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1 **PreBötzing complex neurons drive respiratory modulation of blood pressure and**
2 **heart rate**

3

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17

18 **Summary**

19 Heart rate and blood pressure oscillate in phase with respiratory activity. A component of
20 these oscillations is generated centrally, with respiratory neurons entraining the activity of
21 pre-sympathetic and parasympathetic cardiovascular neurons. Using a combination of
22 optogenetic inhibition and excitation *in vivo* and *in situ* in rats, as well as neuronal tracing, we
23 demonstrate that preBötzing Complex (preBötC) neurons, which form the kernel for
24 inspiratory rhythm generation, directly modulate cardiovascular activity. Specifically,
25 inhibitory preBötC neurons modulate cardiac parasympathetic neuron activity whilst
26 excitatory preBötC neurons modulate sympathetic vasomotor neuron activity, generating
27 heart rate and blood pressure oscillations in phase with respiration. Our data reveal yet more
28 functions entrained to the activity of the preBötC, with a role in generating cardiorespiratory
29 oscillations. The findings have implications for cardiovascular pathologies, such as
30 hypertension and heart failure, where respiratory entrainment of heart rate is diminished and
31 respiratory entrainment of blood pressure exaggerated.

32

33 **Keywords:** preBötzing complex; master oscillator; cardiorespiratory control; respiratory
34 sinus arrhythmia; respiratory-sympathetic modulation; optogenetic inhibition; *GtACR2*.

35 **Introduction**

36 The respiratory and cardiovascular systems act in synergy to maintain blood gas homeostasis
37 in animals. Beyond their respective separate roles, these systems are also functionally
38 coupled, with oscillations in blood pressure (BP), called Traube-Hering waves, and in heart
39 rate (HR), called respiratory sinus arrhythmia (RSA), that are in phase with the respiratory
40 cycle. This respiratory entrainment of cardiovascular activity is a highly conserved
41 physiological property present in vertebrates, including mammals, fish, amphibians and
42 reptiles (Taylor et al., 2010, 1999), and was recently found in the early air-breathing primitive
43 lungfish (Monteiro et al., 2018). When within physiological range, Traube-Hering waves are
44 important contributors to vascular tone changes (Briant et al., 2015), RSA reduces cardiac
45 energetic cost (Ben-Tal et al., 2012), and both are believed to optimize blood tissue perfusion
46 and gas exchange. However, an alteration in the amplitude of these oscillations, typically with
47 enlargement of Traube-Hering waves and concurrent abolishment of RSA, occurs in common
48 cardiovascular diseases (e.g. hypertension and heart failure) and contributes to their
49 pathogenesis (Machado et al., 2017; Menuet et al., 2017; Palatini and Julius, 2009; Task
50 Force of the European Society of Cardiology and the North American Society of Pacing and
51 Electrophysiology, 1996). The pathological relevance of these oscillations extends to
52 numerous neurological diseases, not superficially linked to cardiovascular function, including
53 depression (Brush et al., 2019) and amyotrophic lateral sclerosis (Merico and Cavinato,
54 2011).

55 Traube-Hering waves and RSA are generated by multiple mechanisms, including
56 changes in thoracic pressure and viscerosensory afferent feedback, but their origin has a
57 central component, as they remain in heart- and lung-denervated humans, and in paralyzed
58 and deafferented animal preparations (Dick et al., 2014; Farmer et al., 2016; Menuet et al.,
59 2017; Taha et al., 1995). Traube-Hering waves are generated by bursts of vasomotor
60 sympathetic nerve activity that occur in phase with the respiratory cycle (RespSNA),
61 spanning most of inspiration and the very beginning of post-inspiration (the first phase of
62 expiration). As a result of the slow vascular contraction dynamics, Traube-Hering waves peak
63 during post-inspiration. RespSNA is the result of respiratory modulation of the activity of pre-
64 sympathetic neurons in the rostral ventrolateral medulla oblongata, and in particular of the
65 excitatory inspiratory modulation of adrenergic C1 neurons (Menuet et al., 2017; Moraes et
66 al., 2013). RSA is generated by a cardio-inhibitory vagal parasympathetic tone that decreases
67 during the second part of expiration and into inspiration, resulting in HR oscillations that peak
68 at the end of inspiration. Cardiac vagal preganglionic neurons of the nucleus ambiguus are

69 weakly inhibited during the second part of expiration, and strongly inhibited during
70 inspiration, by both GABAergic and glycinergic inputs (Gilbey et al., 1984; Neff et al., 2003).
71 However, the specific cellular components of the central respiratory pattern generating circuit
72 that are responsible for modulation of both vasomotor pre-sympathetic and cardiac vagal
73 preganglionic neurons are not fully elucidated.

74 Respiratory and cardiovascular neurons are present in adjacent and slightly
75 intermingled cell columns in the ventral medulla oblongata. The respiratory neurons are
76 classified by the relationship between their activity pattern and phase of the respiratory cycle
77 – e.g. inspiratory neurons fire action potentials during inspiration. Core to the respiratory
78 column is a small, yet heterogeneous, group of neurons in the preBötzinger Complex
79 (preBötC), which consists of excitatory (glutamatergic) inspiratory neurons, and inhibitory
80 (GABAergic and glycinergic) neurons that can be inspiratory or expiratory (Baertsch et al.,
81 2018; Koizumi et al., 2013; Morgado-Valle et al., 2010; Sherman et al., 2015). The preBötC
82 is critical for respiratory rhythm generation, and also acts as a master oscillator entraining the
83 activity of other oscillators, like the sniffing and whisking oscillators, in a unidirectional
84 fashion (Deschênes et al., 2016; Moore et al., 2013). Our recent work on the pre-synaptic
85 connectome of C1 neurons shows that preBötC neurons have the anatomical connections to
86 provide the inspiratory input to these pre-sympathetic neurons (Dempsey et al., 2017; Menuet
87 et al., 2017). It is also proposed that the strong inspiratory inhibition of cardiac vagal
88 preganglionic neurons comes from the preBötC (Frank et al., 2009).

89 Using optogenetics, which provides the spatial resolution to restrict opsin expression
90 to a small group of neurons such as those in the preBötC, and temporal control to excite or
91 inhibit neurons during specific phases of the respiratory cycle, we show that the preBötC is a
92 cardiorespiratory hub that drives RespSNA, Traube-Hering waves and RSA.

93 **Results**

94

95 **Injections of lentivirus expressing the light-activated anion channel *GtACR2* are** 96 **targeted to the preBötC.**

97 To induce rapid, reversible and powerful inhibition of neural activity in the preBötC we
98 injected a lentivirus inducing expression of *GtACR2* (Govorunova et al., 2015) bilaterally.
99 Injections were directed by electrophysiological recordings to identify inspiratory neurons in
100 the rostral-most part of the ventral inspiratory cell column in the medulla oblongata. *Post hoc*
101 histological analysis revealed the *GtACR2* expression was targeted to the gap in the
102 rostrocaudal column of parvalbumin-expressing neurons (Figure 1A) (Alheid et al., 2002),
103 dorsal to tyrosine hydroxylase (TH)-expressing A1-C1 neurons, ventral to the caudal tip of
104 the compact part of the nucleus ambiguus, and at the level containing the highest density of
105 neurokinin type 1 receptor (NK₁R)-positive neurons (Figure 1B). Together these markers
106 characterise the preBötC area. *GtACR2* was expressed in both excitatory (glutamatergic) and
107 inhibitory (GABAergic and glycinergic) neurons in the preBötC (Figure 1C-E). A heatmap
108 representation of *GtACR2* expression in all the 12 animals used for the *in situ* Working Heart-
109 Brainstem Preparation (WHBP) experiments shows that intersectional *GtACR2* expression
110 across all animals is restricted to the preBötC area (Figure 1F).

111

112 **The preBötC is necessary for inspiratory rhythm generation.**

113 The preBötC is mostly studied for its critical role in inspiratory rhythm generation.
114 Prolonged *GtACR2*-mediated preBötC photoinhibition, either *in vivo* in anaesthetised rats
115 (n=5) or in the *in situ* WHBP (n=12), produced immediate cessation of inspiratory activity
116 and a long-lasting apnea (Figure 2A-C; Figure 3A-C). Following the apnea, whilst preBötC
117 photoinhibition remained activated, inspiratory activity resumed, but with an altered pattern
118 of intercostal muscle (intC) electromyograph (EMG) activity *in vivo* (Figure 2A-B) or phrenic
119 nerve activity *in situ* (PNA; Figure 3A-C and H; Figure 3S1A-B). In the WHBP, the longest
120 apnea was produced by 10 and 20 Hz photoinhibition (Figure 3A-C). When PNA resumed, 5-
121 10 Hz photoinhibition induced shorter and smaller PNA bursts, whilst PNA burst
122 characteristics were not altered by 20-50 Hz photoinhibition.

123 A single 10 ms light pulse at any time during inspiration was sufficient to immediately
124 stop the PNA burst and trigger post-inspiration (Figure 7A-C). The duration of the following
125 expiratory period was correlated to the duration and amplitude of the stopped inspiration,
126 mostly by adjusting the post-inspiratory phase duration. Photoinhibition of the PreBötC only

127 during the expiratory period prolonged the expiratory time for the duration of the
128 photoinhibition, and PNA activity resumed following a short (~1 s) post-photoinhibition delay
129 (Figure 7E).

130 In whole-cell recordings of *GtACR2*-expressing neurons (n=7) in brainstem slices of
131 the dorsal medulla we observed strong, and sustained, hyperpolarisation with *GtACR2*
132 photoactivation (Figure 2F), induced by large inhibitory conductances (Figure 2E). This
133 hyperpolarisation continued for the entire period of light delivery, and in spontaneously active
134 neurons, complete blockade of action potential firing was obtained with 10ms duration light
135 pulses at 20 Hz. On the basis of these recordings it is not clear why inspiratory activity
136 resumed during prolonged photoinhibition.

137

138 **Prolonged preBötC photoinhibition induces decreased vasomotor sympathetic activity** 139 **and increased cardiac parasympathetic activity.**

140 Due to the profound apnea, which would alter blood gas composition and sensory
141 afferent input, detailed analysis of the cardiorespiratory response to preBötC photoinhibition
142 was not feasible *in vivo* and hence detailed analyses were performed in the WHBP, an
143 oxygenated, perfused preparation devoid of the depressive effects of anaesthesia and where
144 central respiratory perturbations do not influence peripheral blood gases or pH (Menuet et al.,
145 2017; Paton, 1996). During prolonged preBötC photoinhibition, thoracic sympathetic nerve
146 activity (tSNA) showed initial inhibition during apnea, and then a return towards control
147 levels as central respiratory activity resumed (Figure 3A and F; Figure 3S1E and G; Figure
148 3S2A). This was reflected in an initial decrease in perfusion pressure (PP), followed by an
149 acute increase (Figure 3A and G; Figure 3S1F-G) that were maximal with 20 Hz
150 photoinhibition. We also observed a strong increase in vagal nerve activity (VNA) and
151 decrease in HR (bradycardia) (Figure 2A-B and D; Figure 3A-B and D-E; Figure 3S1C-D).
152 Unlike the sympathetic vasomotor effect, the bradycardia lasted the entire photoinhibition
153 period and was not correlated to the apnea duration (Figure 3S1G). The bradycardia was due
154 to increased cardiac parasympathetic drive (measured as non-resp VNA, Figure 3A and D,
155 Figure 3S2B) as it was abolished by bath application of the muscarinic antagonist, atropine
156 (Figure 4). The biphasic tSNA and PP responses to preBötC photoinhibition were maintained
157 during atropine (Figure 4C).

158

159 **The preBötC is sympatho-excitatory and parasympatho-inhibitory.**

160 To avoid secondary circuit effects arising from sustained photoinhibition, we used
161 single light-pulse preBötC photoinhibition to test direct functional connectivity with
162 sympathetic and parasympathetic activities. Laser-triggered averaging of tSNA and VNA
163 following low-frequency (1 Hz) preBötC photoinhibition confirmed opposite sympathetic and
164 parasympathetic effects (Figure 5; Figure 5S1). PreBötC photoinhibition induced a small
165 sympatho-excitation that peaked ~65 ms following the light pulse, and mainly a strong
166 sympatho-inhibition that was maximal at ~135 ms. PreBötC photoinhibition induced dramatic
167 parasympathetic excitation, which could be seen on VNA recordings in response to individual
168 light pulses (Figure 5A-B). This parasympatho-excitation was very fast, peaking at ~7 ms
169 (Figure 5C).

170 We conclude that neurons within the preBötC exert direct excitatory effects on
171 vasomotor sympathetic pathways and inhibitory effects on cardiac parasympathic pathways.
172 We next investigated the role played by PreBötC neurons in the respiratory modulation of
173 tSNA (RespSNA), PP (Traube-Hering waves) and HR (RSA).

174

175 **PreBötC activity contributes to the generation of RespSNA and Traube-Hering waves.**

176 When respiration resumed during prolonged preBötC photoinhibition, the return of
177 RespSNA and Traube-Hering waves mirrored the return of PNA inspiratory activity (Figure
178 3A-C and Figure 6A). Like PNA, both RespSNA and Traube-Hering wave amplitudes were
179 strongly decreased during 10 Hz preBötC photoinhibition, but unaffected during 20 Hz
180 photoinhibition (Figure 6A-B; Figure 6S1A and 6S1D), and overall individual changes in
181 RespSNA area under the curve (AUC) and Traube-Hering wave amplitude correlated closely
182 with changes in PNA amplitude (Figure 6C). Decreased RespSNA duration during 10 Hz
183 photoinhibition was specifically due to a shorter inspiratory component (Figure 6D; Figure
184 6S1A and 6S1C). In contrast, mean tonic tSNA was not altered during preBötC
185 photoinhibition (Figure 6B; Figure 6S1B).

186 Single light pulses delivered during early inspiration arrested PNA bursts (AUC
187 reduced by ~75%) and almost abolished the associated RespSNA and Traube-Hering wave
188 (Figure 7A-D; Figure 7S1A). PreBötC photoinhibition during early post-inspiration had no
189 impact on PNA or RespSNA and induced a small increase in Traube-Hering wave amplitude.
190 Tetanic preBötC photoinhibition during expiration induced exaggerated RespSNA and
191 Traube-Hering wave amplitude, which is likely due to the increased pulse pressure associated
192 with the bradycardia (Figure 7E).

193 Together, these results show that the preBötC exerts a direct phasic inspiratory drive
194 to vasomotor sympathetic activity, with a major involvement in the generation of RespSNA
195 and Traube-Hering waves.

196

197 **PreBötC activity is strongly involved in the generation of RSA.**

198 RSA was almost abolished during both 10 Hz and 20 Hz preBötC photoinhibition
199 (Figure 6A-B; Figure 6S1D), and changes in RSA were independent from changes in PNA
200 amplitude (Figure 6C). The loss of baseline RSA evoked by systemic atropine demonstrates
201 that RSA is generated *via* parasympathetic activity (Figure 4A-B). These results indicate that
202 the preBötC is a source of ongoing respiratory phase-locked inhibitory input to cardiac
203 parasympathetic preganglionic neurons.

204 Baseline RSA is characterised by peaks in HR at the end of inspiration, decreases
205 during the first part of post-inspiration, and increases continuously during the second part of
206 post-inspiration, late-expiration (E2) and inspiration (Figure 7S1B). Single light pulse
207 preBötC photoinhibition during inspiration did not affect the amplitude of the RSA during the
208 respiratory cycle in which stimulation occurred (Figure 7A-D; Figure 7S1), but rather
209 decreased RSA amplitude in the following respiratory cycle due to a smaller post-
210 photoinhibition HR decrease that didn't reach baseline level before HR increased again.
211 Photoinhibition of preBötC during early post-inspiration induced a larger decrease in HR
212 which increased the amplitude of the following cycle of RSA. Prolonged photoinhibition
213 during expiration strongly increased VNA, decreased HR and prevented the tachycardic
214 component of RSA until photoinhibition was stopped, whereupon HR increased abruptly
215 prior to the next inspiration (Figure 7E).

216 Together, these results show that the preBötC exerts a complex cardiac
217 parasympathetic influence that spans both inspiratory and expiratory periods, with a major
218 involvement in the generation of RSA.

219

220 **PreBötC photoexcitation decreases the respiratory command amplitude, resulting in**
221 **decreased RespSNA, Traube-Hering waves and RSA.**

222 The same injection protocol described for the *GtACR2* expression was used to
223 produce restricted expression of channelrhodopsin-2 (ChR2) in preBötC bilaterally (Figure
224 9A). Strong ChR2-positive terminals were found in the facial motor nucleus (Figure 9B),
225 which is characteristic of neurons in the preBötC region, and not those in the inspiratory
226 rostral ventral respiratory group (Deschênes et al., 2016). ChR2-mediated preBötC

227 photoexcitation (n=8) increased respiratory frequency without altering inspiratory duration,
228 and decreased PNA inspiratory and VNA post-inspiratory amplitudes (Figure 8A, 8C and
229 8D), therefore reducing each respiratory cycle intensity. We focused on 20 Hz
230 photoexcitation, as this produced maximal effects across all animals.

231 PreBötC photoexcitation did not alter overall mean tSNA amplitude (which includes
232 both RespSNA and tonic tSNA) (Figure 8F), but disrupted respiratory entrainment of SNA
233 leading to decreased RespSNA amplitude and duration, with shorter inspiratory and post-
234 inspiratory components (Figure 8I-J). As a consequence both Traube-Hering wave amplitude
235 (Figure 8H) and mean PP (Figure 8G) were decreased. Changes in RespSNA magnitude and
236 Traube-Hering wave amplitude induced by preBötC photoexcitation both correlate with
237 changes in PNA inspiratory amplitude (Figure 8K). On the other hand, preBötC
238 photoexcitation increased tonic (non-respiratory) tSNA (Figure 8I), and laser-triggered
239 averaging of 1 Hz preBötC photoexcitation revealed a principal sympatho-excitatory effect
240 (Figure 8L-M).

241 Photoexcitation of preBötC did not alter mean HR (Figure 8E) but decreased RSA
242 amplitude (Figure 8H), due to loss of the post-inspiratory/expiratory bradycardic component.
243 Changes in RSA induced by preBötC photoexcitation were independent of changes in PNA
244 inspiratory amplitude (Figure 8K). Laser-triggered averaging of VNA during continuous low-
245 frequency (1 Hz) preBötC photoexcitation revealed a small short latency parasympatho-
246 inhibition.

247

248 **PreBötC neurons project directly to putative pre-sympathetic vasomotor and** 249 **parasympathetic neurons.**

250 To map axonal projections from preBötC neurons we performed small (5 nl),
251 unilateral virus injections under electrophysiological guidance to induce restricted expression
252 of tdTomato in neurons in the preBötC core (n=2). Successful targeting of respiratory
253 preBötC neurons was indicated by substantial numbers of transduced fibres crossing the
254 midline and terminal fields within the contralateral preBötC (Figure 9C), defining features of
255 functionally identified preBötC neurons (Bouvier et al., 2010; Koizumi et al., 2013).
256 TdTomato-positive preBötC terminals were found in close apposition to both RVLM
257 catecholaminergic C1 neurons, which include pre-sympathetic vasomotor neurons (Figure
258 9D), and nucleus ambiguus neurons, which include cardiac parasympathetic preganglionic
259 neurons (Figure 9E).

260 **Discussion**

261 Respiratory modulation of sympathetic and parasympathetic activity, leading to
262 Traube-Hering waves and RSA, is a well characterized physiological feature but with little
263 mechanistic understanding. Here we demonstrate that this respiratory-cardiovascular
264 entrainment is coded at the core of the brainstem respiratory network. In addition to its role in
265 inspiratory rhythm generation, the preBötC provides inspiratory-locked excitatory drive to
266 sympathetic activity and subsequent generation of Traube-Hering waves, and inhibitory drive
267 to parasympathetic outputs, to generate RSA. Photoinhibition of preBötC exerts profound
268 effects on HR and BP in both anesthetized *in vivo* and *in situ* rat models, highlighting the
269 importance of this ongoing activity under basal conditions. These data reinforce other recent
270 observations (Deschênes et al., 2016; Moore et al., 2013) supporting the view that oscillatory
271 activity arising from the preBötC provides widespread influences extending well past circuits
272 related to breathing.

273 ***Targeting the preBötC***

274 The preBötC was defined as a group of excitatory inspiratory neurons with intrinsic
275 rhythmogenic properties, located in the ventral respiratory column (Peña et al., 2004; Smith et
276 al., 1991). It remains classified as a region with relatively ambiguous anatomic boundaries
277 that contains a heterogeneous cell population composed of rhythmogenic, mostly inspiratory,
278 excitatory and inhibitory neurons (Baertsch et al., 2019, 2018; Koizumi et al., 2013; Kuwana
279 et al., 2006; Morgado-Valle et al., 2010; Sherman et al., 2015). No single and exclusive
280 marker of the preBötC has been found so far. We studied neurons in the preBötC using the
281 pan-neuronal CBA promoter that targets neurons irrespective of phenotype. Our anatomical
282 validation of correct transduction (Figures 1 and 9) is supported functionally, as
283 photoinhibition caused immediate cessation of inspiratory activity and a prolonged apnea both
284 *in vivo* and *in situ* (Figures 2 and 3), whereas photoexcitation increased respiratory frequency
285 while decreasing inspiratory amplitude (Figure 8). These effects on breathing are consistent
286 with previous optogenetic perturbations of the preBötC (Alsaifi et al., 2015; Cui et al., 2016;
287 Koizumi et al., 2016; Vann et al., 2018). Adjacent to the preBötC are caudal ventrolateral
288 medulla (CVLM) GABAergic neurons, which form part of the sympathetic vasomotor circuit
289 and inhibit rostral ventrolateral medulla pre-sympathetic neurons (Guyenet, 2006), as well as
290 some cardiac parasympathetic preganglionic neurons of the nucleus ambiguus (Dergacheva et
291 al., 2010; Gourine et al., 2016). Some of these neurons could have been transduced with our
292 viral approach, however our physiological observations do not support this being a substantial
293 influence, as *GtACR2*-mediated photoinhibition caused inverse effects to those expected if

294 these neurons were transduced (which would be sympathoexcitation/increase in blood
295 pressure and parasympatho-inhibition/tachycardia) (Figures 3 and 5).

296 When inspiration resumed during photoinhibition, we observed a crenelated activity
297 pattern in inspiratory intC EMG and PNA bursts (Figures 2 and 3). This is likely to result
298 from photoinhibition of some rostral ventral respiratory group phrenic premotor neurons,
299 which intermingle with the caudal pole of the preBötC. Importantly, these neurons are not
300 thought to project to respiratory/cardiovascular neurons other than phrenic motoneurons and
301 lateral reticular nucleus (Wu et al., 2017), so they are unlikely to mediate the cardiac
302 parasympathetic and vasomotor sympathetic effects found in this study.

303 *Caveats related to optically-activated chloride channels*

304 To interrogate interactions between preBötC respiratory circuits and autonomic
305 outputs we used the high temporal resolution of the natural light-activated anion channel
306 *GtACR2*, which induces large chloride photocurrents with faster kinetics than other inhibitory
307 opsins (Govorunova et al., 2015) (Figure 2). Interpretation of our results requires careful
308 consideration, as the chloride electrochemical gradient is heterogeneous among subcellular
309 compartments, and axonal activation of chloride channels can lead to terminal
310 neurotransmitter release (Messier et al., 2018). This phenomenon is unlikely to explain the
311 current results, which are consistent with neural inhibition and were opposed by optogenetic
312 excitation, highlighted by the strong, perfectly inverse sympathetic (tSNA) and
313 parasympathetic (VNA) effects observed with single light pulse ChR2-mediated
314 photoexcitation (Figures 5 and 8). We conclude that in this study and with this cell group,
315 *GtACR2* reliably inhibited neuronal activity.

316 *The preBötC provides powerful, ongoing inhibition of cardiac parasympathetic activity*

317 Cardiac parasympathetic preganglionic neurons are located in the nucleus ambiguus
318 and the dorsal motor nucleus of the vagus nerve (Gourine et al., 2016). Among these, only
319 nucleus ambiguus neurons show respiratory-related phasic patterns of activity, with an
320 inhibition of their activity during inspiration, an excitation during post-inspiration and a weak
321 inhibition during late expiration (E2) (Gilbey et al., 1984; Neff et al., 2003). Respiratory
322 inputs into nucleus ambiguus cardiac preganglionic neurons could be widely distributed,
323 although the strongest evidence indicates they come from the pontine Kölliker-Fuse nucleus
324 for the post-inspiratory excitation (Farmer et al., 2016), and from GABAergic and glycinergic
325 neurons that could be located in the preBötC for the inspiratory inhibition (Frank et al., 2009;
326 Neff et al., 2003).

327 Single light pulse preBötC photoinhibition induced intense vagal activation (Figure 7),
328 whilst prolonged photoinhibition, both *in vivo* and *in situ*, caused profound bradycardia, with
329 up to a 70% decrease in HR (Figures 2 and 3), which was blocked by of the
330 parasympatholytic agent atropine (Figure 4). The simplest pathway that could explain this
331 result is a direct inhibitory connection between preBötC and cardiac vagal motoneurons, a
332 scheme supported by our tracing studies (Figure 9) and the short latencies of vagal responses
333 to optical excitation and inhibition (Figures 5 and 8). The preBötC contains both GABAergic
334 and glycinergic neurons (Koizumi et al., 2013; Kuwana et al., 2006; Morgado-Valle et al.,
335 2010) that display four activity patterns: tonic, inspiratory, pre-inspiratory and expiratory
336 (Baertsch et al., 2018; Morgado-Valle et al., 2010; Sherman et al., 2015). It is likely that most
337 of these functional subgroups are involved in cardiac parasympatho-modulation.

338 Bradycardia occurs independent of when in the respiratory cycle photoinhibition
339 commences, showing that tonic neurons must be involved. Most interestingly, RSA, which is
340 mediated by a progressive decrease of vagal parasympathetic drive during expiration that
341 peaks during inspiration (Bouairi et al., 2004; Dergacheva et al., 2010; Gilbey et al., 1984;
342 Neff et al., 2003), was nearly abolished by preBötC photoinhibition, even after the re-
343 commencement of PNA during prolonged photoinhibition. RSA was also abolished with
344 prolonged preBötC photoexcitation, while there was a tendency for an increase in HR (Figure
345 8). This shows that prolonged photostimuli of the preBötC, that are likely to suppress phasic
346 activities in targeted neurons and render them either inactive (photoinhibition) or tonically
347 active (photoexcitation), are able to suppress RSA. This could be explained by suppression of
348 the phasic activity of inhibitory preBötC neurons, composed of a small (~10%) proportion of
349 inhibitory expiratory neurons (Baertsch et al., 2018; Carroll et al., 2013), and a larger
350 proportion of inhibitory pre-inspiratory and inspiratory neurons. As a result, phasic late
351 expiratory and inspiratory inhibition of cardiac vagal parasympathetic activity would be
352 suppressed, therefore suppressing RSA. The preBötC is therefore critically involved in tonic
353 regulation of HR as well as the generation of RSA, through a combination of phasic
354 inhibitory neurons.

355 The decrease in HR just after inspiration, during the post-inspiratory phase, likely
356 involves an excitatory drive to cardiac parasympathetic preganglionic neurons external to the
357 preBötC, most likely coming from the pontine Kölliker-Fuse nucleus (Farmer et al., 2016;
358 Gilbey et al., 1984). Still, preBötC neurons exert an indirect influence on this, as a single
359 photoinhibitory light pulse during inspiration modulated both the inspiratory and post-

360 inspiratory phases, as well as amplitude of the following cycle of RSA, by reducing the post-
361 inspiratory decrease in HR (Figure 7).

362 ***The preBötC provides excitatory drive to vasomotor sympathetic activity***

363 Respiratory modulation of neurons involved in generating vasomotor sympathetic
364 activity is found from spinal sympathetic preganglionic neurons of the intermediolateral cell
365 column (Zhou and Gilbey, 1992) to medullary bulbospinal pre-sympathetic neurons of the
366 RVLM (McAllen, 1987; Moraes et al., 2013) and caudal raphe (Gilbey et al., 1995), as well
367 as in baroactivated CVLM neurons (Mandel and Schreihofner, 2006). Neurons in each of these
368 regions display three main types of respiratory modulation, inspiratory activated, inspiratory
369 inhibited, and post-inspiratory activated, which could be acquired mono-synaptically or poly-
370 synaptically from respiratory neurons.

371 Prolonged preBötC photoinhibition reduced SNA and PP, but only for the apneic
372 period (Figure 3). Single pulse preBötC photoinhibition revealed a fast inhibition of SNA,
373 peaking ~140ms after the light pulse (Figure 5). This effect on SNA is delayed compared to
374 the latencies of responses recorded in VNA, and cannot be attributed to the relatively slow
375 conduction velocity of sympathetic pathways, as SNA responses to photostimulation of
376 sympathetic premotor neurons are also lower than those seen here (Abbott et al., 2009;
377 Menuet et al., 2014). These observations are consistent with a polysynaptic pathway prior to
378 the recorded SNA, which could potentially include recruitment of neurons in the intermediate
379 reticular nucleus (Toor et al., 2019). Also, inhibitory, GABAergic CVLM neurons are
380 respiratory modulated (Mandel and Schreihofner, 2006) and could mediate inspiratory
381 modulation of RVLM pre-sympathetic neurons arising from inhibitory or excitatory
382 inspiratory preBötC neurons. Microinjection of the GABA_A receptor agonist muscimol into
383 the CVLM/preBötC abolishes RespSNA and respiratory modulation of RVLM neurons, but it
384 is not clear whether this is a direct effect to CVLM inhibition, or due to the associated total
385 loss of respiratory drive and consequent network effects (Koshiya and Guyenet, 1996).
386 Microinjections of GABA_A receptor antagonists in the RVLM enhance RespSNA (Guyenet et
387 al., 1990; Menuet et al., 2017), which, together with our single pulse preBötC photoinhibition
388 experiments, favour the hypothesis that any respiratory phasic input from the CVLM is likely
389 to inhibit presympathetic RVLM neurons.

390 Direct recordings show that inspiratory modulation of RVLM C1 neurons is mediated
391 by excitatory post-synaptic potentials (Moraes et al., 2013), and we found evidence that
392 neurons in the preBötC, albeit not definitively demonstrated to be inspiratory-modulated
393 neurons, send direct projections onto C1 neurons (Figure 9). This confirms our previous data

394 showing retrograde trans-synaptic labelling of preBötC NK1R neurons from pre-sympathetic
395 C1 neurons (Dempsey et al., 2017; Menuet et al., 2017). Upon resumption of PNA, but during
396 continued photoinhibition, the amplitude of RespSNA and Traube-Hering waves were
397 decreased, strongly correlating with PNA amplitude (Figure 6). Single pulse preBötC
398 photoinhibition during inspiration decreased the concomitant RespSNA burst and subsequent
399 Traube-Hering wave (Figure 7). During preBötC photoexcitation, inspiratory amplitude was
400 decreased and this also correlated with decreased RespSNA and Traube-Hering waves
401 amplitudes (Figure 8). We therefore conclude the preBötC provides ongoing excitatory drive
402 to vasomotor sympathetic activity, most likely via pre-sympathetic C1 neurons whose activity
403 correlates with inspiration (Menuet et al., 2017; Moraes et al., 2013), and drives RespSNA
404 and Traube-Hering waves (Marina et al., 2011; Menuet et al., 2017; Moraes et al., 2017).

405 Single pulse preBötC photoinhibition had no effect on RespSNA when applied during
406 post-inspiration (Figure 7). Part of the post-inspiratory component of RespSNA originates
407 from post-inspiratory neurons in the intermediate reticular nucleus (Toor et al., 2019), and
408 similarly to RSA, the dorsolateral pons could also be involved (Baekey et al., 2008; Dick et
409 al., 2009). Indeed, ponto-medullary transections strongly decreased RespSNA and Traube-
410 Hering waves. Yet, this also caused a switch from eupnea to an apneustic breathing pattern,
411 with prolonged and square shaped phrenic nerve activity. This highlights the difficulty to
412 interrogate functional circuitry entrained by respiratory neurons, as alteration of the activity of
413 the respiratory network will create rebound network effects and alterations of breathing,
414 making it difficult to disentangle the direct versus indirect mechanisms involving the neurons
415 studied. Ideally, future experiments will enable modulation of individual synapses between
416 respiratory neurons and neurons regulating sympathetic (or parasympathetic) activity.

417 ***Different preBötC respiratory neurons affect SNA compared to cardiac vagal activity***

418 The current view is that excitatory preBötC neurons generate the inspiratory rhythm
419 (Bouvier et al., 2010; Vann et al., 2018; Wang et al., 2014), while inhibitory preBötC neurons
420 shape its pattern and frequency (Baertsch et al., 2018; Cregg et al., 2017; Sherman et al.,
421 2015). Here prolonged preBötC photoinhibition induced apnea but not for the entire
422 photoinhibition period. Previous investigators have reported prolonged apnea upon preBötC
423 inhibition induced by pharmacogenetic (Tan et al., 2008) or (brief) optogenetic neural
424 inhibition (Vann et al., 2018). Like the inspiratory activity, tSNA also recovered respiratory
425 modulation when inspiration resumed. By stark contrast, vagal activation and bradycardia
426 remained affected for the entire period of photoinhibition. This observation provides strong
427 support for affecting two different cell populations, an excitatory one involved in inspiratory

428 rhythmogenesis and SNA modulation, and an inhibitory one involved in inspiratory
429 patterning and cardiac vagal modulation. The most likely explanation for the return of
430 respiratory activity during continued photoinhibition is that rhythmogenic, excitatory
431 inspiratory neurons of the preBötC escaped *GtACR2*-mediated photoinhibition. The
432 mechanism responsible for the rhythmogenic activity of preBötC is still debated, but involves
433 excitatory pre-inspiratory and inspiratory neurons with cellular and/or micro-network
434 electrophysiological properties particularly prone to burst generation, which could confer
435 particular propensity to escape prolonged inhibition. If this is the mechanism, it appears to be
436 a property of the excitatory neurons.

437 ***Respiratory cardiovascular interactions in the clinical setting***

438 Assessment of the short-term variability of HR, and to a lesser extent BP, is common
439 in clinical evaluation of cardiovascular health. The main spectral density found in both HR
440 and BP variability is in phase with the respiratory frequency, in the high frequency band, and
441 is actually a quantification of RSA and Traube-Hering waves, respectively. These
442 measurements provide an invaluable means for non-invasive assessment of parasympathetic
443 and sympathetic drives to the cardiovascular system and have attracted general community
444 interested due to the connection between HR variability and “healthiness” scores. However,
445 views on the analysis and interpretation of these data vary substantially (Hayano and Yuda,
446 2019). At the core of uncertainty regarding the value of HR and BP variability lies a lack of
447 understanding of the mechanisms driving RSA and Traube-Hering waves.

448 Regardless of how it is measured, substantial evidence links altered respiratory
449 modulation of autonomic activity with cardiovascular diseases. Exaggerated inspiratory
450 modulation of sympathetic activity leads to increased Traube-Hering waves and drives the
451 development of hypertension (Menuet et al., 2017). This study identifies a key source of this
452 excitatory inspiratory modulation, and while modifying preBötC activity is not conceivable
453 given its critical role, targeted therapeutics that only impact the preBötC to C1 neuron
454 connection could be developed to prevent hypertension development. A common feature of
455 many cardiovascular diseases, and prognostic indicator of poor survival, is reduced RSA,
456 often in concert with increased Traube-Hering waves (Palatini and Julius, 2009; Task Force of
457 the European Society of Cardiology and the North American Society of Pacing and
458 Electrophysiology, 1996). Our study also identifies the preBötC as the likely source of this
459 dual alteration of cardiac parasympathetic and vasomotor sympathetic respiratory drives.

460 ***Conclusion***

461 Whilst initially characterized as a key oscillator for driving inspiratory activity, the
462 preBötC has already been shown to have more widespread functions, acting as a master
463 oscillator driving other activities, such as sniffing and whisking (Deschênes et al., 2016;
464 Moore et al., 2013). Redundancy is usually a characteristic feature of neuronal networks, as it
465 provides robustness to perturbations (Li et al., 2016). It is therefore surprising to find that the
466 preBötC is so critical for respiratory, cardiovascular and other oscillations. Our study
467 provides evidence for another layer of influence for the preBötC as a hub oscillator that
468 synchronizes cardio-respiratory function. This coordination is a fundamental physiological
469 phenomenon observed across many different phyla that is likely to be of key importance for
470 optimal perfusion, and function, of tissues.
471

Key Resources Table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
strain, strain background (male Sprague Dawley rats)	WT Sprague Dawley rats	Biomedical Science Animal Facility of the University of Melbourne		
recombinant DNA reagent	pFUGW-hGtACR2-EYFP	Addgene, gift from John Spudich	plasmid # 67877; RRID:Addgene_67877	http://n2t.net/addgene:67877
recombinant DNA reagent	pAAV-CAG-hChR2-H134R-tdTomato	Addgene, gift from Karel Svoboda	plasmid #28017; RRID:Addgene_28017	http://n2t.net/addgene:28017
recombinant DNA reagent	pAM.DCA.spe.tdTomato	Gift from Verena Wimmer		
strain, strain background (lentivirus)	Lv-CBA-GtACR2-YFP-WPRE	In house cloning and virus production		Titre 6.95×10^9 IU/ml
strain, strain background (AAV)	AAV-CBA-GtACR2-YFP-WPRE	In house cloning and virus production		Titre 9.65×10^{11} VP/ml
strain, strain background (AAV)	AAV-CAG-hChR2-H134R-tdTomato	In house virus production		Titre 2.23×10^{10} VP/ml
strain, strain background (AAV)	AAV-CBA-tdTomato	In house virus production		Titre 6.32×10^{12} VP/ml

antibody	Rabbit polyclonal anti-TH	Merck-Millipore Bioscience Research Reagents	AB152	1:5000
antibody	Chicken polyclonal anti-GFP	Abcam	AB13970	1:5000
antibody	Mouse monoclonal anti-parvalbumin	Merck-Millipore Bioscience Research Reagents	MAB1572	1:10,000
antibody	Rabbit polyclonal anti-NK1R	Merck-Sigma-Aldrich	S8305	1:5000
antibody	Rabbit polyclonal anti-DsRed	Takara Bio	632496	1:5000
antibody	Goat polyclonal anti-ChAT	Merck-Millipore Bioscience Research Reagents	AB144P	1:1000
antibody	Rabbit polyclonal anti-GFP	Life Technologies	A-6455	1:500
antibody	Cy3-conjugated donkey polyclonal anti-rabbit	Jackson ImmunoResearch Laboratories	711-165-152	1:500
antibody	Cy3-conjugated donkey polyclonal anti-mouse	Jackson ImmunoResearch Laboratories	715-165-151	1:500
antibody	AlexaFluor-488 donkey polyclonal anti-rabbit	Jackson ImmunoResearch Laboratories	711-545-152	1:500

antibody	AlexaFluor-488 donkey polyclonal anti-mouse	Jackson ImmunoResearch Laboratories	715-545-151	1:500
antibody	AlexaFluor-488 donkey polyclonal anti-chicken	Jackson ImmunoResearch Laboratories	703-545-155	1:500
antibody	AlexaFluor-488 donkey polyclonal anti-rabbit	Life Technologies	A21206	1:500
sequence-based reagent	PCR primers for generation of cRNA probe for glycine transporter 2 (GlyT2)			SP6 forward primer: GGATCCATTTAGGT GACACTATAGAAGa agcgtcttgccactagaa T7 reverse primer: GAATTCTAATACGA CTCACTATAGGGAG Aagcctgagcttgctttcag
sequence-based reagent	PCR primers for generation of cRNA probe for glutamic acid decarboxylase 67 (GAD67)			SP6 forward primer: GGATCCATTTAGGT GAC ACTATAGAAGTtatgtc aatgcaaccgc T7 reverse primer: GAATTCTAATACGA CTCAC TATAGGGAGAccaac ctctctatttctcC
sequence-based reagent	PCR primers for generation of cRNA probe for vesicular glutamate transporter 2 (VGluT2)			SP6 forward primer: GGATCCATTTAGG TGACACTATAGA AGtcaatgaaatccaacgt cca T7 reverse primer: GAATTCTAATACG ACTCACTATAGG GAGAcagagcacagg

				acaccaaa
chemical compound, drug	meloxicam	Boehringer Ingelheim		1mg/kg, s.c.
chemical compound, drug	isoflurane	Rhodia Australia Pty. Ltd.,		5% induction, 3% maintenance
chemical compound, drug	ketamine	Lyppard		60mg/kg, i.m.
chemical compound, drug	medetomidine	Pfizer Animal Health		250 µg/kg, i.m.
chemical compound, drug	atipamazole	Pfizer Animal Health		1 mg/kg, i.m.
chemical compound, drug	urethane	SigmaAldrich		1.2 mg/kg i.v.
chemical compound, drug	vasopressin acetate	Sigma-Aldrich	V9879	0.5 nM
chemical compound, drug	vecuronium bromide	Organon Teknika		2–4 µg.ml ⁻¹
chemical compound, drug	atropine	Sigma Aldrich	A0257-25G	0.2µM
software, algorithm	Spike2	Cambridge Electrical Design		
software, algorithm	pClamp 10.3	Molecular Devices		

software, algorithm	ZEN 2.6	Carl-Zeiss		
software, algorithm	Affinity Designer	Serif Ltd.		
software, algorithm	Image J	NIH		
software, algorithm	SigmaPlot v12	Systat Software Inc.		
software, algorithm	Prism v2.0	GraphPad		

473

474 **Animal Experiments**

475 Experiments were conducted in accordance with the Australian National Health and Medical
476 Research Council ‘Code of Practice for the Care and Use of Animals for Scientific Purposes’
477 and were approved by the University of Melbourne Animal Research Ethics and Biosafety
478 Committees (ethics ID #1413273, #1614009, #1814599 and Florey 16-040). All experiments
479 were performed on male Sprague Dawley (SD) rats. Animals were housed with a 12-h light-
480 dark cycle (06H30 to 18H30), at a constant temperature ($22 \pm 1^\circ\text{C}$) with *ad libitum* access to
481 standard rat chow and water.

482

483 **Viruses**

484 To perform optogenetic inhibition or excitation of preBötC neurons, we used viral-mediated
485 transgenesis to drive the expression of two opsins, the guillardia theta anion channelrhodopsin
486 2 (*GtACR2*) and the humanized ChR2 H134R, fused to the enhanced yellow fluorescent
487 protein (YFP) and tdTomato reporter genes, respectively. To trace preBötC terminals, a virus
488 driving only tdTomato expression was used. We used the pan-neuronal promoter chicken β -
489 actin (CBA), either alone, or as part of the larger synthetic promoter CAG. Indeed, CAG
490 induces the same expression profile as CBA, since CBA is the promoter component (A) of

491 CAG (C being the cytomegalovirus early enhancer element, G being the splice acceptor of the
492 rabbit β -globulin gene). The viral vectors used were: Lv-CBA-*Gt*ACR2-YFP-WPRE (titre
493 6.95×10^9 IU/ml), AAV-CBA-*Gt*ACR2-YFP-WPRE (titre 9.65×10^{11} VP/ml), AAV-CAG-
494 hChR2-H134R-tdTomato (titre 2.23×10^{10} VP/ml), AAV-CBA-tdTomato (6.32×10^{12} VP/ml).
495 The *Gt*ACR2 constructs were cloned in-house from the pFUGW-hGtACR2-EYFP plasmid,
496 which was a gift from John Spudich (Addgene plasmid # 67877 ;
497 <http://n2t.net/addgene:67877> ; RRID:Addgene_67877) (Govorunova et al., 2015), by
498 replacing the UbiquitinC promoter with CBA. The ChR2-expressing virus was made from the
499 pAAV-CAG-hChR2-H134R-tdTomato plasmid, which was a gift from Karel Svoboda
500 (Addgene plasmid #28017; <http://n2t.net/addgene:28017> ; RRID:Addgene_28017) (Mao et
501 al., 2011). The tdTomato-expressing virus was made from pAM.DCA.spe.tdTomato plasmid.

502

503 **Brainstem virus microinjections**

504 Thirty to sixty minutes prior to surgery, 21 day old (P21) rats were injected with a non-
505 steroidal, anti-inflammatory drug (meloxicam, 1mg/kg, s.c., Metacam, Boehringer Ingelheim,
506 Sydney, Australia) and then lightly anesthetized by inhalation of isoflurane in an induction
507 box (Rhodia Australia Pty. Ltd., Notting Hill, Australia) prior to intramuscular injection of a
508 mixture of ketamine (60mg/kg, i.m.; Lyppard, Dingley, Australia) and medetomidine (250
509 μ g/kg, i.m.; Pfizer Animal Health, West Ryde, Australia). Surgery was initiated once a deep
510 surgical level of anesthesia was obtained, as evidenced by loss of the pedal withdrawal and
511 corneal reflexes. Body temperature was maintained at 37.5°C with a heat pad (TC-1000
512 Temperature controller, CWE Inc., USA) that was covered with an autoclaved absorbent pad.
513 Rats were then placed in a stereotaxic frame with the nose ventro-flexed (40°). A midline
514 incision was made over the occipital bone, portions of the bone overlying the cerebellum were
515 removed with a dental drill, and the atlanto-occipital membrane was opened to expose the
516 dorsal brainstem. Glass micropipettes (~30 μ m tip diameter) were back-filled with injectate
517 and connected via a silver wire to an electrophysiology amplifier (NeuroLog system,
518 Digitimer Ltd., Welwyn Garden City, UK) to enable recording of neuronal activity. The
519 pipette (20° angled in the rostro-caudal axis, tip pointing forward) was descended into the
520 brainstem 1.5 mm lateral to the midline and extracellular recordings of multiunit activity used
521 to functionally map the preBötC, defined as the most rostral point at which vigorous
522 inspiratory-locked activity was isolated (typically 0.2 ± 0.3 mm rostral, 1.4 ± 0.3 mm ventral
523 to the *calamus scriptorius*). Bilateral injections were performed for *Gt*ACR2 and ChR2
524 expressions, unilateral injection for tdTomato expression. For *in vitro* slice electrophysiology,

525 *GtACR2* expression was induced in the solitary tract nucleus (NTS). NTS injection
526 coordinates, relative to *calamus scriptorius* for antero-posterior and medio-lateral coordinates,
527 with all injections performed 0.4mm ventral to the dorsal surface of the brainstem at each
528 injection coordinate: 0.4mm and 0.1mm caudal (midline); 0.1mm rostral, 0.25mm lateral
529 (both sides); 0.4mm rostral, 0.4mm lateral (both sides). A picospritzer (World Precision
530 Instruments, Sarasota, USA) was used to microinject viral vectors driving *GtACR2*-YFP (50
531 nl per injection site over 5 min), *ChR2*-tdTomato (100 nl per injection site over 1 min) or
532 tdTomato alone (5 nl per injection site over 1 min). Pipettes were left in place for 5 minutes
533 after injection, the pipette withdrawn, and wounds closed with sterile sutures, and anesthesia
534 reversed with atipamazole (1 mg/kg, i.m., Antisedan, Pfizer Animal Health, West Ryde,
535 Australia). Physiological experiments (working heart and brainstem preparation (WHBP), n =
536 12 *GtACR2* and n = 8 *ChR2*; *in vivo* electrophysiology, n = 5 *Lv-GtACR2* and n=3 AAV-
537 *GtACR2*; and *in vitro* whole cell recordings, n = 7) were performed ten days after viral
538 injections, while animals used for anterograde tracing (n = 2) were sacrificed twenty days
539 after vector injection.

540

541 ***In vivo* electrophysiology**

542 *Lv-GtACR2* injected rats (n = 5), *Figure 2A-D*: Thirty to sixty minutes prior to
543 surgery, rats were injected with a non-steroidal, anti-inflammatory drug (meloxicam, 1mg/kg,
544 s.c., Metacam, Boehringer Ingelheim, Sydney, Australia) and then lightly anesthetized by
545 inhalation of isoflurane in an induction box (Rhodia Australia Pty. Ltd., Notting Hill,
546 Australia) prior to intramuscular injection of a mixture of ketamine (60mg/kg, i.m.; Lyppard,
547 Dingley, Australia) and medetomidine (250 µg/kg, i.m.; Pfizer Animal Health, West Ryde,
548 Australia). Surgery was initiated once a deep surgical level of anesthesia was obtained, as
549 evidenced by loss of the pedal withdrawal and corneal reflexes. Body temperature was
550 maintained at 37.5°C with a heat pad (TC-1000 Temperature controller, CWE Inc., USA) that
551 was covered with an autoclaved absorbent pad. Rats were placed in a stereotaxic frame with
552 the nose slightly dorso-flexed (mouth bar set at 0). A small incision was made over the dorso-
553 lateral thorax and bipolar stainless steel hook electrodes were placed in consecutive
554 intercostal muscles with a ground electrode positioned under the skin. Recordings of
555 inspiratory intercostal activity were amplified, filtered (50–1500 Hz band-pass, NeuroLog
556 system, Digitimer Ltd., Welwyn Garden City, UK), sampled (5 kHz) and digitized (Power
557 1401, Cambridge Electrical Design, Cambridge, UK), and rectified and integrated using
558 Spike2 software (Cambridge Electrical Design, Cambridge, UK). Heart rate (HR) was derived

559 from the same biopotential using a window discriminator to trigger from the R-wave of the
560 electrocardiogram.

561 *AAV-GtACR2 injected rats (n = 3), Figure 2S1:* Thirty to sixty minutes prior to
562 surgery, rats were injected with a non-steroidal, anti-inflammatory drug (meloxicam, 1mg/kg,
563 s.c., Metacam, Boehringer Ingelheim, Sydney, Australia) and then lightly anesthetized by
564 inhalation of isoflurane in an induction box (Rhodia Australia Pty. Ltd., Notting Hill,
565 Australia). The rats were transferred to a surgical table and the nose placed in a nose cone
566 delivering 3-3.5% isoflurane in 100% oxygen at a flow rate of 0.8 L/min. Surgery was
567 initiated once a deep surgical level of anesthesia was obtained, as evidenced by loss of the
568 pedal withdrawal and corneal reflexes. Body temperature was maintained at 37.5°C with a
569 heat pad (TC-1000 Temperature controller, CWE Inc., USA) that was covered with an
570 autoclaved absorbent pad. The femoral artery and femoral vein were cannulated for
571 measurement of arterial pressure and drug administration, respectively (PE10 tubing (ID 0.28
572 x OD 0.61 mm) connected to PE50 (0.7 x 1.45 mm)). For diaphragm electromyography
573 (DiaEMG) recordings, a lateral abdominal incision was made and two nylon-insulated
574 stainless-steel wire electrodes ending with suture pads were placed in the costal diaphragm.
575 Isoflurane was slowly replaced by urethane (1.2 mg/kg i.v., SigmaAldrich, St Louis, USA),
576 following which rats were tracheotomized, and oxygen (100%) directed over the tracheotomy
577 cannula. Rats were placed in a stereotaxic frame with the nose slightly dorso-flexed (mouth
578 bar set at 0). The arterial catheter was connected to a pressure transducer (P23 Db, Statham
579 Gould, USA), and recordings of DiaEMG were amplified and filtered (500-1000 Hz band-
580 pass, NeuroLog System, Digitimer Ltd., UK). Both signals were sampled (5 kHz) and
581 digitized (Power 1401, Cambridge Electrical Design, Cambridge, UK), and monitored using
582 Spike2 software (Cambridge Electrical Design, Cambridge, UK).

583 *Then, for both groups:* A midline incision was made over the occipital bone, portions
584 of the bone overlying the cerebellum were removed with a dental drill, and bilateral optical
585 fibers (200 µm diameter; Doric Lenses, Quebec, Canada) connected to a 473nm DPSS Laser
586 (Shanghai Laser & Optics Century Co., Shanghai, China) were descended vertically. Optical
587 fibers were positioned 1.5 mm lateral (lambda level), and descended slowly while
588 photostimulating at 20 Hz (10 ms pulses) until maximal respiratory and bradycardic effects
589 were observed. The experimental protocol was then started after a 15 min period of
590 acclimatization.

591

592 ***In vitro* slice electrophysiology**

593 Rats were deeply anaesthetised with 5% isoflurane and the medulla removed, blocked and
594 rapidly cooled in artificial cerebrospinal fluid (aCSF, 2 °C). The medulla was sectioned into
595 250 µm horizontal slices containing the transduced neurons (Leica VT1200s). Slices were
596 continuously perfused in aCSF solution, containing (in mM): NaCl, 125; KCl, 3; KH₂PO₄,
597 1.2; MgSO₄, 1.2; NaHCO₃, 25; dextrose, 10; CaCl₂, 2; (300 mOsmol), bubbled with 95% O₂,
598 5% CO₂, at 32 °C. Recording pipettes (3.5 – 4.5 MΩ) contained a low Cl⁻ internal solution (10
599 mM Cl⁻) containing (in mM): NaCl, 6; NaOH, 4; KOH, potassium gluconate, 130; EGTA, 11;
600 CaCl₂, 1; HEPES, 10; MgCl₂, 1; 0.1% biocytin (pH 7.3, 290 mOsmol). As a consequence
601 ECl⁻ was -68.8 mV. Pipettes were visually guided to NTS transduced YFP-neurons using a
602 fixed stage scope (Zeiss Examiner) and camera (Rolera EM-C2, Q-Imaging). Whole-cell
603 recordings were made in either voltage clamp (holding voltage -60 mV or -40 mV) or current
604 clamp mode (MultiClamp 700B and pClamp 10.3, Molecular Devices). Signals were sampled
605 at 20 kHz and filtered at 10 kHz. Liquid junction potentials were not corrected (-6.2 mV at 32
606 °C). Pulses of light (465 nm, 10 mW at fibre tip) from a light emitting diode (LED, Plexon)
607 were delivered via fibre optic affixed to a micro-manipulator and positioned to illuminate the
608 recorded neurons.

609

610 **Working Heart-Brainstem Preparation**

611 Rats were anaesthetized deeply with isoflurane until loss of the pedal withdrawal reflex,
612 bisected below the diaphragm, exsanguinated, cooled in Ringer solution on ice (composition
613 in mM: 125 NaCl, 24 NaHCO₃, 5 KCl, 2.5 CaCl₂, 1.25 MgSO₄, 1.25 KH₂PO₄ and 10
614 dextrose, pH 7.3 after saturation with carbogen gas (chemicals were purchased from Sigma-
615 Aldrich, Australia)) and decerebrated precollicularly. The lungs were removed and the
616 descending aorta was isolated and cleaned. Retrograde perfusion of the thorax and head was
617 achieved via a double-lumen catheter (ø 1.25mm, DLR-4, Braintree Scientific, Braintree,
618 USA) inserted into the descending aorta. The perfusate was Ringer solution containing Ficoll
619 (1.25%), vasopressin acetate (0.5 nM, V9879, Sigma-Aldrich, Australia) and vecuronium
620 bromide (2–4 µg.ml⁻¹, Organon Teknika, Cambridge, UK) warmed to 31°C and gassed with
621 carbogen (closed loop reperfusion circuit, 25-30 ml/min). The second lumen of the cannula
622 was connected to a transducer to monitor perfusion pressure (PP) in the aorta. The head of the
623 preparation was fixed with ear and mouth bars with the nose ventro-flexed (40°).
624 Simultaneous recordings of phrenic nerve activity (PNA), cervical vagus nerve (left side)
625 activity (VNA) and thoracic sympathetic chain activity (tSNA, between T8-10) were obtained
626 using glass suction electrodes. Some WHBPs for both *GtACR2* and *ChR2* experiments were

627 performed without VNA recordings, to preserve a fully functional vagal cardiac
628 parasympathetic modulation by keeping both vagus nerves intact. No differences in HR and
629 respiratory sinus arrhythmia were found between WHBPs with either unilateral or bilateral
630 intact vagus nerves, therefore experiments were pooled for analysis. Neurograms were
631 amplified, filtered (50–1500 Hz band-pass, NeuroLog system, Digitimer Ltd., Welwyn
632 Garden City, UK), rectified and integrated using Spike2 (Cambridge Electrical Design,
633 Cambridge, UK). HR was derived by using a window discriminator to trigger from the R-
634 wave of the electrocardiogram recorded simultaneously through the phrenic nerve electrode.
635 Bilateral optical fibers (200µm diameter; Doric Lenses, Quebec, Canada) connected to a 473
636 nm DPSS Laser (Shanghai Laser & Optics Century Co., Shanghai, China) were descended,
637 20° angled in the rostro-caudal axis, tip pointing forward, to a position 400 µm dorsal to the
638 injection coordinates. Pilot mapping experiments indicated that light delivery to these
639 coordinates was associated with the largest physiological effects.

640

641 **Optogenetic stimulation**

642 A light meter (PM20A fiber power meter, Thorlabs, Newton, NJ, USA) was used to calibrate
643 light output from each optical fiber to 10 mW as this was found to induce maximal effects in
644 our experimental set-up without any adverse effect on the tissue (Menuet et al., 2014).
645 Photostimulation consisted of: i) frequency-response trials to compare physiological effects
646 induced by *GtACR2* photoactivation in the WHBP (5, 10, 20 and 50 Hz, 10 ms pulses, with
647 respectively 300, 500, 1000 and 1500 pulses at each frequency) and at the single cell level *in*
648 *vitro* (1, 10, 20 and 50 Hz; 1, 10 and 100 ms pulses, 30s each). The results of these trials
649 indicated that maximal physiological effects were evoked by 10 ms light pulses delivered at
650 20 Hz; in subsequent experiments *in vivo* or in the WHBP we used tetanic (20 Hz, 300-600
651 pulses), intermittent (1 Hz, 300 pulses), or single triggered 10ms pulses delivered at specific
652 phases of the respiratory cycle. All photostimulation protocols were repeated multiple times,
653 in a randomized manner, in each animal (technical replication). We found strong intra-
654 preparation reproducibility for all protocols.

655

656 **Histology**

657 At the end of WHBP experiments, brainstems were removed, fixed by immersion in 4%
658 formaldehyde in 0.1M sodium phosphate buffer (FA), for 24 h at 4°C and cryoprotected in
659 20% sucrose. At the end of the *in vivo* and tracing experiments animals were perfused
660 transcardially with 200 ml phosphate-buffered saline followed by 400 ml of 4% FA.

661 Brainstems were removed, post-fixed for 12 h in 4% FA at 4°C and cryoprotected in 20%
662 sucrose. Brainstems were mostly cut coronally (40 µm sections), except for parvalbumin
663 labelling where they were cut sagittally (50 µm sections), using a cryostat. Fluorescence
664 immunohistochemistry was performed as previously described (Chen et al., 2010; Menuet et
665 al., 2014; Sevigny et al., 2012). Primary antibodies used were rabbit anti-TH (1:5000, Merck-
666 Millipore Bioscience Research Reagents, Bayswater, VIC, Australia, AB152), chicken anti-
667 GFP (1:5000, Abcam, Melbourne, VIC, Australia, AB13970), mouse anti-parvalbumin
668 (1:10,000, Merck-Millipore Bioscience Research Reagents, Bayswater, VIC, Australia,
669 MAB1572), Rabbit anti-NK1R (1:5000, Merck-Sigma-Aldrich, Castle Hill, NSW, Australia
670 S8305), Rabbit anti-DsRed (1:5000, Takara Bio, Mountain View, CA, USA, 632496) and
671 Goat anti-ChAT (1:1000; Merck-Millipore Bioscience Research Reagents, Bayswater, VIC,
672 Australia, AB144P). The secondary antibodies used were Cy3-conjugated donkey anti-rabbit
673 (1:500, Jackson ImmunoResearch Laboratories, West Grove, USA), Cy3-conjugated donkey
674 anti-mouse (1:500, Jackson ImmunoResearch Laboratories, West Grove, USA), AlexaFluor-
675 488 donkey anti-rabbit (1:500, Jackson ImmunoResearch Laboratories, West Grove, USA),
676 AlexaFluor-488 donkey anti-mouse (1:500, Jackson ImmunoResearch Laboratories, West
677 Grove, USA) and AlexaFluor-488 donkey anti-chicken (1:500, Jackson ImmunoResearch
678 Laboratories, West Grove, USA).

679

680 **In situ hybridization**

681 cRNA probes for glycine transporter 2 (GlyT2) and glutamic acid decarboxylase 67 (GAD67)
682 were synthesized as previously described, permitting identification of glycinergic and
683 GABAergic neurons respectively by *in situ* hybridization (Bowman et al., 2013; Le et al.,
684 2016). A digoxigenin-labelled cRNA probe for vesicular glutamate transporter 2 (VGluT2), a
685 marker of glutamate synthesis, was synthesized as follows:

686 cDNA template (886bp, Genebank reference sequence NM_053427) was amplified using the
687 PCR primers (lower case) with T7 and Sp6 RNA polymerase promoters (upper case) attached
688 F:GGATCCATTTAGGTGACACTATAGAAGtcaatgaaatccaacgtcca;

689 R: GAATTCTAATACGACTCACTATAGGGAGAcaagagcacaggacacccaaa.

690 Following purification of the DNA template with gel extraction, antisense riboprobes
691 were *in vitro* transcribed (Epicentre Technologies, Madison, WI) incorporating digoxigenin-
692 11-UTP (Roche Applied Science) and validated as previously described (Kumar et al., 2012).

693 Sections containing GtACR2-YFP-labelled neurons were incubated in pre-
694 hybridization buffer (50% formamide, 100 µg/ml heparin, 5 x SSC, pH 7.0, 1 x Denhardt's

695 solution, 250 µg/ml herring sperm DNA, 100 µg/ml yeast tRNA, 5% dextran sulphate, 0.1%
696 Tween-20, reagents obtained from Sigma, Australia unless otherwise indicated) at 37 °C (30
697 minutes), then 58 °C (1 h) before hybridization with gentle agitation with GlyT2, GAD67 or
698 VGluT2 riboprobe (1000 ng/ml) at 58 °C (12-18 h). Sections were washed in 2 x SSC buffer
699 with 0.1% Tween-20, followed by 0.2 x SSC buffer with 0.1% Tween-20, and then maleic
700 acid buffer (0.1 M maleic acid, 0.15 M NaCl, 0.1% Tween-20). The tissue was then blocked
701 in maleic acid buffer containing 2% Boehringer blocking reagent (Roche Applied Science,
702 Mannheim, Germany) and 10% normal horse serum.

703 Rabbit anti-GFP (1:500, Life Technologies, Scoresby, Australia, A-6455) was added
704 to the blocking buffer and incubated at 4 °C (24 h), then at room temperature (4 h). Sections
705 were washed in TPBS (Tris-HCl 10 mM, sodium phosphate buffer 10 mM, 0.9% NaCl, pH
706 7.4, 3 x 30 min) and incubated overnight with AlexaFluor-488 donkey anti-rabbit IgG
707 secondary antibody (1:500, Life Technologies, Scoresby, Australia, A21206) with 2% normal
708 horse serum. DIG-labelled neurons were revealed by incubation in NTMT (0.1 M NaCl, 0.1
709 M Tris-HCl pH 9.5, 0.1 M MgCl₂, 0.1% Tween-20, 2 mM tetramisole HCl) containing nitro
710 blue tetrazolium (Roche Applied Science) and 5-bromo-4-chloro-3-indolyl phosphate salts
711 (Roche Applied Science). The reaction was stopped by washing (0.1M Tris, 1 mM EDTA, pH
712 8.5, 3 x 15 min) when DIG-labelling was intense with minimal background staining. No
713 labelling was seen when the sense probe was substituted for the anti-sense probe.

714

715 **Mapping of reporter expression**

716 A complete map of the distribution of *GtACR2*-YFP expression from the 12 WHBP
717 experiments was generated from 1:4 series of sections co-labelled with ChAT, TH and NK1R
718 to assist with alignment of the sections into the Paxinos-Watson atlas (Paxinos and Watson,
719 2004). Heatmaps of pooled *GtACR2*-YFP expression, imaged using Zeiss Axio Imager.M2
720 microscope with an Apocromat 20x/0.8 air objective (Carl-Zeiss, NSW, Australia) and
721 captured using Axiocam 506 mono camera and ZEN 2.6 (blue edition) imaging software,
722 were made using Affinity Designer software (Serif Ltd., UK). Sections from four
723 representative animals used in the WHBP were processed to examine colocalization of
724 preBötC *GtACR2*-YFP with GAD67, VGluT2 and GlyT2 mRNA. Sections were imaged
725 under epifluorescence and brightfield (Zeiss Z1, 10x objective, 0.3 NA). The expression profile
726 of ChR2-tdTomato and *GtACR2*-YFP used for *in vivo* experiments were similar to that of
727 *GtACR2*-YFP from WHBP experiments, so they were mapped qualitatively rather than

728 quantitatively. The brainstems from two animals from the *GtACR2-YFP in vivo* experiments
729 were cut sagittally for better visualization of rostral-caudal *GtACR2-YFP* expression.

730 For the tracing study, tdTomato expression at the injection site was examined under
731 Zeiss Axio Imager.M2 microscope with an Apocromat 20x/0.8 air objective (Carl-Zeiss,
732 NSW, Australia) and captured using AxioCam 506 mono camera and ZEN 2.6 (blue edition)
733 imaging software. TdTomato terminals were imaged with a Zeiss LSM880, Axio Imager 2
734 confocal microscope, with a Plan-Apochromat 63x/1.4 oil DIC M27 objective, in a Z-stack
735 array, acquiring up to 8 consecutive overlapping slices. All channels were acquired at a
736 consistent pinhole setting of 1.0 μm . Each optical section was assessed for close apposition
737 between axons labelled by tdTomato and ChAT-immunoreactive nucleus ambiguus vagal
738 preganglionic neurons or TH-immunoreactive C1 pre-sympathetic neurons. Maximum
739 intensity projections of 4-6 image stacks were produced using the Image J software (NIH,
740 USA) to better represent the distribution of preBötC terminal labeling.

741

742 **Data analysis and statistics**

743 Nerve signals were rectified and integrated with a 50 ms time constant. For low frequency
744 photostimulation (1 Hz) laser triggered averaging of tSNA and VNA, the signals were
745 rectified and integrated with a 5 ms time constant. For triggered photostimulations, PNA
746 signals were rectified and integrated with a 0.5 ms DC remove time constant and a 100 ms
747 smoothing time constant, to eliminate the ECG background. Phrenic-triggered (end of
748 inspiratory burst) averaging of tSNA, systolic PP and HR were used to quantify respiratory
749 modulation of tSNA (RespSNA), Traube-Hering waves (mmHg) and respiratory sinus
750 arrhythmia (RSA, bpm), respectively. Tonic levels of tSNA were measured using the phrenic-
751 triggered averaging of tSNA, as the average of tSNA during the expiratory period, therefore
752 outside of the burst of respiratory modulation of SNA. Phrenic-triggered (end of inspiratory
753 burst) averaging of VNA was used to quantify the peak post-inspiratory and non-respiratory
754 components of VNA. Details regarding the period analysed are shown on Figure 3S2. For
755 manual photostimulations, to avoid averaging distortion, phrenic-triggered averagings were
756 performed on both consecutive respiratory-related activities to be analysed, and then traces
757 were overlapped and merged at their mean intersection for further analysis. Statistical analysis
758 was done on raw values, and then mean deltas in percentages were quantified
759 (photostimulation vs pre-photostimulation period). Statistics shown on figures presenting only
760 mean delta percentages (for presentation clarity, e.g. Figure 3) were done on the raw values as
761 shown on the associated supplementary figures (e.g. Figure 3S1 and associated data source).

762 Group data are presented as mean \pm SEM. Normal distribution of data was verified with the
763 Shapiro-Wilk test, and equal variance of data was tested (SigmaPlot v12, Systat Software
764 Inc., Erkrath, Germany). Data were analyzed using one-way repeated measures or two-way
765 repeated-measures ANOVA, followed by *post hoc* Holm-Sidak multiple-comparison tests, or
766 paired student's *t* tests, for data with normal distribution and equal variance. Data with failed
767 normal distribution or failed equal variance were analyzed using Friedman repeated measures
768 analysis of variance on ranks followed with pairwise multiple comparison Tukey test, or
769 Wilcoxon signed rank test. Linear regression and correlation analysis was used to analyze
770 parameters relationships (Prism v2.0, GraphPad, La Jolla, USA). When missing values were
771 present, for instance because one photostimulation frequency couldn't be performed or some
772 of the associated data were not appropriate for analysis (noise etc.), it was dealt with
773 accordingly depending on the test used, mostly by excluding the sample from the whole
774 analysis (cf. source data and statistics). All source data and detailed statistics are presented as
775 supplementary information along with each figure. Differences were considered significant at
776 $p < 0.05$.

777 **Acknowledgments**

778 The authors acknowledge the facilities and technical assistance of the Biological Optical
779 Microscopy Platform (University of Melbourne) for the confocal microscopy images.

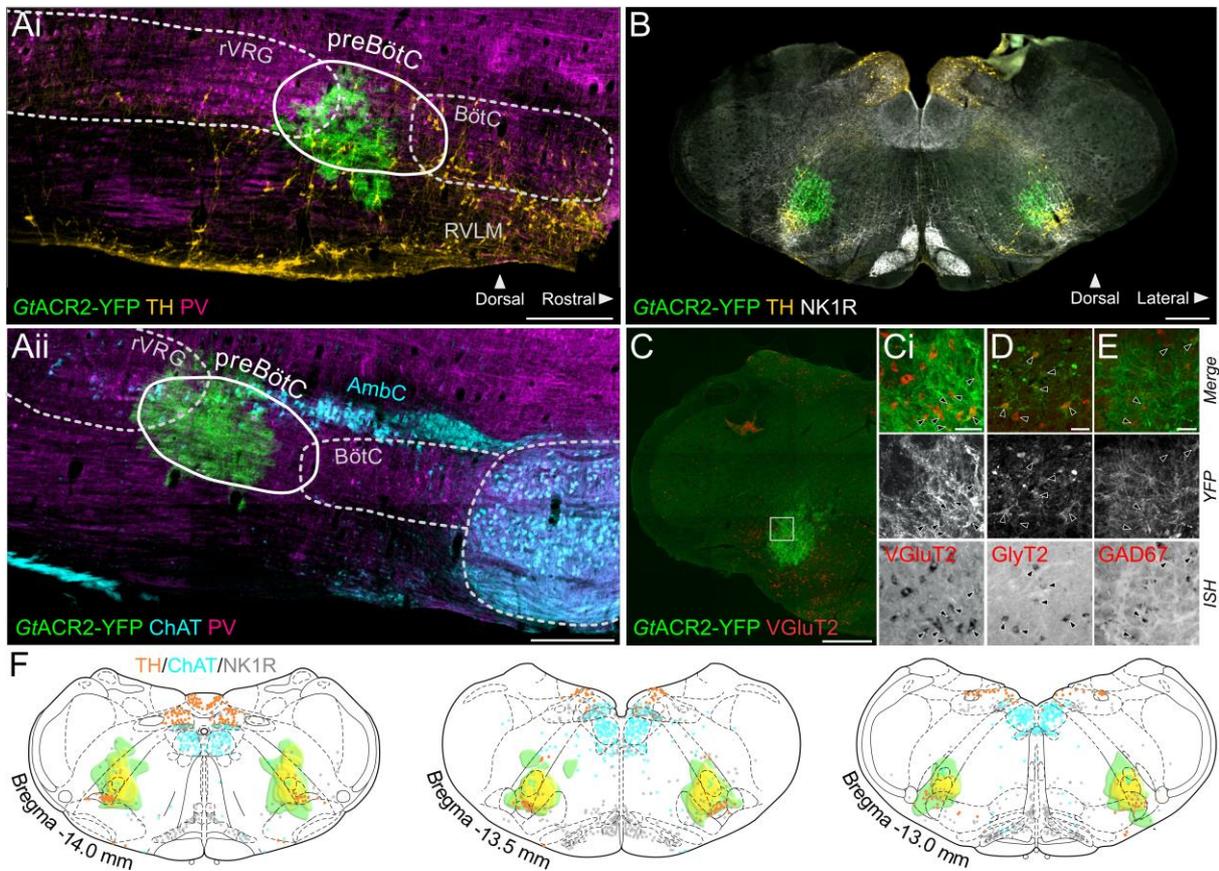
780

781 **Declaration of interests**

782 The authors declare no competing interests.

783 **Figures and legends**

784



785

786 **Figure 1. Expression of *GtACR2*, in the preBötC.** (A) Sagittal and (B) coronal sections
 787 showing expression of *GtACR2*-yellow fluorescent protein (YFP) and parvalbumin (PV),
 788 tyrosine hydroxylase (TH), choline acetyltransferase (ChAT) and neurokinin type 1 receptor
 789 (NK1R) immunoreactivity. (C) *In situ* hybridization showing *GtACR2*-YFP expression in
 790 glutamatergic (Ci), glycinergic (D) and GABAergic (E) neurons in the preBötC, as indicated
 791 by black arrows. (F) Schematic coronal maps, based on atlas of Paxinos and Watson (Paxinos
 792 and Watson, 2004), showing the distribution of *GtACR2*-YFP from all rats (n=12) used in
 793 Working Heart-Brainstem Preparation experiments relative to TH, ChAT and NK1R
 794 expression. Each rat is depicted in green with the gradation to yellow indicating increasing
 795 overlap in expression between animals, and indicating that the common expression area
 796 overlies the preBötC. Scale bars are 500 μ m (Ai, Aii, B, C) or 50 μ m (Ci, D, E).

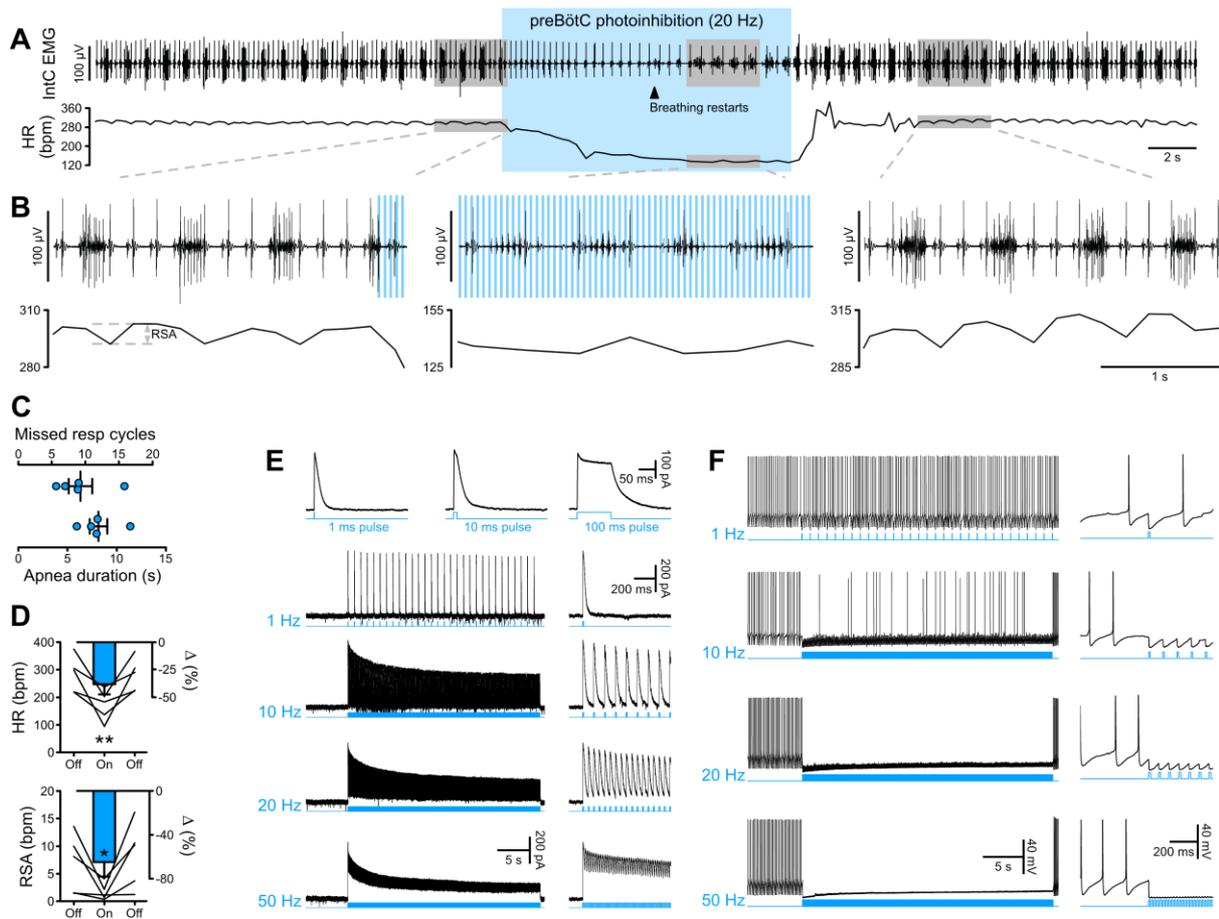
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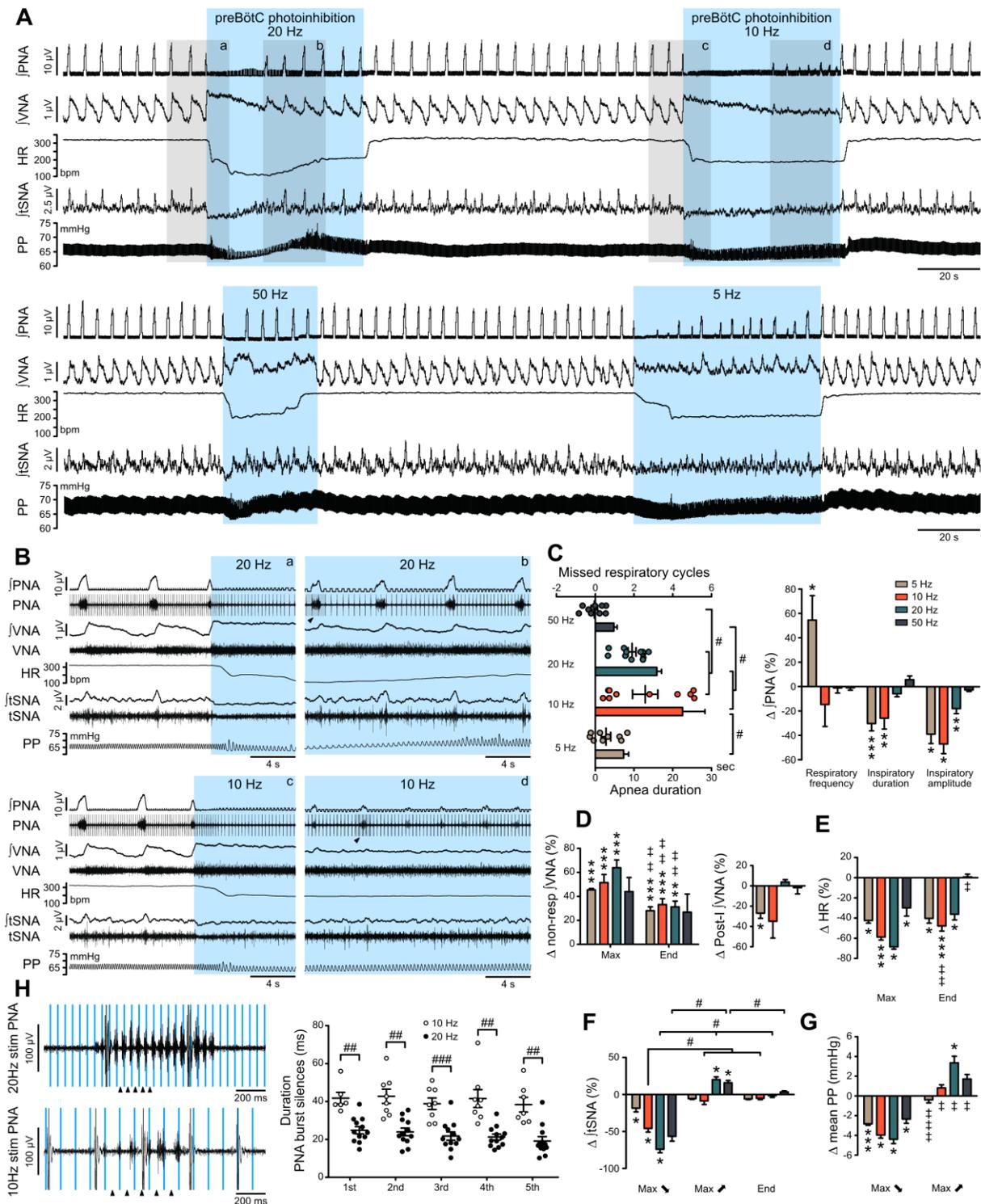
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Figure 2. PreBötC photoinhibition *in vivo* induces respiratory cessation, bradycardia, and suppresses RSA, and *GtACR2* photoactivation *in vitro* causes strong neuronal inhibition. (A) Intercostal (intC) electromyograph (EMG) recording in an anesthetized P31 rat with bilateral *GtACR2* expression in the preBötC, showing bursts of inspiratory activity with the electrocardiogram superimposed, from which heart rate (HR) is derived. Prolonged (20 Hz, 10 ms pulses, 300 pulses) preBötC photoinhibition, starting during inspiration, immediately stopped the inspiratory burst and induced a long apnea, before respiratory activity resumed even though preBötC photoinhibition continued. PreBötC photoinhibition also induced a profound bradycardia, and a decrease in arterial pressure as shown on Figure 2S1 on a separate animal cohort (n=3). (B) Enlargements of the shaded areas in (A), showing that the first pulse of light immediately stops the intC EMG inspiratory burst, and that when breathing activity resumes during preBötC photoinhibition the intC EMG inspiratory bursts are crenelated with periodic cessations of intC EMG activity after each light pulse. Note that the overall intC EMG ramping discharge pattern is maintained. Respiratory sinus arrhythmia (RSA), the oscillations in HR in phase with respiratory activity, is abolished during preBötC photoinhibition, even when breathing activity resumes. (C-D) PreBötC photoinhibition induced a prolonged cessation of breathing activity, bradycardia and suppression of RSA in

820 all animals tested (n=5, mean \pm SEM for group values; one-way repeated measures ANOVA
821 followed with post hoc Holm-Sidak multiple-comparison test performed on raw data, as
822 shown on the associated source data and detailed statistics; * $p < 0.05$, ** $p < 0.01$,
823 photoinhibition vs. control and recovery conditions). (E-F) Whole-cell recordings (low Cl⁻
824 internal solution, ECl⁻ = -68.8 mV) of *GtACR2*-positive solitary tract nucleus neurons in
825 brainstem slices in voltage clamp ((E), holding voltage -40mV) and current clamp (F) modes.
826 (E) *GtACR2* photostimulation induced large inhibitory conductances that showed fast
827 activation and deactivation kinetics, and little inactivation, as previously shown (Govorunova
828 et al., 2015). The photocurrents persisted throughout the prolonged photostimulation
829 protocols. (F) In a spontaneously active cell, *GtACR2* photostimulation prevented action
830 potential firing when the light pulse was delivered at the appropriate timing, resulting in
831 complete firing cessation during prolonged high frequency photostimulations including and
832 above 20 Hz.
833



834

835 **Figure 3. Effect of prolonged preBötC photoinhibition *in situ* on cardio-respiratory**

836 **function.** (A) Recording of phrenic nerve activity (PNA), vagus nerve activity (VNA) and

837 thoracic sympathetic nerve activity (tSNA), heart rate (HR) and perfusion pressure (PP), in an

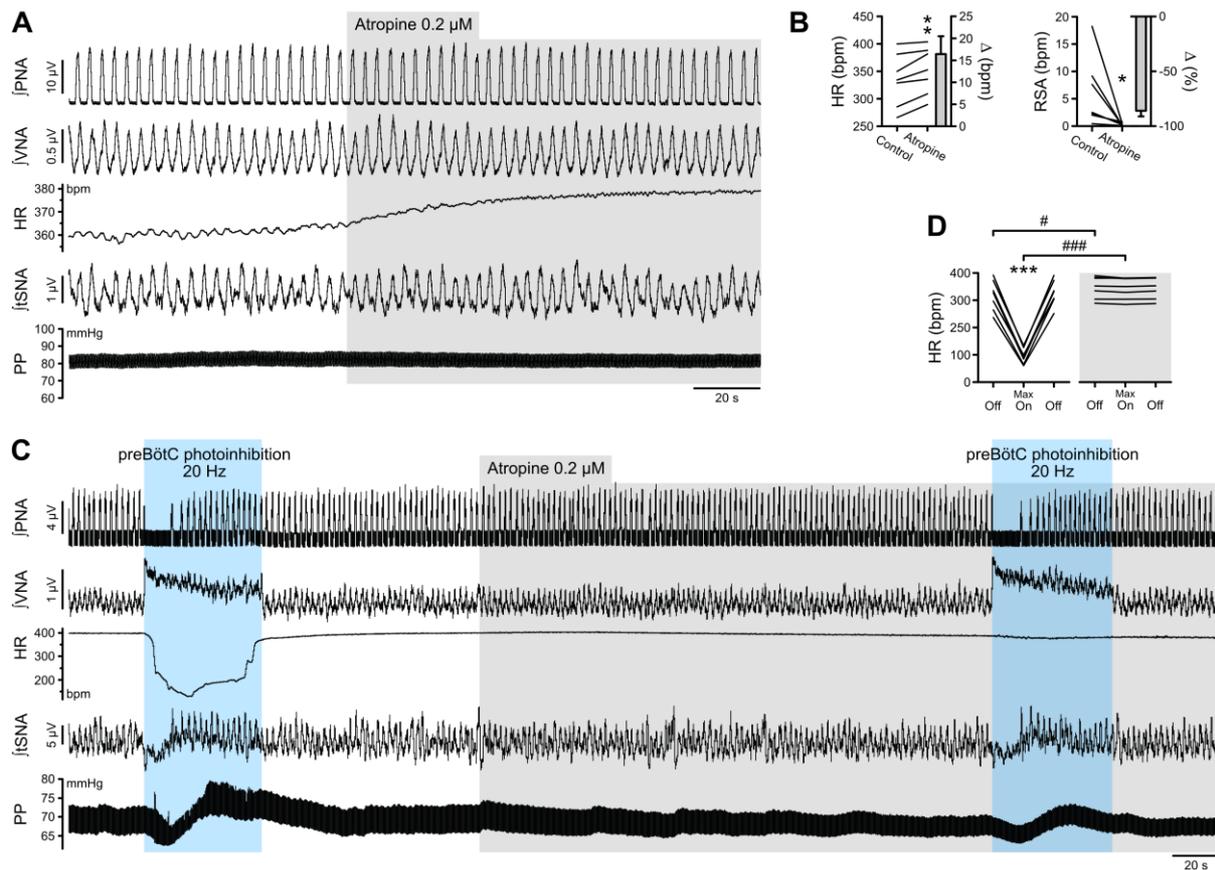
838 *in situ* Working Heart-Brainstem Preparation (WHBP) with bilateral photoinhibition of the

839 preBötC at 5, 10, 20 and 50 Hz. Following the initial apnea, PNA resumed despite continued

840 preBötC photoinhibition. VNA increased and HR decreased for the entire photoinhibition

841 period. A transient decrease in tSNA and PP, concomittent with the apnea, was followed by
842 increases in tSNA and PP with the return of PNA. (B) Higher resolution recordings of the
843 shaded areas in (A) showing the strongest physiological effects occurred with 20 Hz and 10
844 Hz photoinhibition. (C-G) Group data (n=12), expressed as change relative to the pre-
845 photoinhibition control period, examining changes in respiratory parameters, including PNA
846 characteristics following resumption of activity during continued photoinhibition (C); VNA
847 measured outside of inspiratory/post-inspiratory activities (non-resp) and in the post-
848 inspiratory period (D); HR (E) tSNA (F) and PP (G). Details regarding VNA and tSNA
849 analysis are shown on Figure 3S2. (H) Higher temporal resolution recordings of the periods
850 denoted by arrowheads in (B), showing the crenelated PNA burst shape, yet with maintained
851 ramping discharge pattern. Analysis of the first five PNA burst silence periods, denoted by
852 arrowheads in (H), showed the 20 Hz photoinhibition induced shorter silence durations.
853 Group data are presented as mean \pm SEM ; Friedman repeated measures analysis of variance
854 on ranks followed with pairwise multiple comparison Tukey test, one way repeated measures
855 ANOVA followed with *post hoc* Holm-Sidak multiple-comparison test, paired student's t-test
856 or Wilcoxon signed rank test, performed on raw data, as shown on Figure 3-figure
857 supplement 1 and associated source data and detailed statistics ; * $p < 0.05$, ** $p < 0.01$, *** p
858 < 0.001 , photoinhibition vs. control and recovery conditions ; # $p < 0.05$, ## $p < 0.01$, ### $p <$
859 0.001 , between compared data ; ‡ $p < 0.05$, ‡‡ $p < 0.01$, ‡‡‡ $p < 0.001$, intra-photostimulation
860 frequency vs. max change.

861



862

863 **Figure 4. The bradycardia induced by preBötC photoinhibition is mediated by increased**

864 **cardiac parasympathetic drive.** (A) The effect of bath application of the muscarinic

865 antagonist, atropine, in the *in situ* WHBP of a P31 rat. (B) Group data (n=7) showing that

866 atropine induced an increase in HR and abolished RSA, confirming its effective blockade of

867 cardiac parasympathetic modulation. (C) Prolonged 20 Hz preBötC photoinhibition before,

868 and after, bath application of atropine. (D) Atropine didn't affect the increase in VNA induced

869 by preBötC photoinhibition, but totally blocked the decrease in HR, showing that preBötC

870 photoinhibition induces increased cardiac parasympathetic drive. The sympathetic vasomotor

871 effects induced by preBötC photoinhibition were not altered by atropine, showing that they

872 are not mediated by baroreflex afferents. Group data are presented as mean \pm SEM ; paired

873 student's *t* test, Wilcoxon signed rank test or two-way repeated measures ANOVA followed

874 with post hoc Holm-Sidak multiple-comparison test performed on raw data, as shown on the

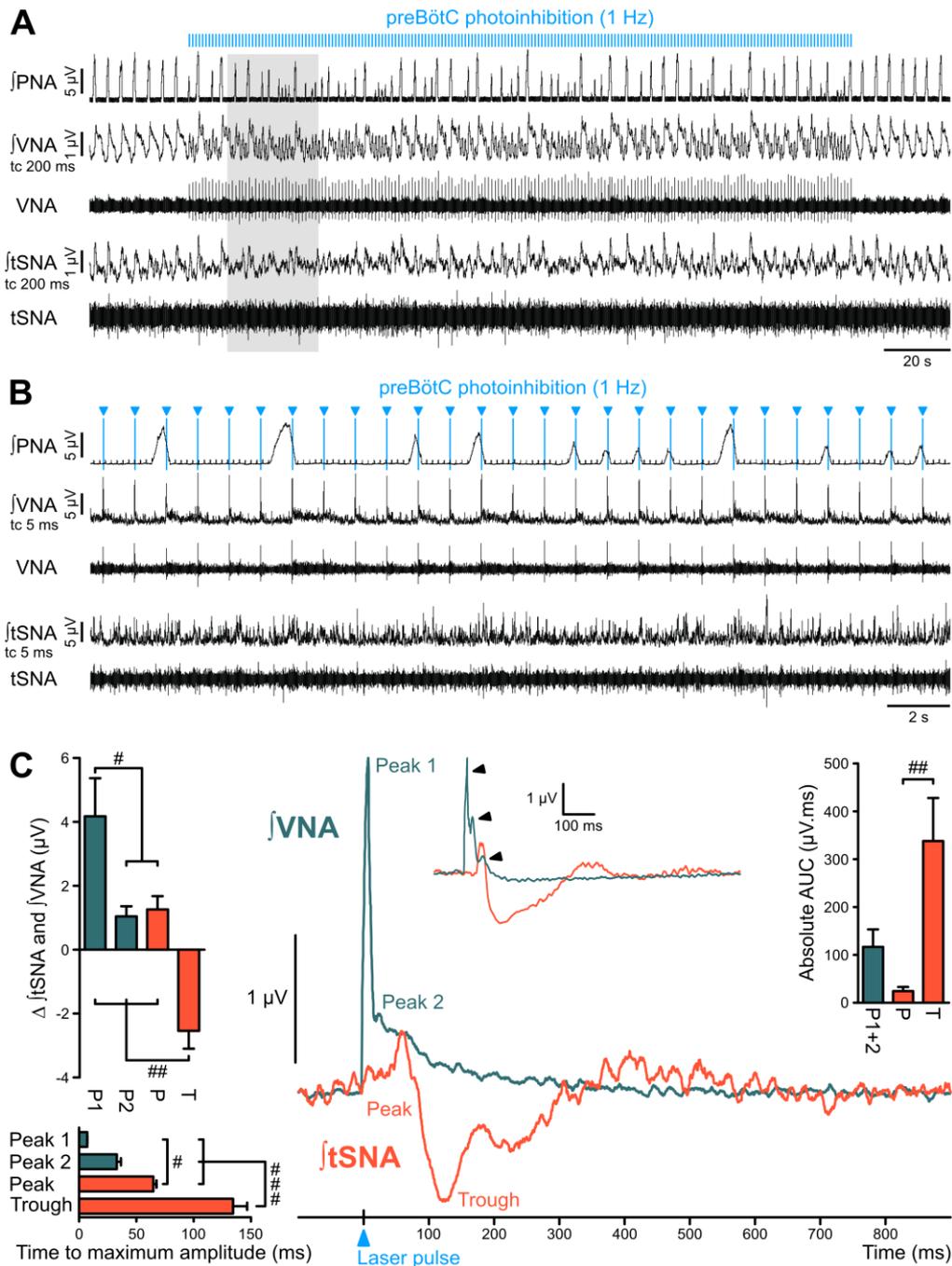
875 associated source data and detailed statistics ; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$,

876 photoinhibition vs. control condition (B), or vs. control and recovery conditions (D) ; # $p <$

877 0.05 , ### $p < 0.001$, between photostimulation conditions.

878

879



880

881 **Figure 5. The preBötC is sympatho-excitatory and parasympatho-inhibitory.** Traces of

882 activity in the WHBP at low (A) and higher (B) time resolution, showing that preBötC

883 photoinhibition at 1Hz disrupts PNA, VNA and tSNA. (C) Laser triggered averages of

884 integrated VNA and tSNA (tc 5 ms, 300 pulses), show that preBötC photoinhibition induced:

885 a rapid excitation of VNA at short latency, sometimes accompanied by a second or third peak

886 (arrowheads in inset); and an initial small tSNA peak and a subsequent larger tSNA trough of

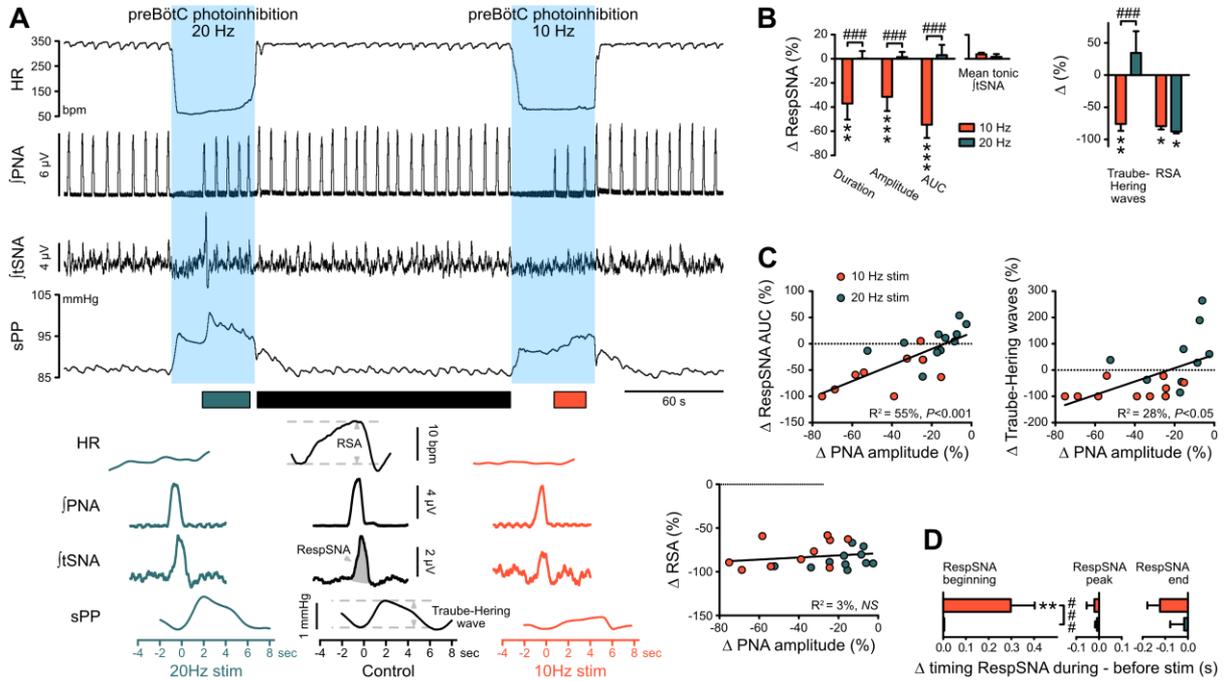
887 longer latency. These effects were found irrespective of the respiratory phase at which the

888 light pulse was delivered. Group data are presented as mean \pm SEM ; one-way repeated

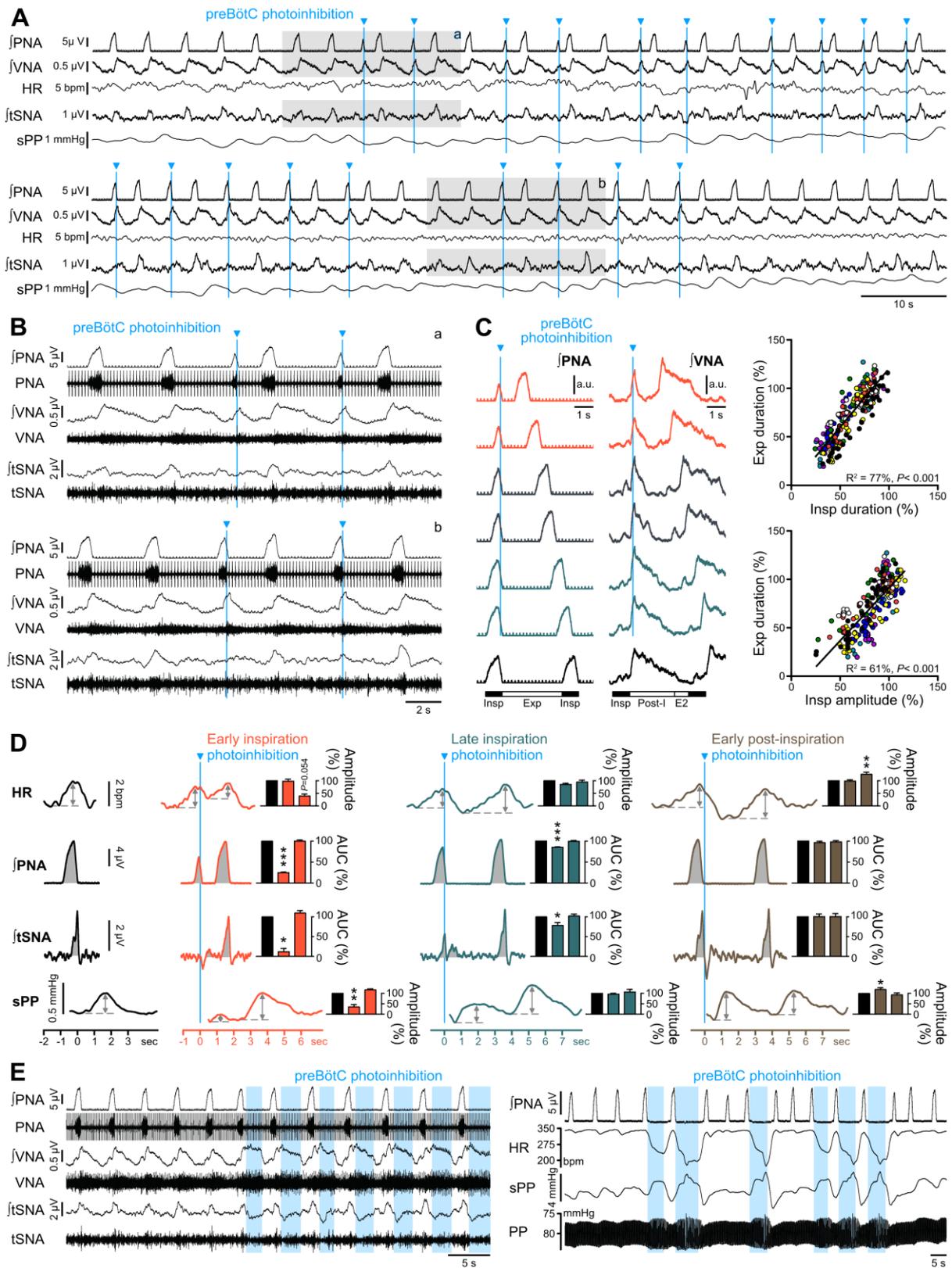
889 measures ANOVA followed with *post hoc* Holm-Sidak multiple-comparison test performed

890 on raw data, as shown on Figure 5-figure supplement 1 and associated source data and
 891 detailed statistics; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, between respective effects.

892
 893



894
 895 **Figure 6. The preBötC drives RespSNA, Traube-Hering waves and RSA.** (A) Traces
 896 from one rat and group analysis of the PNA-related oscillations in HR (RSA), tSNA
 897 (RespSNA) and systolic PP (Traube-Hering waves). PNA burst-triggered averaging (bottom
 898 traces) was performed during control conditions (pre- and post-photoinhibition), and during
 899 10 Hz and 20 Hz photoinhibition when PNA had returned. (B) Quantification of the group
 900 data showing the effect of preBötC photoinhibition on RespSNA duration, amplitude and
 901 area under the curve (AUC), mean tonic tSNA (baseline tSNA outside of RespSNA), Traube-
 902 Hering wave amplitude, and RSA. (C) Correlation analysis indicates that the PNA amplitude
 903 is correlated with RespSNA AUC and Traube-Hering wave amplitude, but not RSA
 904 amplitude. The latter indicates that the preBötC generates RSA by a mechanism unrelated to
 905 the generation of the PNA inspiratory bursts. The incidence of preBötC photoinhibition on the
 906 timing of the onset, peak and end of the RespSNA burst is shown in (D). Group data are
 907 presented as mean \pm SEM ; two-way repeated measures ANOVA followed with *post hoc*
 908 Holm-Sidak multiple-comparison test performed on raw data, as shown on Figure 6-figure
 909 supplement 1 and associated source data and detailed statistics; ** $p < 0.01$, *** $p < 0.001$,
 910 photoinhibition vs. control and recovery conditions ; ### $p < 0.001$, between photostimulation
 911 frequencies.

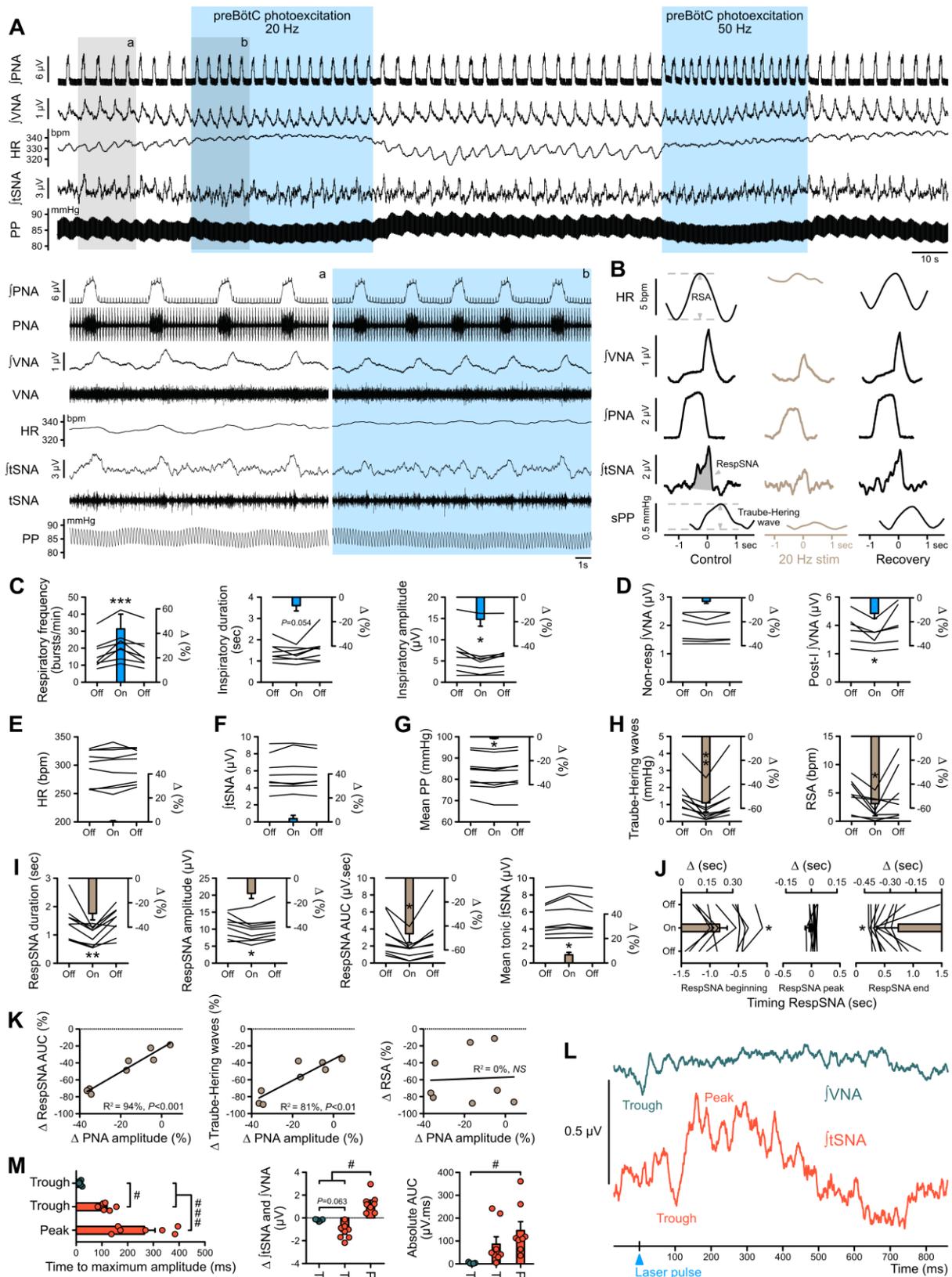


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914 **Figure 7. Effect of single pulse preBötC photoinhibition on respiration, RespSNA,**
 915 **Traube-Hering waves and RSA. (A, B) Traces showing the effect of single light pulse (10**
 916 **ms) preBötC photoinhibition on phrenic (PNA), vagal (VNA) and thoracic sympathetic**

917 (tSNA) nerve activity, HR and systolic perfusion pressure (sPP) at low (A) and high (B) time
918 resolution in the WHBP. Pulses were applied during early (top traces, a) and late (bottom
919 traces, b) inspiration. (C) Triggered averages of PNA and VNA showing that single pulse
920 photoinhibition of preBötC delivered during early (orange, 40% and 50% inspiratory
921 duration), mid- (grey, 60% and 70% inspiratory duration) or late inspiration (green, 80% and
922 90% inspiratory duration) immediately stopped inspiration (Insp) and triggered post-
923 inspiration (Post-I). For analysis, inspiratory burst duration was normalized to the bottom
924 (black) control trace. The correlograms, showing analysis across multiple respiratory cycles in
925 all animals, with each animal represented by a single colour, indicate that the duration of the
926 expiratory phase (Exp, the addition of Post-I and late expiration, E2) is determined by the
927 duration and amplitude of the preceding inspiratory period. Data are expressed in % relative
928 to intra-preparation averaged control values, to normalize variability in respiratory phase
929 durations between preparations. (D) PNA burst-triggered averages of HR, PNA, tSNA and
930 sPP were used to derive RSA amplitude, mean PNA AUC, RespSNA AUC and Traube-
931 Hering wave amplitude during control (black) and preBötC photoinhibition in early (orange),
932 late (green) or early post-inspiration (brown). Data are expressed relative to intra-preparation
933 control conditions, and were analysed for the respiratory cycle during which preBötC
934 photoinhibition occurred and the following, to evaluate direct and indirect photoinhibition
935 effects. (E) Traces showing that when delivered during expiration, preBötC photoinhibition
936 increased VNA for the entire photoinhibition period, but only transiently decreased tSNA.
937 The latter effect was amplified with successive bouts of expiratory preBötC photoinhibition,
938 also inducing increases in RespSNA. PNA bursting always occurred following a short (~1 s)
939 post-photoinhibition delay. Group data are presented as mean \pm SEM; one-way repeated
940 measures ANOVA followed with *post hoc* Holm-Sidak multiple-comparison test or Friedman
941 repeated measures analysis of variance on ranks followed with pairwise multiple comparison
942 Tukey test performed on raw data, as shown on Figure 7-figure supplement 1 and associated
943 source data and detailed statistics; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, different vs. the two
944 other conditions.

945



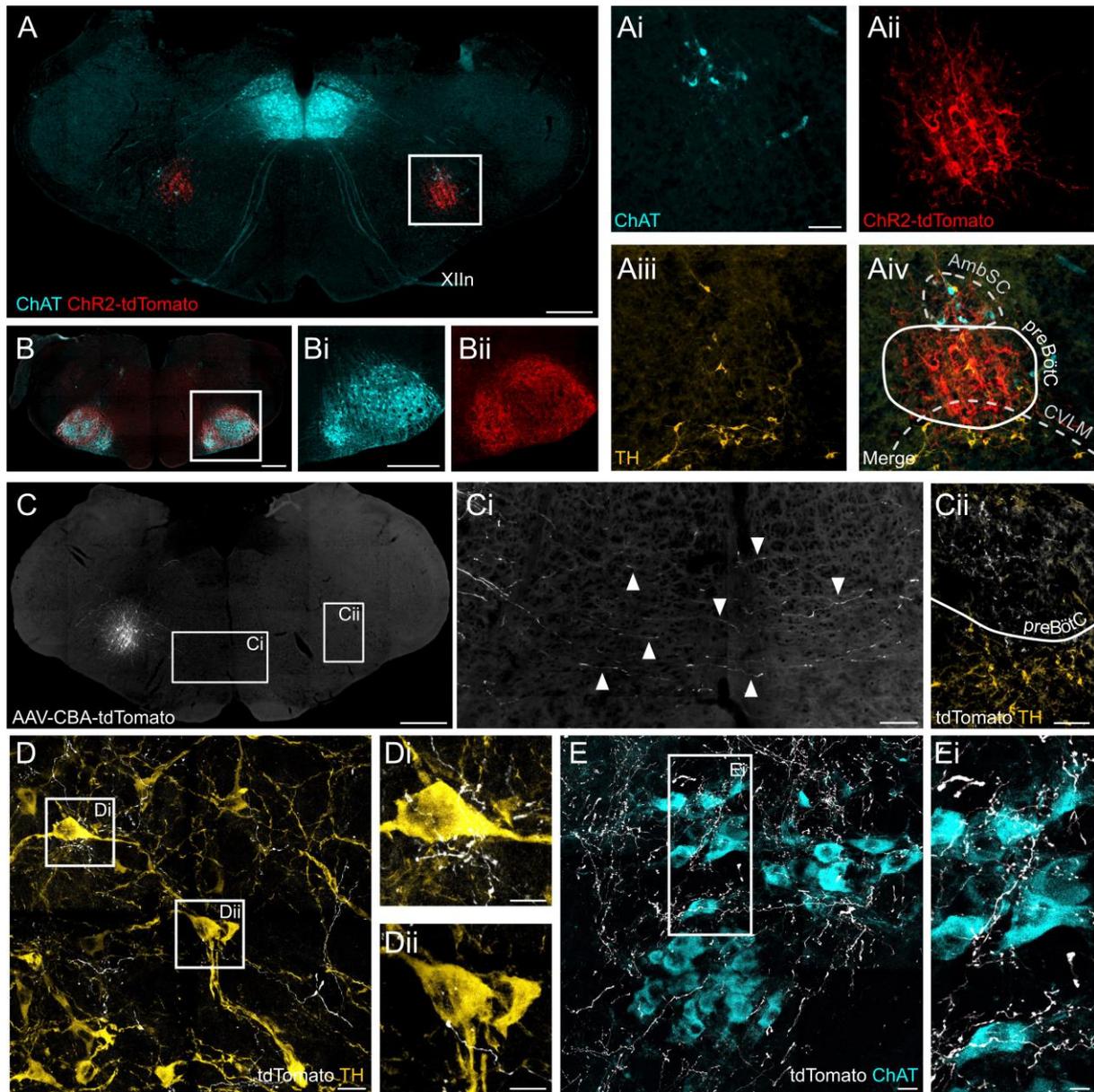
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947 **Figure 8. Photoexcitation of preBötC decreases amplitude of the respiratory command,**
 948 **RespSNA, Traube-Hering waves and RSA.** Traces of activity recorded in the WHBP at low
 949 (A) and higher (lower traces from the shaded areas) time resolution showing the effect of
 950 bilateral photoexcitation of the preBötC with Chr2. (B) PNA burst-triggered averaging was

951 used to quantify the effect on RespSNA AUC, Traube-Hering wave amplitude and RSA
952 amplitude. (C-J) Individual and group data (n=8) quantifying the effect of preBötC
953 photoexcitation (20 Hz) on: (C) respiratory parameters; (D) VNA; (E) HR; (F) tSNA; (G)
954 mean PP; (H) Traube-Hering wave and RSA; and (I) RespSNA. (J) Further analysis shows
955 the decrease in duration of RespSNA was due to both shorter inspiratory and post-inspiratory
956 components. (K) Correlograms showing that RespSNA AUC and Traube-Hering wave
957 amplitude were both correlated with PNA amplitude, whilst RSA was not. (L) Traces from
958 one animal showing laser-triggered averaging of integrated VNA and tSNA in response to
959 1Hz preBötC photoexcitation. (M) Quantification of this response in all animals showing
960 photoexcitation induces inverse responses to that of photoinhibition, and of smaller
961 amplitude. Group data are presented as mean \pm SEM ; one-way repeated measures ANOVA
962 followed with *post hoc* Holm-Sidak multiple-comparison test or Friedman repeated measures
963 analysis of variance on ranks followed with pairwise multiple comparison Tukey test
964 performed on raw data, as shown on the associated source data and detailed statistics ; * $p <$
965 0.05, ** $p <$ 0.01, *** $p <$ 0.001, photoinhibition vs. control and recovery conditions ; # $p <$
966 0.05, ## $p <$ 0.01, between respective effects.

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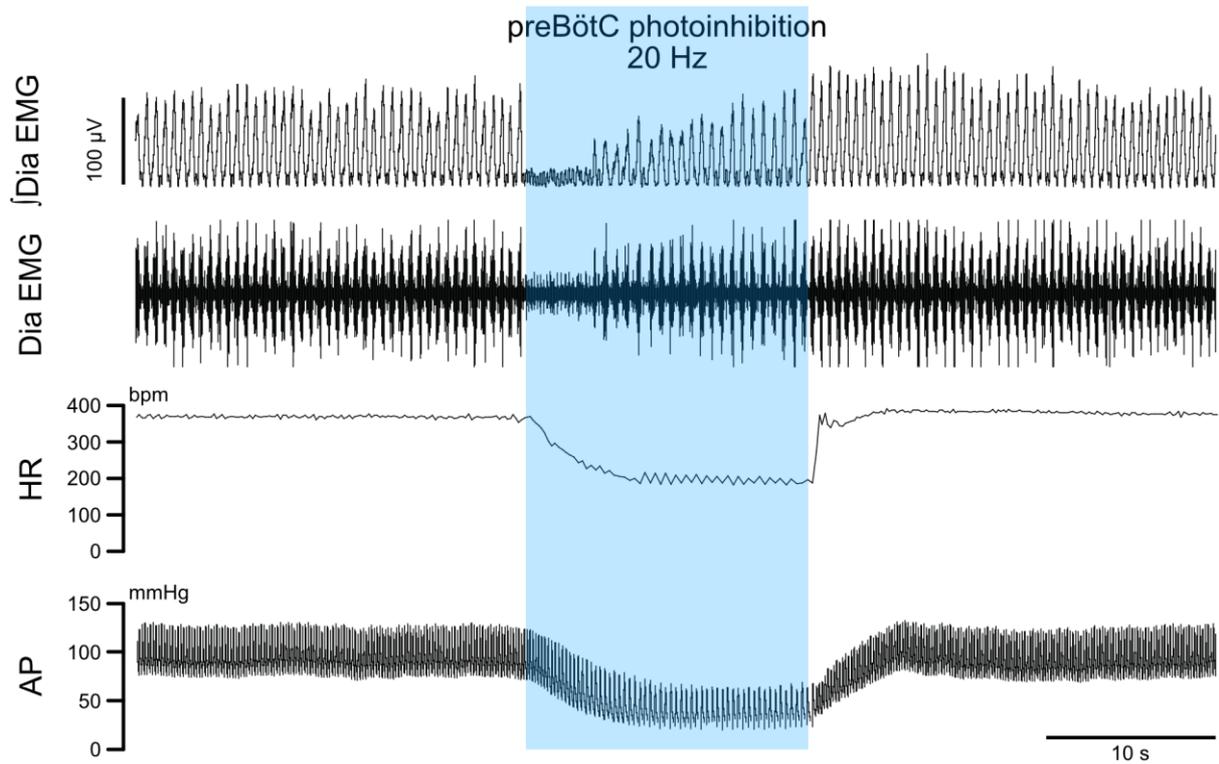
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970 **Figure 9. ChR2 expression in the preBötC, and direct projections to adrenergic C1 and**
 971 **nucleus ambiguus neurons.** (A) Coronal section showing immunoreactivity for tdTomato,
 972 fused to ChR2, choline acetyltransferase (ChAT) and tyrosine hydroxylase (TH) at low and
 973 high magnification (white box in A, shown as individual colour channels in Ai-iv). The ChR2
 974 expression profile is restricted to the preBötC, ventral to the semicompact formation of the
 975 nucleus ambiguus (AmbSC), and dorsal to the caudal ventrolateral medulla oblongata
 976 (CVLM). (B) ChR2-positive terminals were found in the facial nucleus, a characteristic
 977 feature of preBötC projections (Deschênes et al., 2016). (C) Small volume (5 nl) unilateral
 978 viral injections (n=2) restricted tdTomato expression to core preBötC neurons, with
 979 substantial numbers of tdTomato-positive fibers crossing the midline (Ci) to the contralateral
 980 preBötC (Cii). (D) TdTomato-positive preBötC terminals were found in close apposition to

981 TH-positive C1 neurons in the rostral ventrolateral medulla (Di and Dii), and (E) ChAT-
982 positive neurons in the compact formation of the nucleus ambiguus (AmbC) (Ei). Scale bars
983 are 500 μm (A, B, Bi, C), 100 μm (Ai, Ci, Cii), 20 μm (D, E) or 10 μm (Di, Dii, Ei).
984

985 **Supplemental figures and legends**

986

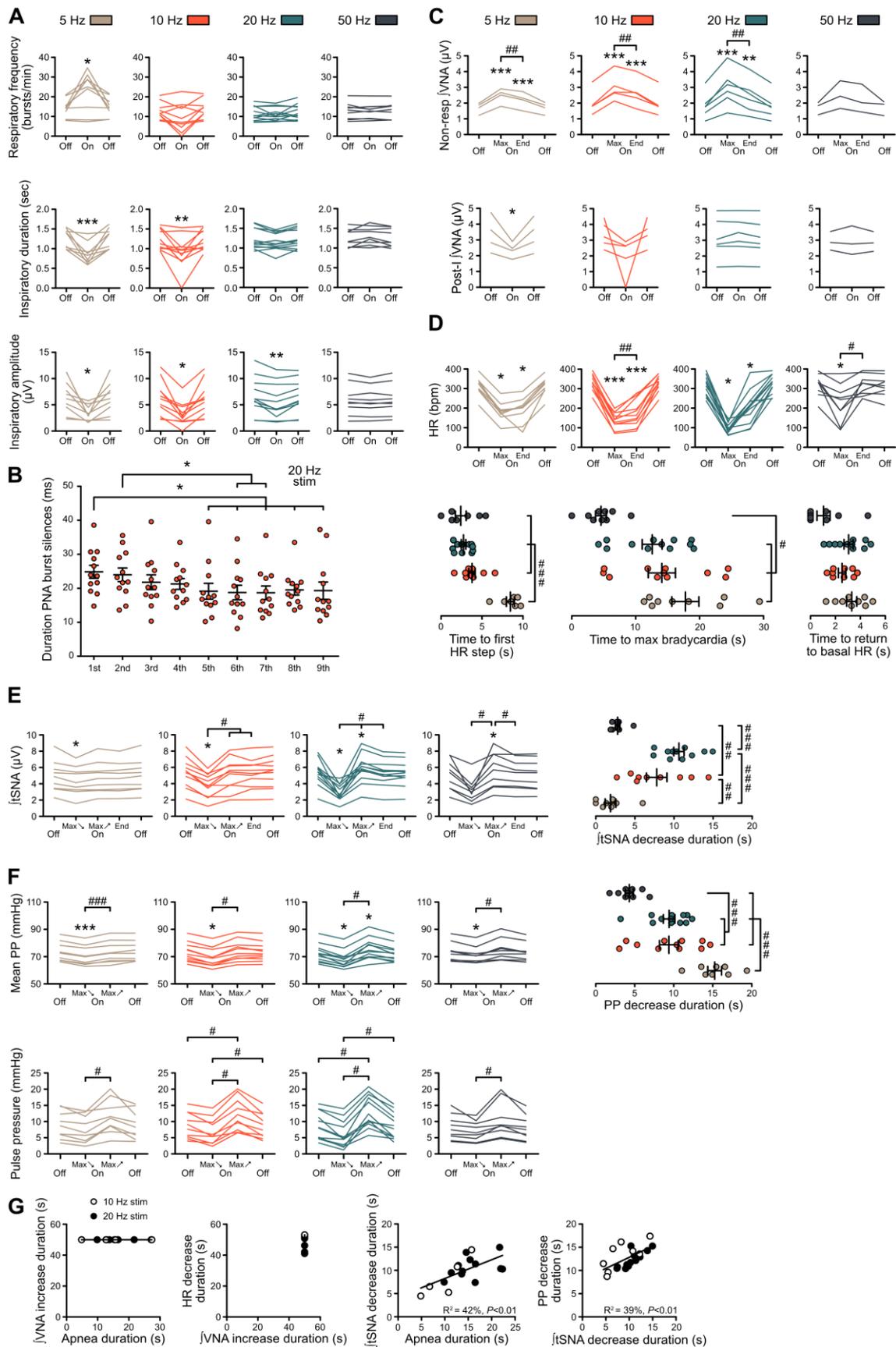


987

988 **Figure 2-figure supplement 1. PreBötC photoinhibition *in vivo* induces respiratory**
989 **cessation, bradycardia, and decreases arterial pressure.** In a separate cohort (n=3) from
990 the one shown on Figure 2, we recorded diaphragm (Dia) electromyograph (EMG) activity,
991 heart rate (HR) and arterial pressure (AP) in anesthetized P31 rats with bilateral *GtACR2*
992 expression in the preBötC. Prolonged preBötC photoinhibition induced a long apnea, before
993 respiratory activity resumed even though preBötC photoinhibition continued. PreBötC
994 photoinhibition also induced a profound bradycardia and decreased AP.

995

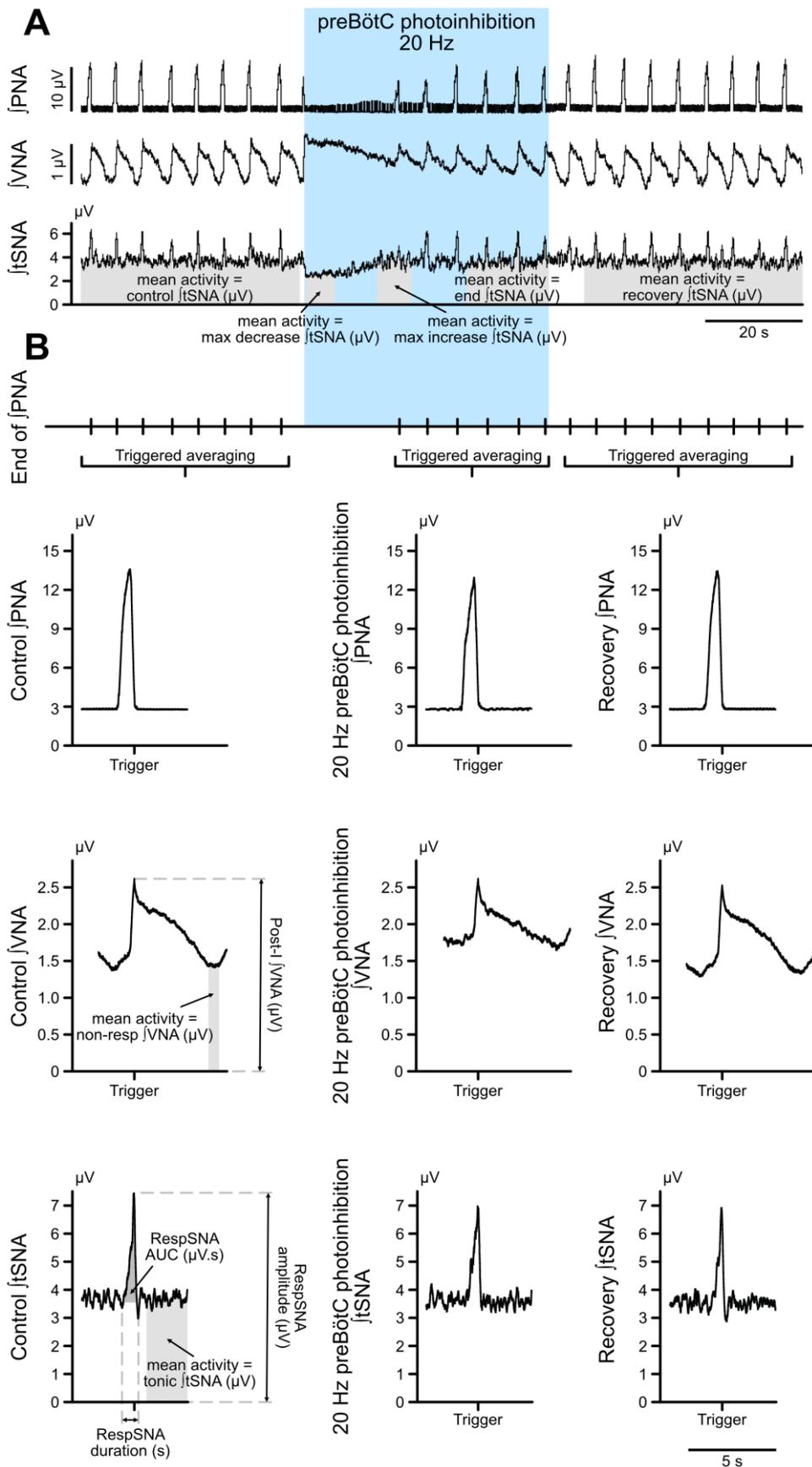
996 **Figure 2-source data 1. Source data and statistics for Figure 2.**



997

998 **Figure 3-figure supplement 1. Individual data showing alterations of respiratory,**
 999 **sympathetic vasomotor and cardiac parasympathetic activities during prolonged**

1000 ***GtACR2*-mediated preBötC photoinhibition *in situ*.** (A-F) Individual data for the *in situ*
1001 WHBP of P31 rats with bilateral *GtACR2* expression in the preBötC, presented as group data
1002 in Figure 3. (A) Individual data for respiratory frequency, and PNA burst inspiratory duration
1003 and amplitude, presented as group data in Figure 3C. During prolonged 20 Hz preBötC
1004 photoinhibition, PNA bursts were transiently silenced after each light pulse as shown on
1005 Figure 3H. (B) The duration of PNA burst silences during an individual burst decreased with
1006 continued photoinhibitory pulses. The abscissae shows successive PNA bursts with the
1007 duration of silences on the ordinate, starting from the first pulse of light that impacted the
1008 analysed PNA burst. (C) Individual data for VNA measured outside (non-resp) the
1009 inspiratory/post-inspiratory activities, and for the post-inspiratory component of VNA,
1010 presented as group data in Figure 3D. (D) Individual data for HR, presented as group data in
1011 Figure 3E. HR decreased in steps at the beginning of preBötC photoinhibition, the first step
1012 being reached earlier in the high frequency preBötC photoinhibitions. Similarly, the time to
1013 reach maximum bradycardia from the beginning of preBötC photoinhibition trials was shorter
1014 with high frequency preBötC photoinhibition. The time to return to basal HR, during the
1015 recovery period, was only shorter for the 50 Hz photoinhibition compared to other
1016 frequencies. (E) Individual data for mean tSNA, presented as group data in Figure 3F. The
1017 mean tSNA decrease was more pronounced for 10 Hz and 20 Hz preBötC photoinhibition,
1018 both in amplitude and duration. (F) Individual data for mean PP, presented as group data in
1019 Figure 3G, and for pulse pressure. PP decrease duration for 10 Hz, 20 Hz and 50 Hz preBötC
1020 photoinhibitions followed the same timing as mean tSNA decrease, but not for 5 Hz preBötC
1021 photoinhibition, likely due to the decreased RespSNA and Traube-Hering waves that lasted
1022 the entire 5 Hz preBötC photoinhibition period. (G) Linear regression analysis of data,
1023 showing that the overall cardiac parasympathetic drive elicited by preBötC photoinhibition is
1024 unrelated to the presence of respiratory activity. In contrast, the vasomotor sympathetic tone
1025 is directly related to it, suggesting different mechanisms of action. Group data are presented
1026 as mean \pm SEM ; Friedman repeated measures analysis of variance on ranks followed with
1027 pairwise multiple comparison Tukey test, one way repeated measures ANOVA followed with
1028 *post hoc* Holm-Sidak multiple-comparison test, paired student's t-test or Wilcoxon signed
1029 rank test, performed on raw data, as shown on the associated source data and detailed
1030 statistics ; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, photoinhibition vs. control and recovery
1031 conditions ; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, between photostimulation frequencies ; ‡
1032 $p < 0.05$, ‡‡ $p < 0.01$, ‡‡‡ $p < 0.001$, intra-photostimulation frequency vs. max change.
1033



1034

1035 **Figure 3-figure supplement 2. Method for analysis of the different components of VNA**

1036 **and tSNA. (A) Prolonged photoinhibition of the preBötC induced three different effects on**

1037 total tSNA, depending on the respiratory status. To demonstrate this we analysed, as a
1038 repeated measures comparison, the mean integrated tSNA during a pre-photoinhibition
1039 control period; the initial photoinhibition period with the maximal tSNA decrease during the
1040 apneic period; the photoinhibition period where PNA restarted and there was a maximal tSNA
1041 increase; the end of the photostimulation period; and in the recovery post-photoinhibition
1042 period. These periods are shown in grey on the integrated tSNA trace in A. Analysis of total
1043 tSNA is shown on Figure 3F and Figure 3S1E. (B) To assess whether preBötC prolonged
1044 photoinhibition could have specific effects on the phasic respiratory-associated and tonic non-
1045 respiratory-associated components of tSNA and VNA, we performed respiratory (end of
1046 PNA) triggered averaging of integrated tSNA and VNA. The phasic burst of respiratory
1047 modulation of tSNA, called here RespSNA, was analysed for its duration, amplitude, and
1048 AUC above tonic tSNA. Non-respiratory-modulated tSNA was called tonic tSNA, and
1049 analysed as the mean activity outside the burst of RespSNA. RespSNA analysis is presented
1050 on Figure 6B and Figure 6S1A, tonic tSNA analysis is presented on Figure 6B and Figure
1051 6S1B. Both respiratory and non-respiratory components of integrated VNA were also
1052 quantified. Efferent VNA was measured from the cervical level, where it contains activities
1053 going to multiple targets, including respiratory activity going to the upper airways, and
1054 parasympathetic activity going to the heart. The peak of post-inspiratory activity was
1055 quantified and called Post-I VNA. Mean VNA activity during the late-expiratory phase,
1056 which does not contain respiratory-related activity on this nerve, was quantified and called
1057 non-resp VNA, as shown on Figure 3D and Figure 3S1C. The same analysis was performed
1058 for preBötC prolonged photoexcitation.

1059

1060 **Figure 3-source data 1. Source data and statistics for Figures 3 and 3S1.**

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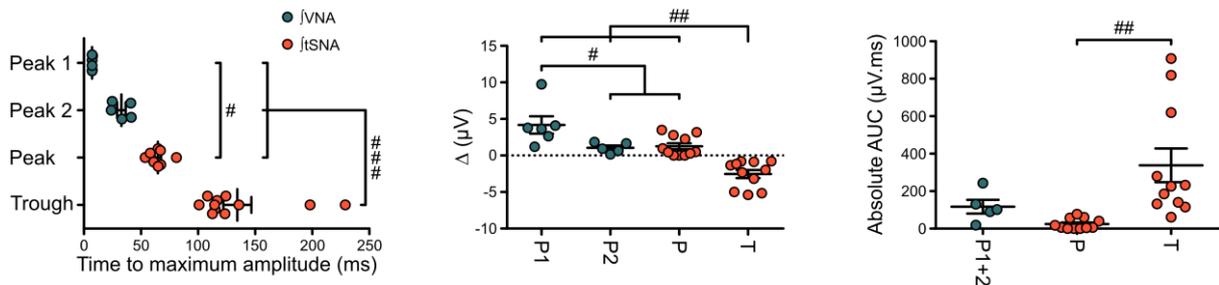
1062 **Figure 4-source data 1. Source data and statistics for Figure 4.**

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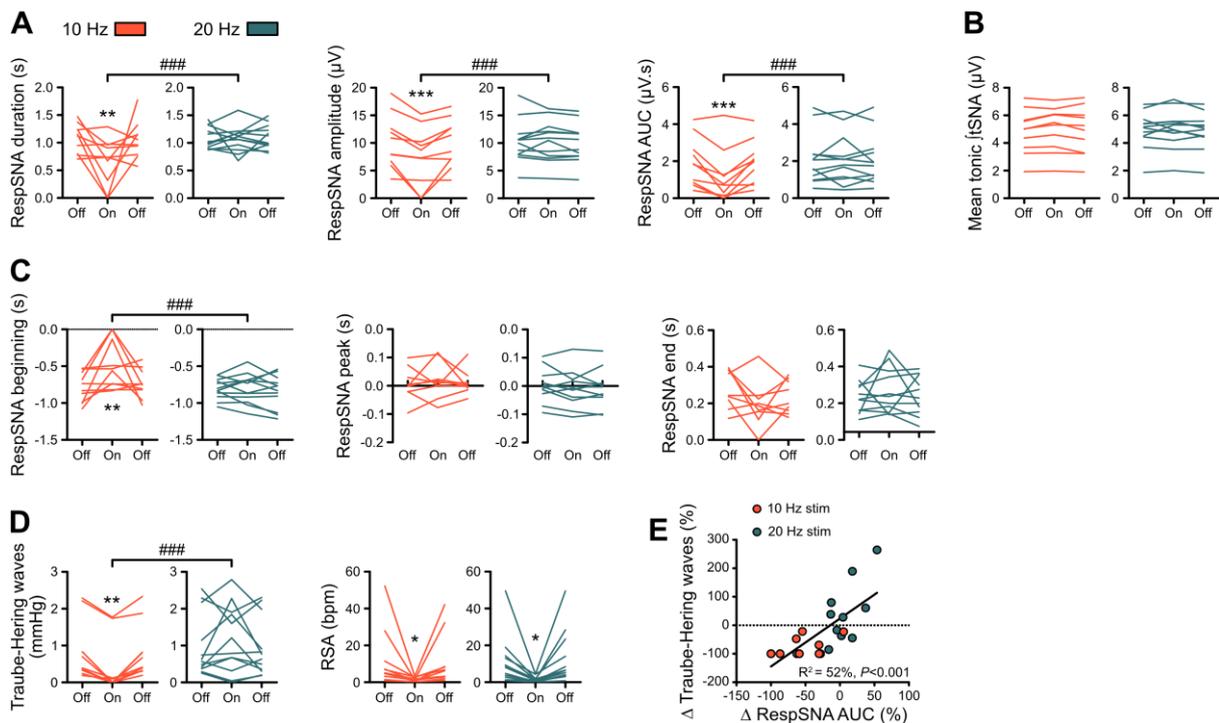
1068 **Figure 5-figure supplement 1. Individual data showing sympathetic and**
 1069 **parasympathetic effects of 1Hz preBötC photoinhibition.** Individual data for the laser
 1070 triggered averaging of integrated VNA and tSNA (tc 5 ms, 300 pulses) presented in Figure
 1071 5C. Group data are presented as mean \pm SEM ; one-way repeated measures ANOVA
 1072 followed with *post hoc* Holm-Sidak multiple-comparison test performed on raw data, as
 1073 shown on the associated source data and detailed statistics; # $p < 0.05$, ## $p < 0.01$, ### $p <$
 1074 0.001 , between respective effects.

1075

1076 **Figure 5-source data 1. Source data and statistics for Figures 5 and 5S1.**

1077

1078



1079

1080 **Figure 6-figure supplement 1. Individual data showing alterations in RespSNA, Traube-**
 1081 **Hering waves and RSA during prolonged *GtACR2*-mediated preBötC photoinhibition *in***
 1082 ***situ*.** (A-D) Individual data for the RespSNA, tonic tSNA, Traube-Hering wave and RSA
 1083 group data presented in Figure 6B. (E) The amplitude of Traube-Hering waves during 10 Hz

1084 and 20 Hz preBötC photoinhibition is correlated to RespSNA AUC. Two-way repeated
1085 measures ANOVA followed with post hoc Holm-Sidak multiple-comparison test, as shown
1086 on the associated source data and detailed statistics ; ** $p < 0.01$, *** $p < 0.001$,
1087 photoinhibition vs. control and recovery conditions ; ### $p < 0.001$, between photostimulation
1088 frequencies.

1089

1090 **Figure 6-source data 1. Source data and statistics for Figures 6 and 6S1.**

1091

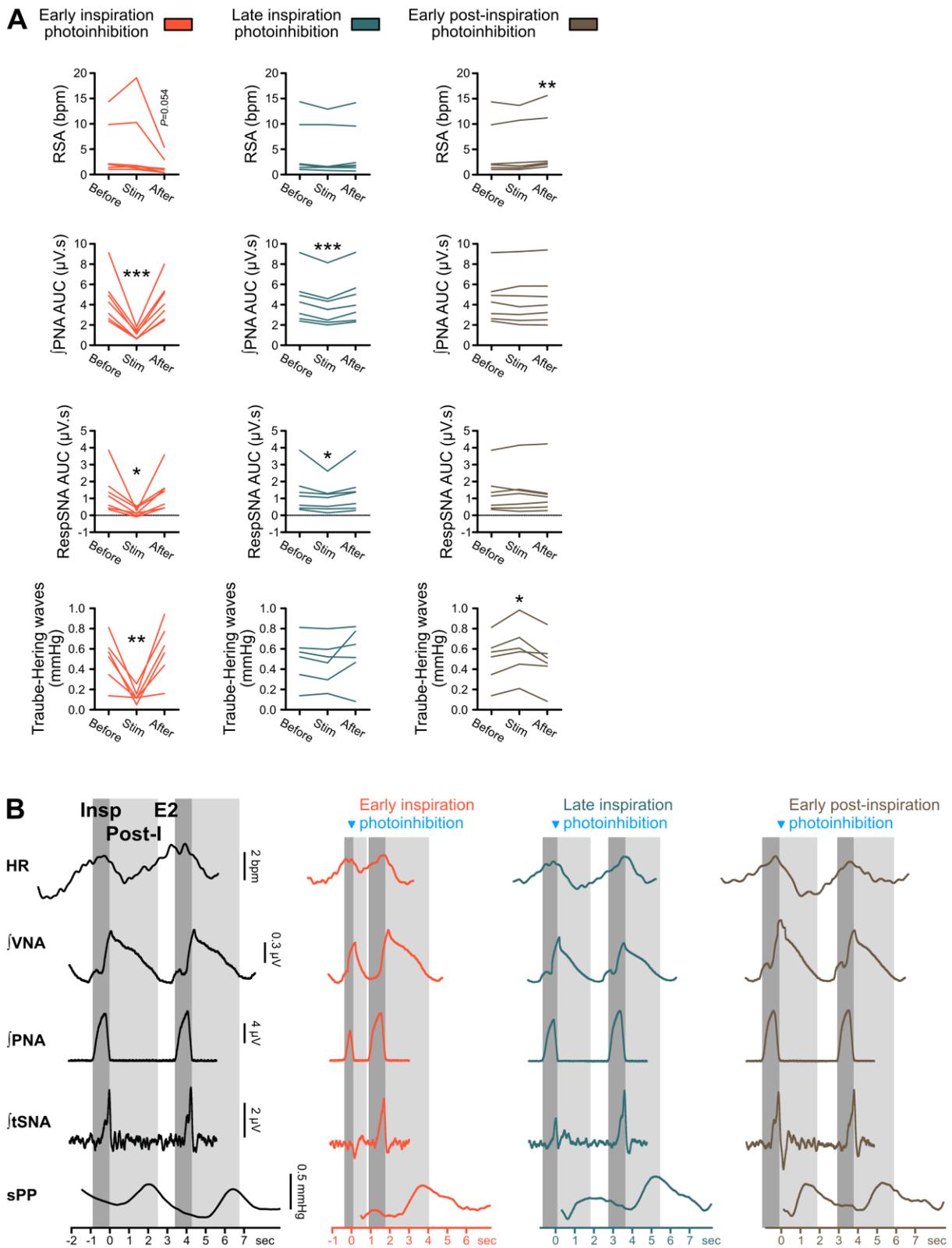


Figure 7-figure supplement 1. Individual data showing respiratory phase specific alterations in PNA AUC, RSA, RespSNA and Traube-Hering waves during single pulse preBötC photoinhibition. (A) Individual data for the PNA AUC, RSA, RespSNA and Traube-Hering waves presented in Figure 7D. (B) Representation of the three phases of the

1097 respiratory cycle as observed in PNA burst-triggered averages of HR, sPP and integrated
1098 VNA, PNA, and tSNA, with inspiration (dark gray) defined by PNA activity, post-inspiration
1099 (light gray) defined by the post-inspiratory burst of VNA activity, and late expiration (E2,
1100 white) defined as the period of non-respiratory-related activity on both PNA and VNA. RSA
1101 is characterized by a peak in HR at the end of inspiration, a decrease in HR at the beginning
1102 of post-inspiration, and a progressive increase in HR from mid-post-inspiration, through late
1103 expiration and into inspiration. RespSNA peaks at the transition between inspiration and post-
1104 inspiration, and the RespSNA burst is mainly composed of an inspiratory component, and a
1105 smaller post-inspiratory component. Traube-Hering waves, induced by RespSNA, peak
1106 during post-inspiration and therefore with a delay compared to RespSNA, due to the slow
1107 vascular contraction dynamics. The duration of the respiratory phases was impacted by
1108 preBötC photoinhibition, which also impacted the duration of RSA, RespSNA and Traube-
1109 Hering waves, yet their timing relative to the respiratory cycle remained unaltered. One-way
1110 repeated measures ANOVA followed with *post hoc* Holm-Sidak multiple-comparison test or
1111 Friedman repeated measures analysis of variance on ranks followed with pairwise multiple
1112 comparison Tukey test, as shown on the associated source data and detailed statistics ; * $p <$
1113 0.05 , ** $p < 0.01$, *** $p < 0.001$, different vs. the two other conditions.

1114

1115 **Figure 7-source data 1. Source data and statistics for Figures 7 and 7S1.**

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1117 **Figure 8-source data 1. Source data and statistics for Figure 8.**

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