherty et al., 2013; Talpalar et al., 2013; Caldeira et al., 2017). Our results  
371 also do not speak to the possible involvement of other central locomotor descending pathways (Jordan et  
372 al., 2008). However, the absent phasing between respiratory and locomotor drives or movements reported  
373 here during CnF-evoked locomotion and that we have previously described during running (Hérent et al.,  
374 2020) together with the persistence of a normal ventilatory response to exercise following the removal of  
375 peripheral signals in multiple species (Eldridge et al., 1981; Eldridge et al., 1985; Fernandes et al., 1990), make  
376 it very unlikely that phasing signals arising from proprioceptive limb afferences or sensory modalities  
377 reporting visceral oscillations are important components of the respiratory adaptation to exercise.

## 378 Different respiratory nodes integrate distinct locomotor drives

379 Another intriguing observation is that the two revealed locomotor drives target different nuclei in  
380 the rhythm generating respiratory network. The CnF connects to the preBötC, the main site of inspiratory  
381 rhythm generation (Smith et al., 1991; Del Negro et al., 2018), while the lumbar CPG contacts the pF  
382 respiratory region, and possibly the RTN<sup>Phox2b/Atoh1</sup> previously implicated in central CO<sub>2</sub> chemoreception (Abbott  
383 et al., 2009; Abbott et al., 2011; Ramanantsoa et al., 2011b; Ruffault et al., 2015) that in turn projects onto  
384 the preBötC.

385 For the former, the connectivity is first demonstrated anatomically by the detection of anterogradely-  
386 labelled fibers, and a transynaptic labelling approach initiated from glutamatergic preBötC neurons (Figure  
387 1). This connectivity is also supported functionally, by the capacity of CnF neurons or their projections in the  
388 preBötC to impact respiratory rhythm mechanisms (Figure 2). In contrast, we found that ascending  
389 projections from the lumbar spinal cord were virtually absent in the preBötC but were dense in the pF (Figure  
390 4), in an area compatible with that of the RTN. The synaptic nature of these ascending projections was  
391 ascertained using a synaptic labelling (Figure 4g) and an anterograde transynaptic strategy (Figure 4h, Zingg  
392 et al., 2017). The functionality of these ascending projections and the identity of their neuronal targets as  
393 RTN<sup>Phox2b/Atoh1</sup> neurons were evaluated by loss of function experiments *ex vivo* (Figure 5) and *in vivo* (Figure  
394 6). Therefore, while other pF neurons as well as neighboring adrenergic C1 neurons (Abbott et al., 2011;  
395 Guyenet et al., 2013) may also be targeted by the locomotor spinal circuits, the RTN<sup>Phox2b/Atoh1</sup> subset may be  
396 the prominent integrator of the ascending locomotor drive, at least for the setting of respiratory frequency.

397 We also show that the RTN<sup>Phox2b/Atoh1</sup> neurons in turn project to the preBötC (Figure 7). This makes  
398 the preBötC inspiratory generator a final integrator of both the descending (from the CnF) and the ascending  
399 (from the lumbar spinal cord) locomotor drives and raises the question of the identity of the targeted  
400 neurons. Our transynaptic tracing scheme from the preBötC demonstrates that the CnF targets glutamatergic  
401 preBötC neurons (Figure 1h-j). Comparatively however, the number of transynaptically labeled neurons in  
402 the pF area was much lower (Figure S1d, e). Moreover, brief CnF photostimulations were most effective to  
403 shorten the respiratory cycle when delivered during early expiration but failed when delivered during  
404 inspiration (Figure 2). This is reminiscent to what was observed when directly stimulating glutamatergic  
405 preBötC neurons collectively (Baertsch et al., 2018) or the *Dbx1*-expressing V0 subset (Cui et al., 2016), the  
406 main rhythmogenic candidates (Bouvier et al., 2010; Gray et al., 2010). In contrast, when stimulating the  
407 RTN<sup>Phox2b/Atoh1</sup>, the shortening of the respiratory cycle is most efficient during inspiration and a significant  
408 lengthening of the respiratory cycle is observed when photoactivations are delivered in late expiration  
409 (compare Figure 2c with Figure 7f). This permissive action in inspiration and the lengthening in expiration  
410 recalls what others have reported when specifically activating inhibitory, but not excitatory, preBötC neurons  
411 (Baertsch et al., 2018). The RTN<sup>Phox2b/Atoh1</sup> and the CnF might thus preferentially target different cell-types in  
412 the preBötC: inhibitory neurons for the former, and glutamatergic ones for the latter. Note that such a bias

413 towards preBötC inhibitory target neurons for the RTN<sup>Phox2b/Atoh1</sup> compared to the CnF could would be  
414 mechanistically compatible with switching from CnF-induced “pre-loco” moderate respiratory frequency  
415 increase, to the higher “loco” respiratory frequency range associated to actual locomotion. Indeed, the  
416 current model ascribes a limited ability of preBötC excitatory neurons compared to inhibitory neurons at  
417 entraining high frequency rhythms (Baertsch et al., 2018). Although our data are compatible with such a  
418 working model, the proposed connectivity will need to be investigated directly by future work.

## 419 **Methods**

### 420 **Mice**

421 C57BL/6J wild-type mice were obtained by Janvier Labs (Le Genest-Saint-Isle, France). *Vglut2-IRES-*  
422 *Cre* animals (thereafter *Vglut2<sup>Cre</sup>*, Vong et al., 2011) and *Ai32(RCL-ChR2(H134R))/EYFP* (thereafter *ChR2<sup>flox</sup>*,  
423 Madisen et al., 2012) were obtained from Jackson Laboratories. To manipulate RTN<sup>Phox2b/Atoh1</sup> neurons we  
424 used the following mouse lines: *Egr2<sup>Cre</sup>* (Voiculescu et al., 2000) crossed with *Phox2b<sup>27AlaCK1</sup>* (Ramanantsoa et  
425 al., 2011a) and *Atoh1<sup>FRTCre</sup>;Phox2b<sup>Flpo</sup>* (Ruffault et al., 2015) mouse lines. Animals were group-housed with  
426 free access to food and water in controlled temperature conditions and exposed to a conventional 12-h  
427 light/dark cycle. Experiments were performed on animals of either sex, aged 2 to 3 months at the time of  
428 first injection. All procedures were approved by the French Ethical Committee (authorization 2020-  
429 022410231878) and conducted in accordance with EU Directive 2010/63/EU. All efforts were made to reduce  
430 animal suffering and minimize the number of animals.

### 431 **Viruses used**

432 For anterograde tracing and photostimulation of the CnF and its projection in the preBötC, we used  
433 a Cre-dependent AAV9-Ef1a-DIO-hChR2(E123T/T159C)-eYFP (Addgene #35509, titer 7.7e12 vp/ml, Mattis et  
434 al., 2011) unilaterally in the CnF (50 to 100 nL). For anterograde tracing from the lumbar spinal cord, the same  
435 virus was injected bilaterally (600-750 nL each side) in the lumbar segment, and for RTN<sup>Phox2b/Atoh1</sup>  
436 photostimulation, the injection was unilateral (350-500 nL). For reversible silencing of RTN<sup>Phox2b/Atoh1</sup> neurons  
437 we used bilateral injections (350-500 nL each side) of an AAV8.2-hEF1a-DIO-hM4Di-mCherry-WPRE obtained  
438 from Dr. Rachael Neve (Gene Delivery Technology Core, Massachusetts General Hospital, USA, titer: 5.6e12  
439 vp/ml). For transynaptic labelling of inputs onto preBötC neurons, we used 500 nL of a HSV1-hEF1a-LS1L-  
440 TVA950-T2A-rabiesOG-IRES-mCherry obtained from Dr. Rachael Neve (Gene Delivery Technology Core,  
441 Massachusetts General Hospital, USA), and 200 nL of EnvA-ΔG-rabies-GFP obtained by the GT3 core (Salk  
442 Institute, USA, titer: 9.59e9 vp/ml). For anterograde transynaptic tracing, we used injection of the AAV2/1-  
443 hSyn-Cre-WPRE-hGH (UPenn Vector Core, titer: 6.68e13 vg/ml, Zingg et al., 2017) bilaterally in the spinal cord  
444 (600-750 nL each side) and AAV-DJ-EF1-DIO-hChR2(E123T/T159C)-p2A-eYFP-WPRE (Addgene # 35509, titer:  
445 0.78e12 vg/ml) bilaterally in the pF area (300 nL). For anterograde synaptic tracing from the RTN<sup>Phox2b/Atoh1</sup> we

446 used a unilateral injection (350-500 nL) of an AAV8.2-hEF1a-DIO-synaptophysin-eYFP obtained from Dr.  
447 Rachael Neve (Gene Delivery Technology Core, Massachusetts General Hospital, USA, titer: 5.4e12 vp/ml).

## 448 **Surgical procedures**

### 449 *Injections and implants in the brainstem*

450 Animals were anesthetized with isoflurane throughout the surgery (4 % at 1 L/min for induction and  
451 2-3 % at 0.2 L/min for maintenance). Buprenorphine (0,025 mg/kg) was administered subcutaneously for  
452 analgesia before the surgery. The temperature of the mice was maintained at 36 °C with a feedback-  
453 controlled heating pad. Anesthetized animals were placed on a stereotaxic frame (Kopf) and the skull was  
454 exposed. Viral vectors were delivered using a pulled glass pipette connected to a syringe pump (Legato 130,  
455 KD Scientific, customized by Phymep, France). The infusion flow was set to 100 nL/min. Coordinates (in mm)  
456 used to target CnF neurons (Figures 1-3) were: 4.4 caudal to bregma, 1.3 lateral, and 2.8 mm from the skull  
457 surface. RTN<sup>Phox2b/Atoh1</sup> neurons were targeted unilaterally (Figure 7) or bilaterally (Figures 4, 6) using the  
458 following coordinates: 6.0 caudal to bregma, 1.25 lateral, and 200 µm above the ventral surface. Sequential  
459 bilateral injection in the preBötC (Figure 1) were performed at the following coordinates: -7.2 from bregma,  
460 1.25 lateral, and 500 µm above the ventral surface. After the injection, the pipette was held in place for 5 to  
461 10 min before being slowly retracted. For optogenetic activations of the CnF, the CnF fibers in the preBötC  
462 and the RTN<sup>Phox2b/Atoh1</sup>, a 200 µm core 0.39 NA optic fiber connected to a 1.25 mm diameter ferrule (Thorlabs)  
463 was implanted 0.4 mm above the injected site (CnF, Figures 2-3) or 0.8 mm (RTN<sup>Phox2b/Atoh1</sup>, Figure 7) or 1.5  
464 mm (preBötC, Figure 2) above the ventral surface. Optic fibers were secured to the skull with dental cement  
465 (Tetric Evoflow). Animals were followed daily after the surgery.

### 466 *Injections in the spinal cord*

467 Animals were anesthetized as described above and spinal injections were performed as previously  
468 done (Bouvier et al., 2015; Usseglio et al., 2020). A two cm incision of the skin was performed dorsally on  
469 anesthetized animals and the exposed spinal column was fixed with two holders on the left and right sides  
470 to a stereotaxic frame to minimize movements. Vertebral spinous processes were used as landmarks to  
471 target specific segments (Harrison et al., 2013). A small incision of the ligamentum Flavum allowed access to  
472 the spinal cord. A pulled glass pipette connected to a motorized syringe pump injector (Legato 130, KD  
473 Scientific, customized by Phymep, France) was positioned into the ventromedial area of the L2 (between the  
474 11<sup>th</sup> and 12<sup>th</sup> vertebral body) using the following coordinates: 350 µm laterally from the dorsal artery and 800  
475 µm depth from the dorsal surface. This lateral positioning ensures that the injection pipette does not pass  
476 through the lateral funiculus where ascending and descending axons travel. For Cholera Toxin B (CTB)  
477 experiments (Figure S6), we injected on each side of the spinal cord 600-750 nL of CTB-AF647 conjugate  
478 (ThermoFisher Scientific Reference C-34778) diluted at 0.5 % in sterile water. After each injection, the pipette



479 was held in place for 5 to 10 min before being slowly retracted. The skin was sutured, and animals were  
480 followed daily after the surgery. All animals recovered without motor impairments.

#### 481 *Diaphragm EMG recordings*

482 The protocol was described previously (Hérent et al., 2020). In brief, a 12 cm pair of electrodes was  
483 prepared from Teflon-coated insulated steel wires with an outside diameter of 0.14 mm (A-M systems, #  
484 793200). Two wires were lightly twisted together, and a knot was placed 5 cm from one end. At 1 cm from  
485 the knot, the Teflon insulation was stripped over 1 mm from each wire so that the two bared regions were  
486 separated by about 2 mm. The ends of the two wires were soldered to a miniature dissecting pin. The free  
487 ends of the electrodes, as well as a 5 cm ground wire, were soldered to a micro connector (Antelec). Nail  
488 polish was used to insulate the wires at the connector.

489 To implant the diaphragm as previously reported (Hérent et al., 2020), animals were anaesthetized,  
490 placed in a stereotaxic frame and hydrated by a subcutaneous injection of saline solution (0.9 %). Their  
491 temperature was maintained at 36°C with a feedback-controlled heating pad. This step was crucial to ensure  
492 post-surgery survival. The skull was exposed and processed to secure the micro connector using dental  
493 cement (Tetric Evoform). The ground wire was inserted under the neck's skin and the twisted electrodes were  
494 tunneled towards the right part of the animal guided by a 10 cm silicon tube of 2 mm inner diameter. The  
495 animal was then placed in supine position, the peritoneum was opened horizontally under the sternum,  
496 extending laterally to the ribs, and the silicon tube containing the electrodes was pulled through the opening.  
497 The sternum was clamped and lifted upwards to expose the diaphragm. A piece of stretched sterile parafilm  
498 was placed on the upper part of the liver to avoid friction during movement of the animal and to prevent  
499 conjunctive tissue formation at the recording sites. The miniature dissecting pin was pushed through the  
500 right floating ribs. The pin was then inserted through the sternum, leaving the bare part of the wires in  
501 superficial contact with the diaphragm. The position of the electrodes was secured on both sides of the  
502 floating ribs and sternum using dental cement. The pin was removed by cutting above the secured wires. The  
503 peritoneum and abdominal openings were sutured, and a head bar was placed on the cemented skull to  
504 facilitate animal's handling when connecting and disconnecting EMG cables during behavioral sessions.  
505 Buprenorphine (0.025 mg/kg) was administered subcutaneously for analgesia at the end of the surgery and  
506 animals were observed daily following the surgery and treated with Buprenorphine if needed.

#### 507 **Histology**

508 Adult mice were anesthetized with Euthazol Vet (140 mg/kg) and perfused with 4 %  
509 paraformaldehyde (PFA) in 1X Phosphate Buffered Saline (PBS). Brains and spinal cords were dissected out  
510 and fixed overnight in 4 % PFA at 4 °C. After fixation, tissues were rinsed in 1X PBS. Brain and spinal cord were  
511 cryoprotected overnight at 4 °C, respectively in 16 % and 20 % of sucrose in PBS. Tissues were rapidly cryo-  
512 embedded in OCT mounting medium and sectioned at 30 µm using a cryostat. Sections were blocked in a

513 solution of 1X Tris Buffered Saline (TBS), 5 % normal donkey serum and 1 % Triton X-100. The primary  
514 antibodies, carried out 48 hours at 4 °C, were: goat anti-ChAt (1:500, ref. # AB144P, Merck Millipore), chicken  
515 anti-GFP (1:500, ref. # 1020, Aves Labs), rabbit anti-RFP (1:500, ref. # 600-401-379, Rockland), rabbit anti-SST  
516 (1:500, ref. # T-4103, BMA Biomedicals), and sheep anti-TH (1:500, ref. # AB1542, Merck Millipore). Primary  
517 antibodies were detected after 2 hours of incubation at room temperature with appropriate secondary  
518 antibodies coupled to Alexa-Fluor 488, 647, Cy-3 or Cy-5 (1:500, Jackson ImmunoResearch). Sections were  
519 counterstained with a fluorescent Nissl stain (NeuroTrace 435/445 blue, ref. # N21479, 1:200 or NeuroTrace  
520 640/660 deep-red, ref. # N21483, 1:1000, Thermo Fisher Scientific) and mounted in Prolong Diamond  
521 Antifade Montant (P36970, Thermo Fisher Scientific) or ibidi Mounting Medium (50001, Ibidi). Sections were  
522 acquired with a Leica TCS SP8 confocal microscope (NeuroPICT imaging platform of the NeuroPSI Institute)  
523 with 10x and 25x objectives.

524 The preBötC was defined as located ventrally to the cholinergic ChAT<sup>+</sup> neurons of the nucleus  
525 ambiguus (na) where somatostatin<sup>+</sup> (SST) neurons are detected (Stornetta et al., 2003, Figures 1, 4, 7,  
526 corresponding to areas from 7.0 to 7.4 mm caudal to bregma). The pF respiratory region was defined as  
527 immediately ventral, ventro-median and ventro-lateral to the facial motor neurons (7N, Guyenet and Mulkey,  
528 2010, Figures 1, 4, 6, 7, corresponding to areas from 6.5 to 5.7 mm caudal to bregma).

## 529 **Behavioral experiments**

### 530 *Optogenetic activations*

531 Behavioral experiments started 4 to 5 weeks after the viral injection. Implanted animals were  
532 connected to a laser source (473 nm DPSS system, LaserGlow Technologies, Toronto, Canada) through a  
533 mating sleeve (Thorlabs). The laser was triggered by the output of a National Instruments interface (NI-USB  
534 6211) and the timings of light activations were delivered using the NI MAX software. For CnF or RTN<sup>Phox2b/Atoh1</sup>  
535 long photostimulation, light was delivered in trains of pulses of 20 ms (5 to 20 Hz) and of 15 ms (30 and 40  
536 Hz) frequency for a duration of 1 s. Each frequency stimulation was repeated three times with several  
537 minutes of rest between trials. We used the minimal laser power sufficient to evoke a response, which was  
538 measured to be between 5-12 mW at the fiber tip using a power meter (PM100USB with S120C silicon power  
539 head, Thorlabs) to restrict photoactivations unilaterally (Stujenske et al., 2015), prevent heat, and exclude an  
540 unintentional silencing by over-activation. For randomized short light-pulses, 50 ms light stimulations (50-70  
541 pulses/experiment) were applied randomly in the respiratory cycle.

542 *Plethysmography recordings*

543 To analyze the effect of short photoactivations of the CnF and CnF fibers in the preBötC (Figure 2), or  
544 the RTN<sup>Phox2b/Atoh1</sup> neurons (Figure 7) on burst timing, ChR2-injected animals were placed inside a hermetic  
545 whole-body plethysmography (WBP) chamber (Ruffault et al., 2015), customized to allow the passage of the  
546 optical patch-cord, four weeks after viral injection. The plethysmography signal was recorded over a period  
547 of 10 minutes using a National Instruments Acquisition card (USB-6211) and the LabScribe NI software  
548 (iWorxs).

549 *Locomotion in a linear runway*

550 Four to five weeks following the injection of the ChR2-expressing virus in the CnF, animals were  
551 implanted with a diaphragm EMG as explained previously (Figure 3). One week following EMG implantation,  
552 animals were placed in a linear corridor (80 x 10 cm), and familiarized for 1 h/day for 3 days, prior to the  
553 experiments. Implanted animals were filmed from the side at 200 fps and 0.5 ms exposure time using a CMOS  
554 camera (Jai GO-2400-USB) and images were streamed to a hard disk using the 2<sup>nd</sup> LOOK software (IO  
555 Industries). The start of the EMG recordings was hardware-triggered by the start of the video-recordings  
556 using the frame exposure readout of the video camera, so that the two recordings are synchronized. When  
557 animals were immobile at one end of the corridor and the respiration was stable, we delivered CnF  
558 optogenetic activations with frequencies ranging from 5 to 40 Hz. For each frequency, the stimulation was  
559 repeated three times with several minutes of rest between trials.

560 *Chemogenetic silencing and treadmill experiment*

561 Three weeks following the injection of the hM4Di virus in the RTN<sup>Phox2b/Atoh1</sup>, animals were implanted  
562 for diaphragm EMG recordings as explained above (Figure 6). Non-injected C57BL/6J mice were also  
563 implanted as controls to test for CNO side effects. One week following EMG implantation, implanted animals  
564 were familiarized on a stationary custom-made motorized treadmill with adjustable speed range (Scop Pro,  
565 France, belt dimensions: 6 cm x 30 cm) for 30 min/day for 3 days, prior to the experiments. In addition,  
566 implanted animals were exercised during this time for a total of 5 min at 40 cm/s each day. Mice could rest  
567 for 5 min after running before being placed back in their cage. This step was crucial to obtain stable running  
568 animals during experimental sessions. Following this short training, implanted mice were connected with  
569 custom light-weight cables to an AC amplifier (BMA-400, CWE Inc.) and neurograms were filtered (high-pass:  
570 100 Hz, low-pass: 10 kHz), collected at 10 kHz using a National Instruments acquisition card (USB-6211) and  
571 live-integrated using the LabScribe NI software (iWorxs). Animals were first placed on the stationary treadmill  
572 to monitor basal respiration. Animals were then challenged to trot at 40 cm/s for 1.5 min before being  
573 administered intraperitoneally with CNO (Enzo Life science, ref. #: BML-NS105-0005, 10 mg/kg) or saline (0.9  
574 %). Animals were placed again on the treadmill with the same paradigm 2-3 h and 5 h after CNO or saline

575 administration to measure respiration in resting and running conditions. During experiments, animals were  
576 filmed from the side in the same way as above to monitor the stability of running episodes.

### 577 *Ex vivo brainstem-spinal cord experiments*

578 Pups aged 1-2 days were used in all experiments. The pups were anaesthetized with isoflurane,  
579 decerebrated and the brainstem still attached to the spinal cord was dissected and isolated in ice-cold  
580 Ringer's solution that contained (in mM): 111 NaCl, 3 KCl, 25 NaHCO<sub>3</sub>, 1.25 MgSO<sub>4</sub>, 1.1 KH<sub>2</sub>PO<sub>4</sub>, 2.5 CaCl<sub>2</sub> and  
581 11 D-Glucose, and oxygenated in 95 % O<sub>2</sub>, 5 % CO<sub>2</sub> to obtain a pH of 7.4. Isolated brainstem-spinal cords were  
582 transferred into a recording chamber and pinned to a Sylgard 184 resin. Preparations were partitioned in two  
583 compartments at the level of lower thoracic segments (T11) using a Vaseline wall, to restrict bath application  
584 of locomotor drugs on the lumbar spinal cord (Figure 5). The lumbar compartment was continuously perfused  
585 with the above Ringer's solution while the rostral compartment containing the brainstem was superfused  
586 with a Ringer's solution that contained (in mM): 111 NaCl, 8 KCl, 25 NaHCO<sub>3</sub>, 3.7 MgSO<sub>4</sub>, 1.1 KH<sub>2</sub>PO<sub>4</sub>, 1.25  
587 CaCl<sub>2</sub> and 30 D-Glucose. All recordings were done at room temperature (25°C) after allowing 30 min recovery  
588 period after the dissection. Respiratory- and locomotor-like activities were recorded respectively on the 4<sup>th</sup>  
589 cervical (C4) and the 2<sup>nd</sup> lumbar (L2) ventral roots using extracellular suction glass pipettes (120F-10, Harvard  
590 Apparatus). Drug-evoked locomotor-like activities were induced by bath-applying 10 to 14 μM of N-methyl-  
591 D-aspartate (NMDA, Tocris) and serotonin (5-HT, Sigma-Aldrich, Figure 5) or using blue light on the lumbar  
592 spinal cord of ChR2-expressing pups (*Vglut2<sup>Cre</sup>;ChR2<sup>flxed</sup>*, Figure S7). Signals were collected and band-passed  
593 filtered at 100 Hz to 1 kHz with an AC amplifier (Model 1700, A-M Systems) and live-integrated (Neurolog  
594 NL703, Digitimer) with a time constant of 100 ms (C4) or 200 ms (L2). Signals were sampled using Clampex  
595 11 (Molecular Devices) at 5 kHz. To control for locomotor drugs leakage, some preparations were transected  
596 at the level of the cervical spinal cord (Figure S7a-c). For brainstem transection experiments, the rostral part  
597 of the brainstem containing the pF respiratory region was physically removed (Figure 5d-f).  
598 *Egr2<sup>Cre</sup>;Phox2b<sup>27AlaCKI</sup>* pups were used to genetically eliminate RTN<sup>*Phox2b/Atoh1*</sup> neurons (Ramanantsoa et al.,  
599 2011a; Ruffault et al., 2015, Figure 5g-i).

## 600 **Quantifications and statistical analysis**

### 601 *Phase-shift analysis*

602 We measured the duration of the respiratory cycle containing the light stimulus (perturbed cycle,  $\theta$ )  
603 and the previous respiratory cycle (control cycle,  $\phi$ , Figures 2 and 7). One respiratory cycle was defined as  
604 from the onset of inspiration to the subsequent inspiratory onset. The phase of light-stimulation  $\phi_s$  was  
605 defined as from the onset of the perturbed cycle to the onset of the light pulse. The perturbed cycle  $\theta$  was  
606 defined as from the onset of the inspiration that precedes the light stimulation to the onset of the subsequent  
607 inspiration. The perturbed phase (phase-shift) was calculated as the ratio of the perturbed cycle divided by  
608 the control cycle ( $\theta/\phi$ ). The light phase was defined as the ratio of the stimulated cycle divided by the control

609 cycle ( $\phi_s/\phi$ ). The perturbed phase was then plotted against the light phase for all events from all animals.  
610 The number of events (N) and animals (n) are given in the corresponding figures for all tested condition. In  
611 addition, the average perturbed phase was plotted against the average light phase in 0.1 ms bins as mean  $\pm$   
612 SD. Inspiratory time (I) was measured, averaged for each animal and a grand average was calculated and  
613 annotated in the corresponding figures for all tested condition. Expiratory time (E) was calculated from  
614 respiratory cycle and inspiratory (I) times.

#### 615 *Locomotor parameters analysis*

616 To track the mouse displacement and measure its speed, we used DeepLabCut (version 2.1.5.2,  
617 Mathis et al., 2018) and manually labelled the positions of the head from 50 frames of each video. We then  
618 used 95 % of the labelled frames to train the network using a ResNet-50-based neural network with default  
619 parameters for 3 training iterations. This network was then used to analyze videos from similar experimental  
620 settings. For treadmill experiments, the head X coordinate was used as a control for running stability on the  
621 treadmill. For CnF stimulations on the corridor, the head X coordinate was used to calculate the animal's  
622 speed  $s_x$  using the gradient over time.

$$623 \quad \vec{s}_x = \frac{\partial \vec{x}}{\partial t}, \vec{x} \text{ being the displacement of the head along the } x \text{ axis}$$

624 Head X coordinate (treadmill) and calculated velocity (corridor) were then exported to Clampfit  
625 (Molecular Devices) and interpolated to 10 kHz to match the acquisition framerate of diaphragmatic EMG  
626 recordings. Both sets of signals (head X and diaphragm, or speed and diaphragm) were merged in single files,  
627 before being processed offline in Clampfit. The animal's instantaneous speed is illustrated on figures (Figure  
628 3). The mean speed, defined from the onset of the movement to the end of the photostimulation, was then  
629 calculated using the statistic function in Clampfit for each CnF stimulation (5 to 40 Hz). All values were  
630 averaged across trials for each animal (3 trials/animal), and a grand mean  $\pm$  SD across n animals was  
631 calculated per stimulation frequency (Figure S4b). Locomotor onset latencies (Figure S4c) were defined as  
632 the delay between the onset of the CnF stimulation and the onset of movement for each CnF stimulations.  
633 All values were averaged across trials (3 trials/animal) and a grand mean  $\pm$  SD across n animals was calculated  
634 per stimulation frequency.

635 For gait analysis during CnF photostimulations (Figure S4d), we manually annotated the paw of a  
636 reference hindlimb (ipsilateral) and registered the timings of footfalls (when the paw first touches the floor).  
637 Each reference locomotor cycle was then defined as the duration from one footfall ( $t_{\text{ipsi\_FF}_n}$ ) to the next  
638 ( $t_{\text{ipsi\_FF}_{n+1}}$ ). The time of occurrence of the contralateral hindlimb footfall within the reference locomotor  
639 cycle was annotated manually ( $t_{\text{contra\_FF}}$ ) and the synchronicity rate was then computed as follows:

$$640 \quad \text{synchronicity rate} = \frac{t_{\text{contra\_FF}} - t_{\text{ipsi\_FF}_n}}{t_{\text{ipsi\_FF}_{n+1}} - t_{\text{ipsi\_FF}_n}}$$

641 A custom MATLAB script was then used to categorize synchronized (synchronicity rate  $\in [0,$   
642  $0.25 \cup [0.75, 1]$ ) or alternated (synchronicity rate  $\in [0.25, 0.75]$ ) ipsi- and contralateral hindlimb steps.  
643 Synchronicity rates were averaged across animals (3 trials/animal) and a grand mean  $\pm$  SD across n animals  
644 was calculated per stimulation frequency.

#### 645 *Locomotor/respiratory coordination*

646 The temporal coordination of breaths to strides (Figure S5) was represented with circular statistics,  
647 similarly to what we performed recently (Hérent et al., 2020) and imprinting from numerous studies having  
648 investigated the cycle-to-cycle correlations of motor activities (Kjaerulff and Kiehn, 1996; Talpalar et al., 2013;  
649 Skarlatou et al., 2020). The phase of each individual inspiratory burst within the locomotor cycle ( $\Phi_{\text{Insp}}$ ,  
650 around 15 bursts) is represented as the position, from 0 to 1, of the diamond marks on the outer circle (see  
651 Figure S5d). For each animal, we also computed the mean phase of consecutive inspiratory bursts and  
652 represented it as a colored circle (the mean phases of different animals are in different colors). The distance  
653 R of the mean phase to the center of the circle indicates the concentration of individual phase values around  
654 the mean, as established by (Kjaerulff and Kiehn, 1996). If inspiratory and locomotor movements are  
655 temporally correlated, then individual phase values will be concentrated around a preferred phase value (for  
656 instance 0 or 1, at the top of the circle, if the two motor activities were in phase). The mean value would then  
657 be positioned at a significant distance from the center. Conversely, if inspiratory and locomotor movements  
658 are not coupled, individual phases will be evenly distributed across the circle. Consequently, the mean phase  
659 value will be at a short distance from the diagram center, illustrating the dispersion of values around the  
660 mean. The inner circles of the circular diagrams depict the threshold for mean phase values to be considered  
661 significantly oriented ( $R < 0.3$ ) as commonly done (Kjaerulff and Kiehn, 1996; Talpalar et al., 2013; Hérent et  
662 al., 2020; Skarlatou et al., 2020). Circular plots were obtained using a custom macro in Excel.

#### 663 *In vivo respiratory changes analysis*

664 To analyze breathing changes resulting from CnF (EMG recordings, Figure 3) and RTN<sup>Phox2b/Atoh1</sup>  
665 photostimulation (WBP recordings, Figure 7), instantaneous inspiratory frequencies were detected over a 1  
666 s window, using the threshold search in Clampfit before, during and directly after a 1 s light stimulation for  
667 all frequencies (5 to 40 Hz). For CnF stimulations that triggered locomotor episodes (15 to 40 Hz), the recovery  
668 period was measured as soon as the animal returned to immobility. Breaths detected from the onset of the  
669 light stimulus to the onset of the movement were categorized as during the “pre-loco” (Figure 3d). Breaths  
670 detected from the onset of the movement to the offset of the light stimulus were categorized as during the  
671 “loco” phase (Figure 3d, f). All values were averaged across animals (3 trials/animal) and a grand mean  $\pm$  SD  
672 across n animals was calculated per stimulation frequency.

673 For treadmill running (Figure 6), the mean diaphragm frequency was analyzed prior to exercise  
674 (resting condition) and from stable trotting moments, i.e., when the animal’s speed was in phase with the

675 treadmill, inferred by the absence of changes in head's X coordinates (running condition). Instantaneous  
676 respiratory frequency was measured for a total duration of 6 s in each condition: before (CTL), during  
677 (CNO/saline) and after (REC) administration of either CNO or saline. These measurements were done using 2  
678 to 3 windows taken during resting conditions and at any stable moment of the 1.5 min run (excluding the  
679 first 20 seconds to exclude possible stress-induced changes when the treadmill is just engaged).  
680 Measurements were averaged to give the mean value for each animal. Averaged mean values were  
681 expressed as mean  $\pm$  SD across n animals.

#### 682 *Ex vivo respiratory-like activities analysis*

683 Instantaneous respiratory-like frequencies were analyzed offline using the threshold search in  
684 Clampfit (Molecular Devices) before, during and after bath application of NMDA and 5-HT (Figure 5).  
685 Respiratory frequency changes during drug and washout conditions were normalized and expressed as a  
686 percent of the control. A grand mean  $\pm$  SD across n animals was calculated.

#### 687 *Statistical Analysis*

688 All data are expressed as mean  $\pm$  SD. Statistical tests were performed using GraphPad Prism 7 and  
689 are spelled out in the figure legends, as well as the number of trials (N) and animals (n) used for each  
690 experiment. Changes were considered as not significant (ns) when  $p > 0.05$  and as significant when  $p < 0.05$ .  
691 Significance levels are reported as follows: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; and \*\*\*\*,  $p < 0.0001$ .

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697 Gatier for help with DeepLabCut and analytic scripts, Jean-François Brunet for providing the  
698 *Atoh1<sup>FRTCre</sup>;Phox2b<sup>Flpo</sup>* animals, and the animal facility for housing animals.

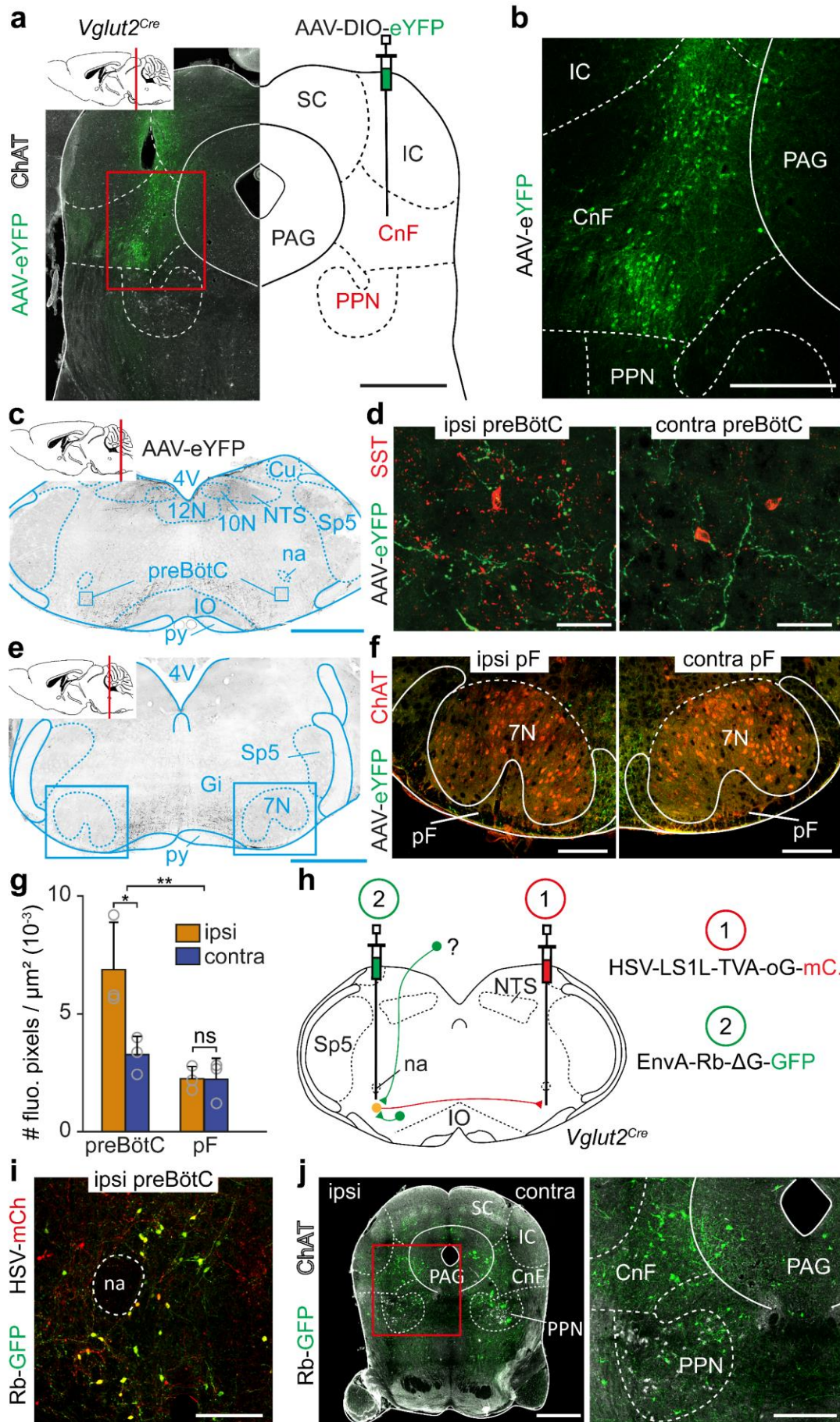
## 699 **Author contribution**

700 J.B. and G.F. designed the study and provided funding. J.B. supervised the work. C.H. performed and analyzed  
701 experiments with contributions from S.D. and J.B. J.B. and C.H. prepared figures. J.B. wrote the paper and all  
702 authors contributed to its editing.

## 703 **Competing interests**

704 The authors declare no competing interests.

705 **Figures and legends**





706 **Figure 1: CnF glutamatergic neurons contact the preBötC inspiratory generator.**

707 **(a)** Transverse section at the level of the injection site in the mesencephalon, showing glutamatergic CnF neurons  
708 transfected unilaterally with an AAV-DIO-eYFP on a *Vglut2<sup>Cre</sup>* adult mouse. The MLR component nuclei CnF and PPN are  
709 in red. Cholinergic PPN neurons are identified by Choline Acetyl Transferase (ChAT) expression. Scale bar, 1 mm.

710 **(b)** Magnification of the boxed area in **(a)**, showing transfected eYFP<sup>+</sup> cells concentrated in the CnF. Scale bar, 400  $\mu$ m.

711 **(c)** Transverse section in the caudal medulla showing the distribution of CnF projections in the ventral reticular  
712 formation including the preBötC. Scale bar, 1 mm.

713 **(d)** Magnifications of the boxed areas in **(c)**, over the ipsilateral and contralateral preBötC containing SST<sup>+</sup> cells and  
714 processes. Note that the CnF projects to both sides with an ipsilateral predominance. Scale bar, 40  $\mu$ m.

715 **(e)** Transverse section in the rostral medulla showing the distribution of CnF projections at the level of the parafacial  
716 (pF) respiratory area. Scale bar, 1 mm.

717 **(f)** Magnifications of the red boxed areas over the ipsilateral and contralateral pF respiratory areas, delineated as  
718 ventral, ventro-median and ventrolateral to the ChAT-positive facial motor nucleus (7N). Note that the CnF projects very  
719 little to the pF area on either side. Scale bar, 250  $\mu$ m. Panels **(a-f)** are representative of n=3 animals.

720 **(g)** Bar-graphs showing the mean density  $\pm$  SD across mice of eYFP<sup>+</sup> fluorescent pixels located ipsilaterally and  
721 contralaterally in the preBötC and the pF. Grey open circles are the mean values of individual mice. \*\*, p < 0.01; \*, p <  
722 0.05; ns, not-significant (Wilcoxon matched-pairs tests; preBötC, 9 sections per side; pF, 9 sections per side; from n = 3  
723 mice).

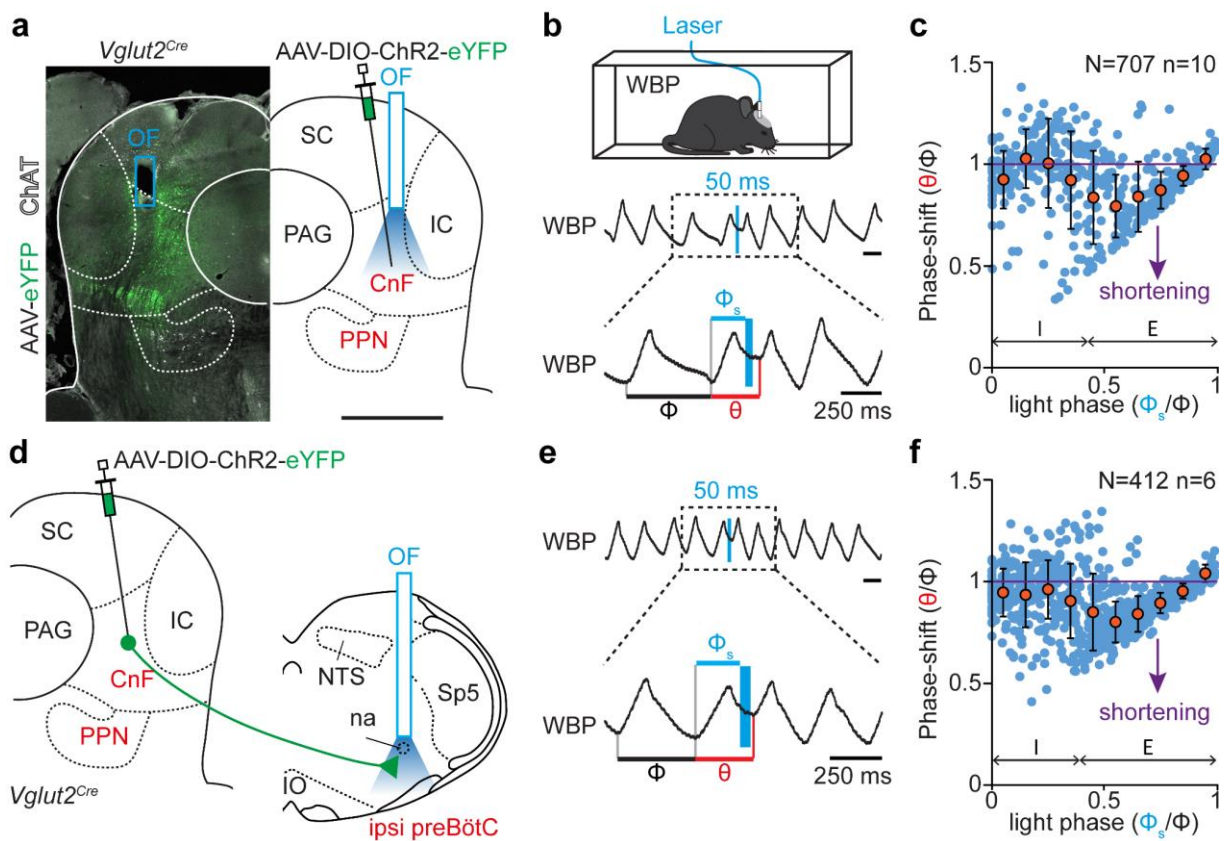
724 **(h)** Retrograde transynaptic monosynaptic tracing from glutamatergic commissural preBötC neurons. An HSV-LS1L-TVA-  
725 oG-mCherry is first injected in the contralateral preBötC followed by a G-deleted and EnvA pseudotyped Rb virus (EnvA-  
726  $\Delta$ G-Rb-GFP) in the ipsilateral preBötC in a *Vglut2<sup>Cre</sup>* adult mouse.

727 **(i)** Magnification over the ipsilateral preBötC from a transverse section showing double-transfected “starter” cells. Scale  
728 bar, 200  $\mu$ m.

729 **(j)** Left: Transverse section at the MLR level showing putative presynaptic cells (Rb-GFP<sup>+</sup>) on a ChAT background. Scale  
730 bar, 1 mm. Right: magnification of the boxed area showing the presence of transynaptically labelled cells in the CnF and  
731 PPN. Scale bar, 250  $\mu$ m. Panels **(h,j)** are representative of n= 3 animals.

732 See also Figure S1.

733 Abbreviations used in all figures: PAG: periaqueductal gray; IC: inferior colliculus; SC: superior colliculus; PPN:  
734 pedunculo-pontine nucleus; CnF: cuneiform nucleus; 4V: fourth ventricle; 10N: dorsal motor nucleus of vagus ; 12N:  
735 hypoglossal motor nucleus; NTS: nucleus tractus solitarius; py: pyramidal tract; IO: inferior olive; Cu: cuneate nucleus;  
736 na: nucleus ambiguus; preBötC: pre-Bötzinger complex; pF: parafacial respiratory area; Sp5: spinal trigeminal nucleus;  
737 7N: facial nucleus; Gi: gigantocellular reticular nucleus.



738 **Figure 2: Photoactivation of glutamatergic CnF neurons impacts respiratory rhythm generation in the preBötC.**

739 **(a)** Left: low magnification of a transverse section at the level of the injection and optic fiber (OF) implantation sites,  
 740 showing glutamatergic CnF neurons transfected with an AAV-DIO-ChR2-eYFP in a *Vglut2<sup>Cre</sup>* adult mouse. The MLR  
 741 component nuclei CnF and PPN are in red. Cholinergic PPN neurons are identified by Choline Acetyl Transferase (CHAT)  
 742 expression. Scale bar, 1 mm.

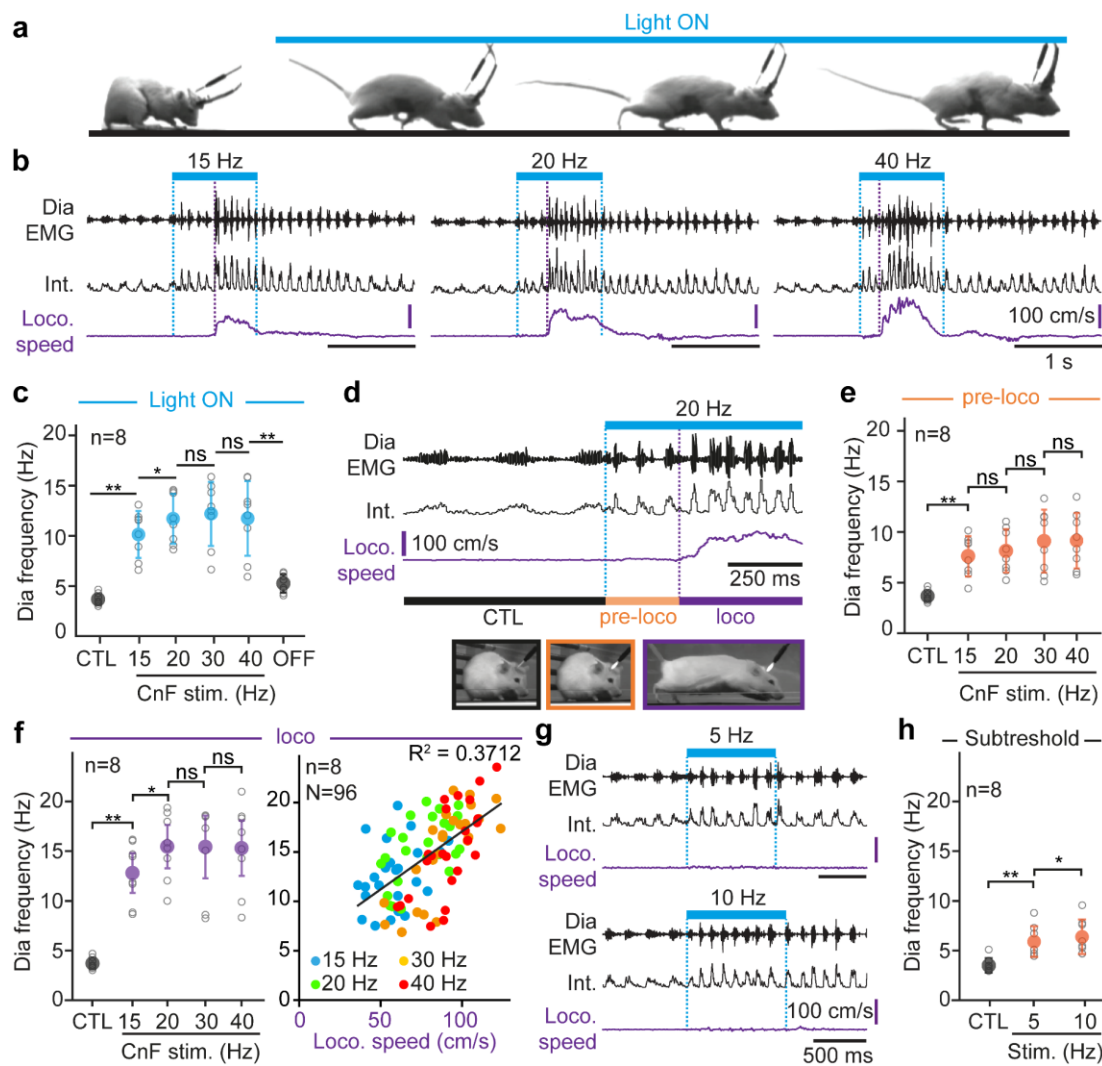
743 **(b)** Top: setup for recording ventilation using whole-body plethysmography (WBP) during optogenetic activations.  
 744 Middle: WBP recordings around a single 50 ms light-stimulation pulse of glutamatergic CnF neurons during the  
 745 expiratory phase of one respiratory cycle (inspirations are upwards, expirations are downwards). Note that the  
 746 stimulation shortens the respiratory cycle. Bottom: magnification of the above trace showing the control cycle ( $\phi$ , black),  
 747 the phase of light-stimulation ( $\phi_s$ , blue), and the perturbed cycle ( $\theta$ , red).

748 **(c)** Plot of the phase-shift (perturbed cycle normalized to the control cycle:  $\theta/\phi$ ) as a function of the phase of light-  
 749 stimulation normalized to the control cycle ( $\phi_s/\phi$ ). Values < 1 (purple line) indicate a shortening of the perturbed cycle.  
 750 Note that the phase-shift is shortened when the light pulse occurs in the expiratory phase of the respiratory cycle.  
 751 Inspiration (I) and expiration (E) mean durations are indicated. Blue circles represent individual data from N = 707  
 752 random trials from n = 10 mice. Orange circles are averages  $\pm$  SD across all trials within 0.1 ms bins.

753 **(d)** Experimental strategy for photostimulating glutamatergic CnF neurons fibers in the preBötC: the injection is done as  
 754 above but a second optic fiber is implanted above the preBötC on the ipsilateral side.

755 **(e-f)** Same as in (c-d) during a single 50 ms light-stimulation of the preBötC area showing again a shortening of the  
 756 respiratory cycle when the light pulse is delivered during expiration. N = 412 random trials from n = 6 mice.

757 See also Figures S2, S3.



758 Figure 3: Glutamatergic CnF neurons upregulate breathing in synergy with, and even in the absence of, locomotion.

759 **(a)** Example snapshots showing that photostimulation of the glutamatergic CnF neurons for 1 s (20 Hz pulses) triggers  
760 running. The injection and implantation strategy is similar to that described in Figure 2a.

761 **(b)** Raw (Dia EMG) and integrated (Int.) electromyogram of the diaphragm and instantaneous locomotor speed during  
762 1 s CnF photostimulations at 15, 20 and 40 Hz. Dotted blue lines: onset and offset of light stimulations; purple dotted  
763 line: onset of the locomotor episode. Note that breathing frequency increases at light onset, before the animal starts  
764 running, and increases further upon running.

765 **(c)** Quantification of the diaphragmatic frequency at rest (CTL), during the entire CnF photostimulation at increasing  
766 frequencies, and following light offset (OFF).

767 **(d)** Top: enlarged EMG recording of one representative animal during rest (CTL) and during the 1 s CnF photostimulation  
768 at 20 Hz which is divided in two phases: “pre-loco” (orange) during which the animal remains still, and “loco” (purple)  
769 when the animal engages in running. Bottom: snapshots of one animal in the 3 states.

770 **(e)** Quantification of the diaphragm frequency in control (CTL) and during the “pre-loco” phase at increasing CnF  
771 photostimulation frequencies showing a significant increase from baseline for 15 Hz photostimulations but no further  
772 increase at higher frequencies.

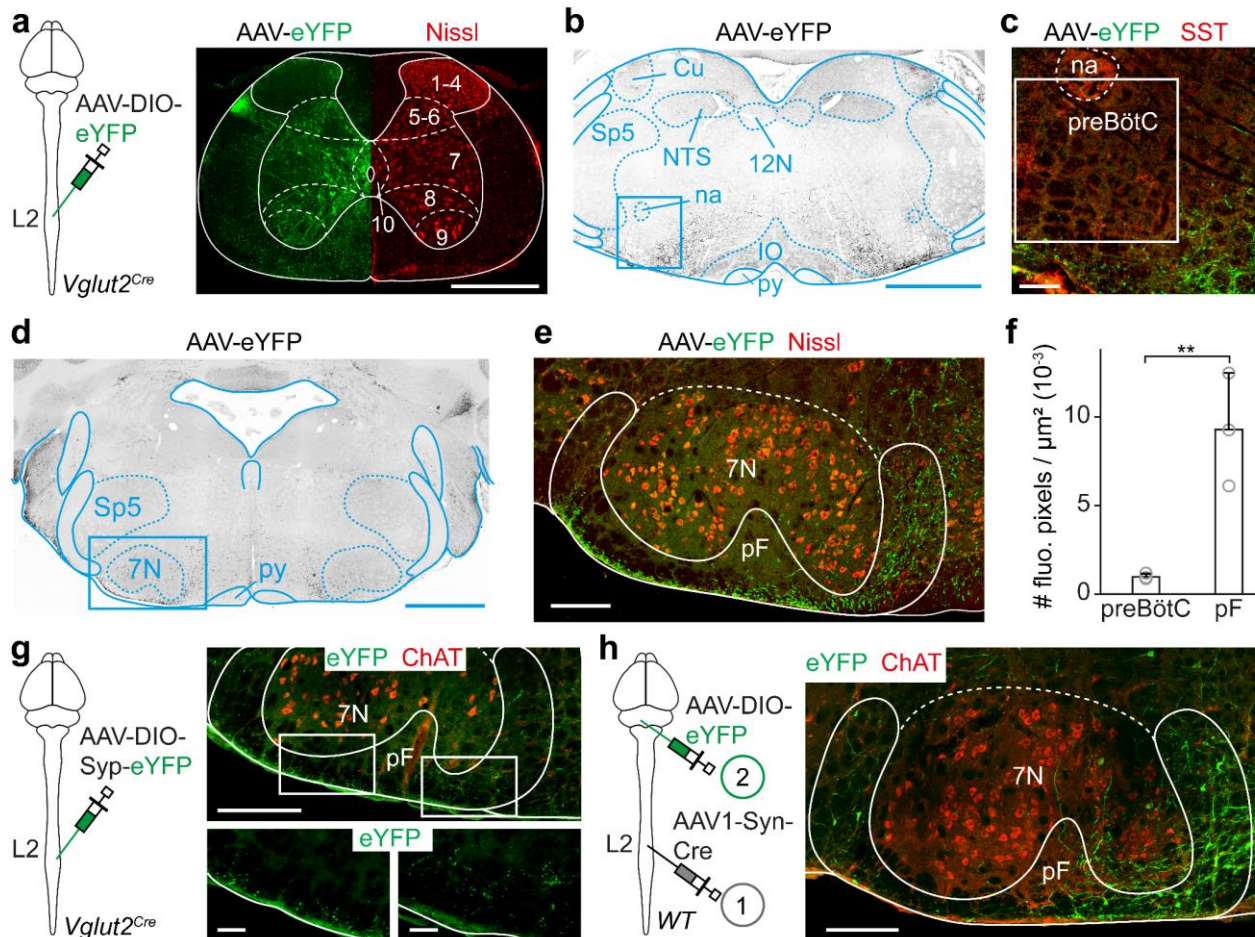
773 **(f)** Left: similar quantifications during the “loco” phase. Right: color plot showing the diaphragm frequency in relation  
774 to locomotor speed. Note that 37 % of the diaphragm frequency correlates with the locomotor speed (linear regression,  
775 black line). N = 96 trials from n = 8 mice.

776 **(g)** Example traces from one representative animal during CnF photostimulations at 5 and 10 Hz. Although these  
777 stimulations are below the threshold for running initiation, breathing frequency is significantly increased.

778 **(h)** Quantifications of the diaphragm frequency in control (CTL) and during subthreshold CnF stimulations.

779 In all graphs, gray open circles are the means of 3 trials for individual animals and colored circles are the means  $\pm$  SD  
780 across n mice. ns, not significant. \*,  $p < 0.05$ , \*\*,  $p < 0.01$  (Wilcoxon matched-pairs tests).

781 See also Figures S3, S4 and S5.



782 **Figure 4: Glutamatergic neurons of the lumbar spinal cord contact the pF respiratory area.**

783 **(a)** Experimental strategy for labelling the projections of glutamatergic lumbar neurons: an AAV-DIO-eYFP is injected  
 784 bilaterally in the 2<sup>nd</sup> lumbar spinal segment (L2) in a *Vglut2<sup>Cre</sup>* adult mouse. Transfected cells are concentrated in ventral  
 785 laminae (delineated using a Nissl counterstain) except lamina 9 containing motoneurons. Scale bar, 500  $\mu$ m.

786 **(b)** Transverse brainstem section at the level of the preBötC showing eYFP<sup>+</sup> projections in grey. Scale bar, 1 mm.

787 **(c)** Magnification of the blue boxed area that depicts the 400 x 400  $\mu$ m square used to delineate anatomically the  
 788 preBötC in this study, with a counterstaining for somatostatin (SST). Note the absence of eYFP<sup>+</sup> projections. Scale bar,  
 789 100  $\mu$ m.

790 **(d)** Transverse brainstem section at the level of the pF showing eYFP<sup>+</sup> projections in grey. Scale bar, 1 mm.

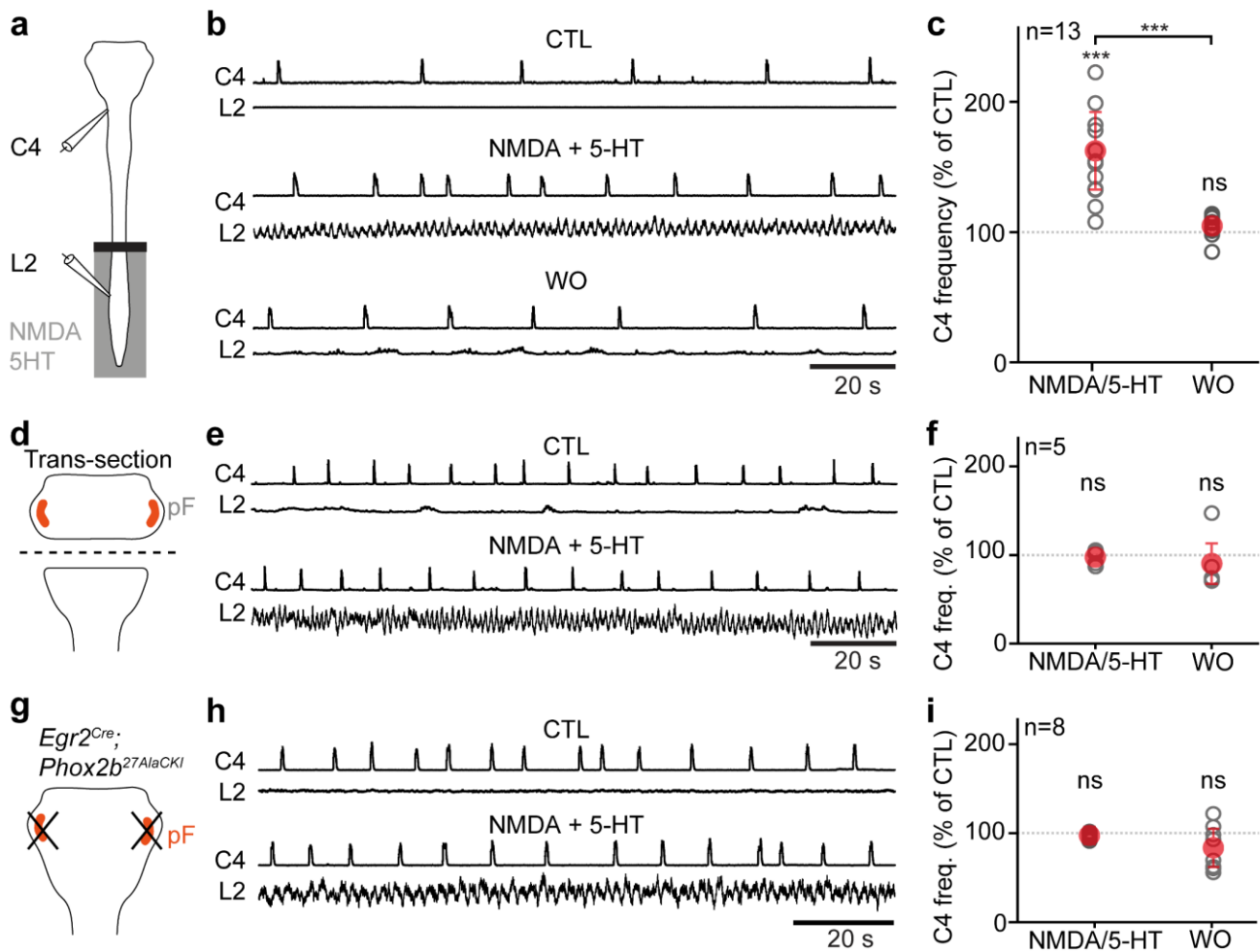
791 **(e)** Magnification of the boxed area. The pF area is framed in white. Note the abundant eYFP<sup>+</sup> projections from the spinal  
 792 cord.

793 **(f)** Bar-graphs showing the mean density  $\pm$  SD across mice of eYFP<sup>+</sup> fluorescent pixels in the preBötC and the pF (both  
 794 sides pooled). Open gray circles are the mean values of individual mice. \*\**p* < 0.01 (Wilcoxon matched-pairs tests; 9  
 795 sections from *n* = 3 mice).

796 **(g)** Bilateral injection, in the L2 segment of a *Vglut2<sup>Cre</sup>* adult mouse of an AAV-DIO-Syp-eYFP. Labelled boutons are  
 797 detected in the pF on transverse sections, ascertaining that ascending projections from the spinal cord form synaptic  
 798 contacts. Scale bars, 250 (top) and 50 (bottom)  $\mu$ m. Representative of *n* = 3 mice.

799 **(h)** Anterograde transsynaptic tracing from the ventral lumbar spinal cord using an AAV1-Syn-Cre injected bilaterally at  
 800 L2 followed by an AAV-DIO-eYFP injected in the pF area in a wild-type adult mouse. The transverse section shows eYFP<sup>+</sup>  
 801 neurons in the pF area, further confirming that spinal ascending projections establish synaptic contacts in this area.  
 802 Representative of *n* = 3 mice. Scale bar, 200  $\mu$ m.

803 See also Figure S6.



804 **Figure 5: Pharmacological activation of locomotor-like activities increases inspiratory-like frequencies *ex vivo*, which**  
 805 **requires the *Atoh1*<sup>+</sup>/*Phox2b*<sup>+</sup> RTN integrity.**

806 **(a)** Schematics of the isolated *ex vivo* brainstem-spinal cord preparation from neonates (1-2 days) used throughout the  
 807 figure. Respiratory- and locomotor-like activities are recorded using glass suction electrodes attached to the 4<sup>th</sup> cervical  
 808 (C4) and the 2<sup>nd</sup> lumbar (L2) motor nerve roots respectively. The preparation is separated in two compartments using a  
 809 Vaseline barrier (black bar). The lumbar spinal cord is superfused with control or locomotor drugs enriched (NMDA and  
 810 5-HT, 10-14  $\mu$ M each) artificial cerebrospinal fluid (aCSF) while the brainstem compartment remains in control aCSF.

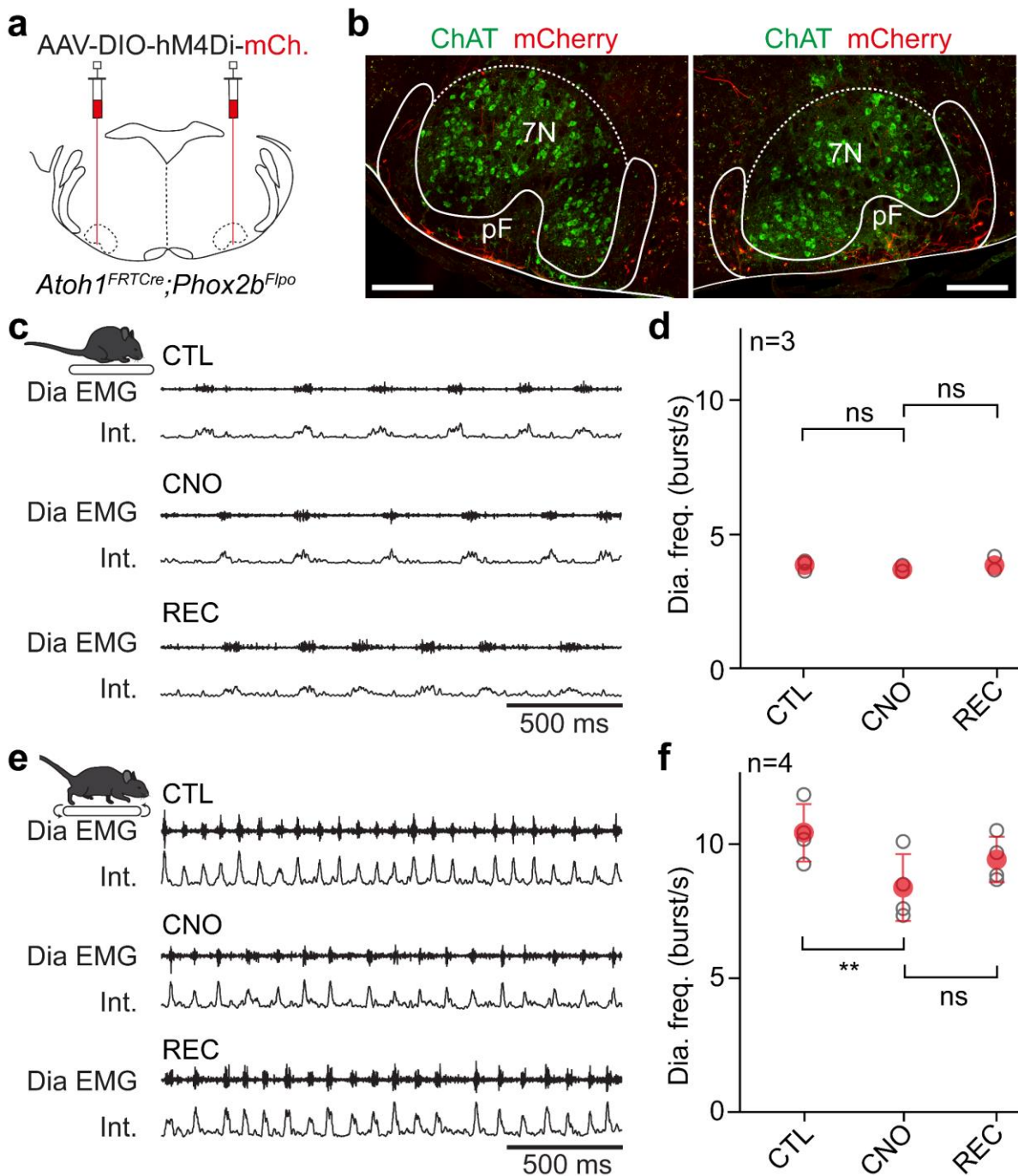
811 **(b)** Recordings of C4 and L2 activities of one representative preparation before (CTL), during, and after (washout, WO)  
 812 perfusion of locomotor drugs on the lumbar spinal cord (only integrated traces are shown). Note the triggering of  
 813 locomotor-like activity on L2 and a concomitant increase in the respiratory-like frequency on C4.

814 **(c)** Quantification of the C4 frequency change during drug-induced locomotor-like activity (NMDA/5-HT) and during  
 815 washout, as a percent change to the CTL condition (100 %, grey dotted line). Grey open circles are the means of  
 816 individual *n* preparations, and red circles are the means  $\pm$  SD across preparations.

817 **(d-f)** Similar experiments as in **(a-c)** but a brainstem transection was performed to physically remove the pF respiratory  
 818 area. Note the absence of frequency change on the C4 root during drug application.

819 **(g-i)** Similar experiments as in **(a-c)** performed in *Egr2*<sup>Cre</sup>;*Phox2b*<sup>27AlaCKI</sup> neonates, which lack pF neurons that express  
 820 *Phox2b* and are derived from rhombomere 5. This intersection recapitulates the genetically-defined RTN<sup>*Phox2b/Atoh1*</sup>. Note  
 821 the absence of frequency change on the C4 root during drug application. \*\*\*, *p* < 0.001; ns, not significant (Wilcoxon  
 822 matched-pairs tests).

823 See also Figures S7 and S8.



824 **Figure 6: Silencing RTN<sup>Phox2b/Atoh1</sup> neurons reduces respiratory frequency during running.**

825 **(a)** Experimental strategy for the bilateral transfection of RTN<sup>Phox2b/Atoh1</sup> neurons with the inhibitory DREADD receptor  
826 hM4Di in an *Atoh1*<sup>FRTCre</sup>; *Phox2b*<sup>Flpo</sup> adult mouse.

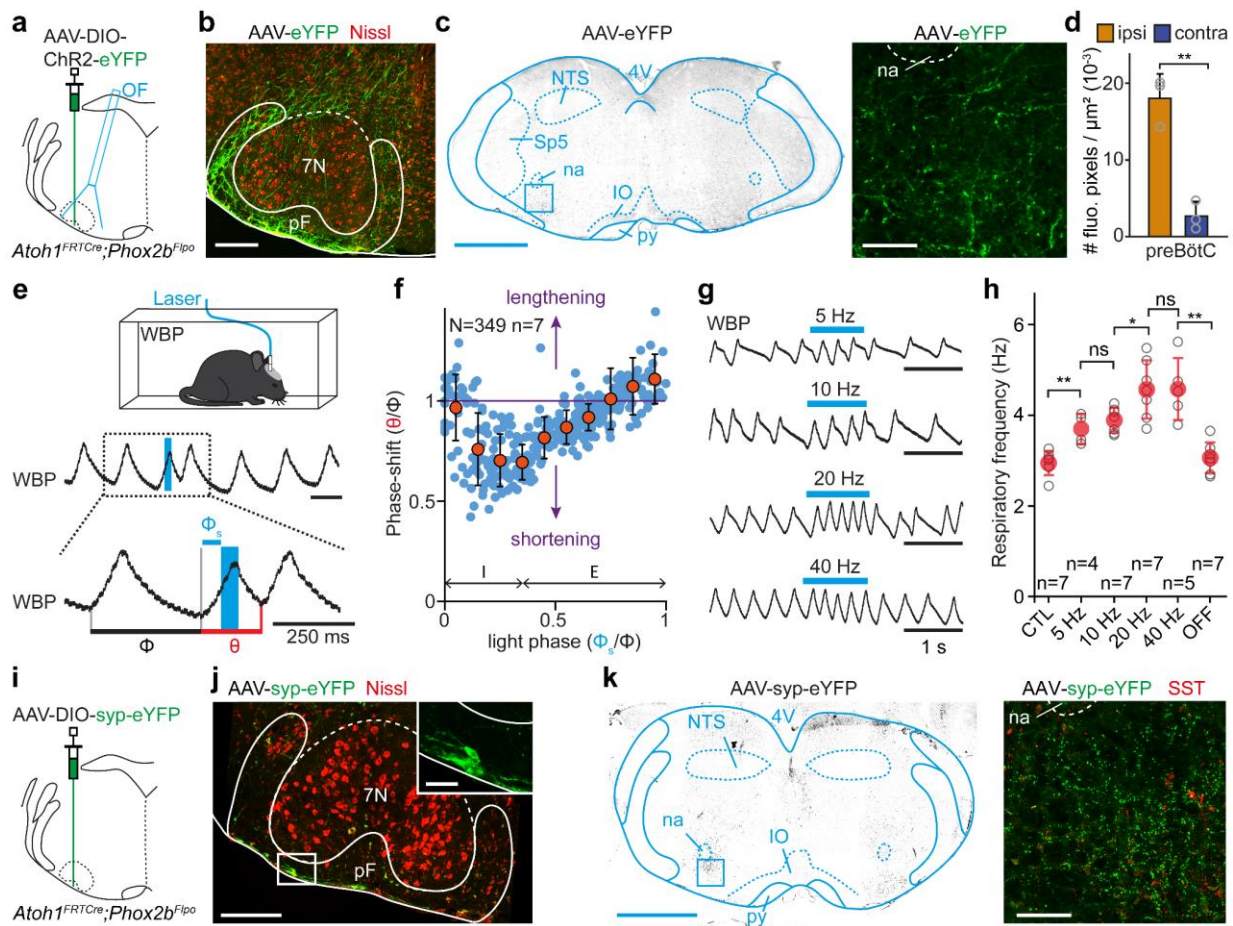
827 **(b)** Example transverse sections showing bilaterally transfected neurons in the pF area ventrally, ventro-laterally, and  
828 ventro-medially to the facial motor nucleus (7N) identified by the expression of Choline Acetyl Transferase (ChAT). Scale  
829 bar, 250  $\mu$ m.

830 **(c)** Raw (Dia EMG) and integrated (Int.) recordings of the diaphragm activity of one representative animal at rest before  
831 (CTL), during (CNO) and after (REC) intraperitoneal injection of CNO at 10 mg/kg.

832 **(d)** Quantification of the diaphragm mean frequency before, during and after CNO administration at rest. Note that  
833 silencing RTN<sup>Phox2b/Atoh1</sup> neurons does not alter basal breathing frequency.

834 **(e, f)** Similar representations as in **(c, d)** in mice running on a treadmill at 40 cm/s. Note the significantly lower diaphragm  
835 frequency following CNO administration compared to non-injected control mice (CTL). In all graphs, grey open circles  
836 are the means of individual mice, and filled circles are the means  $\pm$  SD across n mice. ns, not significant. \*\*,  $p < 0.01$   
837 (Wilcoxon matched-pairs test in d and and paired t-tests in f).

838 See also Figure S9.



839 **Figure 7: RTN<sup>Phox2b/Atoh1</sup> neurons contact the preBötC and impact respiratory rhythm generation.**

840 **(a)** Experimental strategy for labelling and photo-activating RTN<sup>Phox2b/Atoh1</sup> neurons and projections: an AAV-DIO-ChR2-eYFP is injected in the pF area of an *Atoh1*<sup>FRTCre</sup>;*Phox2b*<sup>Flpo</sup> adult mouse and an optic fiber (OF) is implanted above the  
841 injection site.  
842

843 **(b)** Example transverse section showing unilaterally transfected RTN<sup>Phox2b/Atoh1</sup> somata. Scale bar, 250  $\mu$ m.

844 **(c)** Left: transverse section of the medulla at the segmental level of the preBötC. Scale bar, 500  $\mu$ m. Right: magnification  
845 of the 400 x 400  $\mu$ m boxed area containing the preBötC, showing abundant eYFP<sup>+</sup> projections. Scale bar, 100  $\mu$ m.

846 **(d)** Bar-graphs showing the means density  $\pm$  SD across mice of eYFP-labelled pixels ipsilaterally and contralaterally in the  
847 preBötC. Grey open circles are the mean values of individual mice. \*\**p* < 0.01 (Wilcoxon matched-pairs test; 9 sections  
848 from *n* = 3 mice).

849 **(e)** Top: setup for recording ventilation using whole-body plethysmography (WBP) during optogenetic activations.  
850 Middle: WBP recordings around a single 50 ms light-stimulation pulse of RTN<sup>Phox2b/Atoh1</sup> neurons inspiration (inspirations  
851 are upwards, expirations are downwards). Note that the stimulation shortens the respiratory cycle. Bottom:  
852 magnification of the above trace showing the control cycle ( $\phi$ , black), the phase of light-stimulation ( $\phi_s$ , blue), and the  
853 perturbed cycle ( $\theta$ , red).

854 **(f)** Plot of the phase-shift (perturbed cycle normalized to the control cycle:  $\theta/\phi$ ) as a function of the phase of light-  
855 stimulation normalized to the control cycle ( $\phi_s/\phi$ ). Note that the phase-shift is respectively shortened (values < 1, purple  
856 line) or lengthened (values > 1) when the light pulse occurs in the mid-inspiratory/early-expiratory or late-expiratory  
857 part of the respiratory cycle respectively. Inspiration (I) and expiration (E) mean durations are indicated. Blue circles  
858 represent individual data from *N* = 349 random trials from *n* = 7 mice. Orange circles are averages  $\pm$  SD across all trials  
859 within 0.1 ms bins.

860 **(g)** WBP recordings of ventilation during photoactivation of RTN<sup>Phox2b/Atoh1</sup> neurons at increasing train frequencies.

861 **(h)** Quantification of the respiratory frequency before (CTL), during and after (OFF) photostimulations of RTN<sup>Phox2b/Atoh1</sup>  
862 neurons at different frequencies. Gray open circles are the means of 3 trials for individual animals and filled circles are  
863 the means  $\pm$  SD across *n* mice. ns, not significant; \*, *p* < 0.05; \*\*, *p* < 0.01 (Mann-Whitney tests).

864 **(i)** Experimental strategy for labelling the synaptic contacts of RTN<sup>Phox2b/Atoh1</sup> neurons using a virally-driven  
865 synaptophysin-eYFP fusion protein (Syn-eYFP).

866 **(j)** Example transverse section showing unilaterally transfected RTN<sup>Phox2b/Atoh1</sup> somata in the pF area. Scale bar, 250  $\mu$ m;  
867 inset, 50  $\mu$ m.

868 **(k)** Left: transverse section at the level of the preBötC. Scale bar, 1 mm. Right: magnification of the 400 x 400  $\mu$ m boxed  
869 area containing the preBötC (identified by somatostatin expressing cells, SST), showing abundant eYFP<sup>+</sup> synaptic  
870 contacts of RTN<sup>Phox2b/Atoh1</sup> neurons. Scale bar, 100  $\mu$ m. Representative of n=4 animals.

871 See also Figure S10.



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