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Optogenetic perturbation of preBötzing Complex inhibitory neurons modulates respiratory pattern

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Abstract

Inhibitory neurons make up a significant fraction of the neurons within the preBötzing Complex (preBötC), a site critical for mammalian eupneic breathing. The role of glycinergic preBötC neurons in respiratory rhythmogenesis in mice was investigated by optogenetically-targeted excitation or inhibition. Channelrhodopsin-2 (ChR2) or Archaeorhodopsin (Arch) was expressed in glycinergic preBötC neurons of glycine transporter 2 (*GlyT2*)-Cre mice. In ChR2-transfected mice, brief inspiratory-phase bilateral photostimulation targeting the preBötC prematurely terminated inspiration, whereas expiratory-phase photostimulation delayed the onset of the next inspiration. Prolonged photostimulation produced apneas lasting as long as the light pulse. Inspiratory-phase photoinhibition in Arch-transfected mice during inspiration increased tidal volume without altering inspiratory duration, whereas expiratory-phase photoinhibition shortened the latency until the next inspiration. During persistent apneas, prolonged photoinhibition restored rhythmic breathing. We conclude that glycinergic preBötC neurons modulate inspiratory pattern and are important for reflex apneas but that the rhythm can persist after significant dampening of their activity.

Keywords

breathing; preBötzing Complex; optogenetics; respiratory rhythm; adeno-associated virus; inhibition

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Contributions

DS and JLF conceived of the study and designed the experiments. DS performed the experiments with help from JWW and YC, and DS and JWW analyzed data. DS and JLF made the figures and wrote the manuscript with help from JWW.

A supplementary methods checklist is available.

Introduction

Breathing in mammals is a complex behavior that must work (nearly) continuously from birth throughout life and can be rapidly modified, even transiently stopped, to support a wide range of physiological and functional demands. The preBötC, in the ventrolateral medulla, is a critical locus for respiratory rhythmogenesis in the intact eupneic mammal¹ and in reduced rodent *en bloc* and slice preparations². The preBötC contains intermingled heterogeneous populations of excitatory and inhibitory neurons^{3, 4}. Excitatory preBötC neurons are critical components of the respiratory central pattern generator (CPG)^{1, 5–7}. However, the role of inhibitory preBötC neurons is not well understood. Although strong inhibitory currents are observed in intracellular recordings of presumptive preBötC neurons in deeply anesthetized adult cats and rodents^{8, 9}, respiratory rhythm persists after bilateral blockade of fast inhibitory neurotransmission in the preBötC in anesthetized spontaneously breathing adult rats¹⁰. Since there is a substantial population of glycinergic preBötC neurons³, some of which are phasically active during inspiration¹¹, we wanted to determine their contribution to breathing movements by examining the effects of rapid, acute perturbations of their ongoing activity *in vivo*.

We utilized optogenetic tools to probe the functional contribution of glycinergic preBötC neurons to respiratory rhythm and pattern. We used viral transfection to express Channelrhodopsin-2 (ChR2) or Archaeorhodopsin (Arch) in glycinergic preBötC neurons by locally injecting cre recombinase-dependent viruses into the preBötC of transgenic mice expressing Cre driven by the glycine transporter 2 (*GlyT2*) promoter^{12–14}. Since the *GlyT2* promoter is specific to glycinergic neurons¹⁵, this intersectional strategy allowed us to selectively activate and silence these inhibitory neurons in intact, awake or anesthetized, spontaneously breathing mice. We found that perturbing glycinergic neuronal activity in the preBötC profoundly modulated the amplitude and timing of inspiratory motor output and expiratory period with no indications that they were critical for rhythmogenesis.

Results

Targeting opsin expression to preBötC GlyT2 neurons (Fig. 1)

To express ChR2 or Arch in glycinergic preBötC neurons, we used adeno-associated viruses (AAV 2/1) encoding either *ChR2-eYFP* driven by the constitutive promoter *Efla* in a double floxed inverted open reading frame configuration (DIO-ChR2)¹⁶ or *Arch-GFP* driven by the constitutive chicken β -actin (*CBA*) promoter in a FLEX switch (FLEX-Arch). We injected either virus bilaterally into the preBötC of transgenic mice expressing Cre recombinase under the *GlyT2* promoter (Fig. 1a, b). These injections produced protein expression in preBötC neurons that was detectable *post hoc*, with some expression in neighboring neurons outside the preBötC including in the ventral respiratory column (VRC) in the rostral-caudal direction, particularly caudal to the preBötC (Fig 1a–d). We found no evidence of transfected neurons at more distant brainstem sites including those with established projections to preBötC, e.g., nucleus of the solitary tract (NTS), parabrachial nucleus. The peak density of transfected neurons was caudal to the facial nucleus (VII) by 750 – 900 μ m in ChR2-transfected mice and 600 – 750 μ m in Arch-transfected mice and ventral to the compact nucleus ambiguus, which predominately overlapped with the rostrocaudal

distribution of somatostatin (*Sst*)-expressing glutamatergic neurons^{1, 4} (Fig. 1c, d) that demark the preBötC⁴. Within the preBötC, transfected neurons intermingled but rarely colocalized with *Sst* immunoreactivity in either DIO-ChR2 (0.3 ± 0.6 out of 45.5 ± 13.4 *Sst*⁺ neurons; $n = 3$) or FLEX-Arch (2.0 ± 0.0 out of 36.3 ± 9.8 *Sst*⁺ neurons; $n = 3$) transfected mice (Fig. 1e). EYFP/GFP expression colocalized with glycine immunoreactivity in both sets of mice (Fig. 1f).

To efficiently deliver light specifically into the preBötC, we implanted optical cannulae bilaterally from a dorsal approach, about 200 – 400 μm dorsal to the preBötC (Fig 1a–b). By placing the cannulae close to the preBötC, we ensured that the pool of light-responsive neuronal somas able to affect breathing were preBötC *GlyT2* neurons. As delineated in the DISCUSSION, the most parsimonious explanation of our results is that the light-induced responses were due to activation of preBötC *GlyT2* neurons.

Brief photostimulation of ChR2-transfected neurons (Fig. 2)

In anesthetized Cre⁺ mice, brief bilateral photostimulation (100 ms pulse; 473 nm; Fig. 2a) of ChR2-transfected preBötC *GlyT2* neurons generated respiratory phase-dependent changes in breathing ($n = 5$). As revealed by phase response curves (see Methods and Fig. 2a right), ChR2 activation affected respiratory cycle timing, peak inspiratory airflow, and inspiratory duration (Fig. 2b–f).

Bilateral photostimulation during early inspiration (φ_{stim} : $0^\circ - 30^\circ$; see Fig. 2a right) resulted in premature inspiratory burst termination, i.e., a breath with truncated peak inspiratory airflow (decreased to $56.4 \pm 5.9\%$ of control; $P = 10^{-10}$; $n = 5$; Fig. 2c, e) and shortened inspiratory duration (decreased to $67.5 \pm 5.4\%$ of control; $P = 10^{-10}$; $n = 5$; Fig. 2c, f). In contrast, stimulation during expiration (φ_{stim} : $180^\circ - 360^\circ$) delayed the onset of the subsequent inspiration, with the strongest effect during the late expiratory (φ_{stim} : $330^\circ - 360^\circ$; often referred to as the preinspiratory (pre-I)) phase (Fig. 2b, d) with a $233^\circ \pm 20^\circ$ shift ($P = 2 \times 10^{-10}$; $n = 5$), while stimulations falling earlier in expiration, i.e., (φ_{stim} : $150^\circ - 180^\circ$), produced a smaller $94^\circ \pm 16^\circ$ shift ($P = 0.007$; $n = 5$; Fig. 2b). Following this phase shift, the next inspiration had no significant change in amplitude ($101 \pm 3\%$ of control; $P = 1$; $n = 5$) or duration ($104 \pm 3\%$ of control; $P = 1$; $n = 5$).

Prolonged photostimulation of ChR2-transfected neurons (Fig. 3)

In anesthetized ChR2-transfected mice, a 1 s photostimulus (7×100 ms pulses, 50 ms interpulse interval) at any time during the respiratory cycle stopped breathing, i.e., produced an apnea, which continued until after the light shut off (Fig. 3a; $n = 5$). The subsequent respiratory cycle began at a fairly constant delay after the laser turned off regardless of stimulus phase (Fig. 3b, c). The duration of this delay was unique to each mouse (300 – 860 ms, min-max; $n = 3$), presumably due to differences in the precise position of the virus injection and optical fiber placement. When tested in awake mice, a 1 s pulse train consistently produced apneas (Fig. 3d; $n = 5$). We further tested photostimulus responses in anesthetized mice when ventilation was increased during 5 min of either hypoxic (8% O₂, 92% N₂; $n = 5$) or hypercapnic (5% CO₂ in room air; $n = 5$) inspired gas mixtures, finding that ChR2 activation still consistently produced apnea in either of these conditions of

increased respiratory drive (Fig. 3e, f). Prolonged stimulation (up to 20 s pulse trains; $n = 3$) produced an apnea that persisted until the laser turned off (Fig. 3g).

Photoinhibition of Arch-transfected preBötC GlyT2 neurons (Fig. 4)

Preinspiratory or early inspiratory bilateral photoinhibition (φ_{stim} : $-120^\circ - 15^\circ$) of Arch-transfected preBötC *GlyT2* neurons (100 ms pulse; 593 nm; Fig 4a; $n=7$) increased peak inspiratory airflow ($137.0 \pm 3.8\%$ of control; $P = 2 \times 10^{-9}$; $n=7$) and tidal volume (Fig. 4b, c) without a significant change in inspiratory duration ($103.0 \pm 0.9\%$ of control; $P=1$; $n=7$; Fig. 4b, d) or respiratory phase ($P = 1$; $n = 7$; Fig. 4e). Compared to these photoinhibition-augmented breaths, sighs, i.e., larger breaths endogenously generated periodically to hyperinflate the lungs, had greater amplitudes (Fig. 4f). Sighs had peak inspiratory airflows that were $173 \pm 15\%$ of control ($P = 10^{-5}$; $n = 7$) that was also statistically different from photoinhibition-induced augmented breaths ($P = 0.02$; $n = 7$). Bilateral photoinhibition during midexpiration (φ_{stim} : $-210^\circ - -120^\circ$) produced a shift in respiratory phase manifested by an earlier onset of the subsequent inspiration ($52.3^\circ \pm 6.2^\circ$ phase shift; $P = 8 \times 10^{-6}$; $n = 7$; Fig. 4e, g), with the strongest effect at $180^\circ - -150^\circ$. Prolonged photoinhibition (5 s pulse) increased respiratory frequency but the effect was transient and did not last for the full duration of the laser pulse (Fig. 4h; $n = 3$).

Arch activation rescues breathing during a reflex apnea (Fig. 5)

A critical role of inhibition in the preBötC in controlling respiratory pattern is likely in the production of apneas¹⁰, such as during swallowing or breathholding. To determine whether preBötC *GlyT2* neurons participate in generating apneas, we photoinhibited these neurons during Breuer-Hering lung inflation reflex (BHIR)-induced apneas¹⁰. The lungs were inflated with sufficient continuous positive airway pressure (CPAP) to produce apneas that lasted ~ 11 s (~ 4 cm H₂O) during control periods (Fig. 5a, top). Photoinhibiting Arch-transfected preBötC *GlyT2* neurons (1 s pulse) broke the apnea with one or two breaths (Fig. 5a, bottom; $n = 3$). Laser onset at 5.5 ± 0.5 s after onset of CPAP (CPAP + Laser) induced a first breath at 5.8 ± 0.5 s, i.e., a 300 ms delay from the laser onset versus an expected first breath at 11.8 ± 1.4 s during control (CPAP only) periods (ANOVA; $P = 3 \times 10^{-6}$; Fig. 5b; $n = 3$).

Single-unit recording in ChR2- and Arch-transfected mice (Fig. 6)

In ChR2-transfected mice, we unilaterally photostimulated (1 s pulse) and recorded from preBötC neurons within the cone of light ($n = 18$ in 3 mice; Fig. 6a). Unilateral ChR2 photostimulation of preBötC *GlyT2* neurons was sufficiently strong to recreate the effects observed from bilateral photostimulation, i.e., a persistent apnea during the photostimulation period, e.g., Fig 2g. In total, we recorded 18 neurons, of which 8 were respiratory-modulated (5 neurons were pre-I, 2 were inspiratory, and 1 was expiratory; Fig. 6b) and 10 neurons fired tonically. All of the recorded respiratory-modulated neurons and all but two of the tonically firing neurons were silenced during photostimulation. The onset of the next inspiration following photostimulation was delayed relative to the expected phase (black arrows in Fig. 6b depict expected onset of inspiration). Pre-I neurons could be silenced at any point in the respiratory cycle. When silenced early enough in the preinspiratory phase,

initial preBötC activity was not followed by burst firing, motor output, or the onset of inspiratory airflow (Fig. 6b.iv). The delay between the laser shutting off and the onset of the next breath (described above; see Fig. 3b) was also seen in the preBötC neuron firing pattern. While recording within the preBötC, we consistently observed a short-lasting multi-unit field potential after laser onset, consistent with (transient) activation of neighboring *GlyT2* neurons (Fig. 6c inset).

In Arch-transfected mice, we recorded from preBötC neurons during unilateral photoinhibition (1 s pulse; n = 12 units in 2 mice), finding 7 respiratory-modulated neurons (2 pre-I neurons, 2 inspiratory neurons, 1 post-I (or early-E) neuron, and 1 expiratory neuron) (Fig. 6d) and 5 tonic neurons. Unilateral photoinhibition generated shifts in the respiratory cycle, comparable to that observed during bilateral photoinhibition, but did not alter peak inspiratory airflow. During photoinhibition, preBötC neurons exhibited a shorter interval between inspirations, consistent with changes in the breathing pattern (black arrow s in Fig. 6d depict expected inspiratory onset following photoinhibition). We did not find any neurons that increased their firing during delivery of light pulses, which may be reflective of unilateral photoinhibition not altering inspiratory amplitude. We also found 2 tonic neurons that were silenced in response to light from a baseline firing rate of ~16 Hz, e.g., Fig. 6d.i. Following a 1 s light pulse, these neurons exhibited rebound firing after the laser shut off. The remaining neurons did not change their firing pattern or phase relationship to breathing during photoinhibition.

Discussion

Inhibition is an essential element of most neural circuits in the mammalian nervous system. While *GlyT2*⁺ inhibitory neurons are present in the preBötC and in slices *in vitro*, ~20% of these neurons are inspiratory-modulated and ~50% are tonic (we do not know what percentage *in vivo* are respiratory-modulated, tonic or silent, and under which physiological conditions)^{3, 11}, their functional role in the generation of respiratory rhythm and pattern is not well understood and is disputed^{10, 17}. Strong inhibitory currents are observed in intracellular recordings of preBötC neurons in deeply anesthetized rats and cats⁸. While a normal rhythmic breathing pattern remains in the absence of conventional postsynaptic inhibition in the preBötC^{10, 18}, inhibitory modulation plays important roles in preBötC dynamics⁸. By making temporally precise brief bilateral perturbations of preBötC *GlyT2* neurons, we probed their role in the generation of respiratory pattern in spontaneously breathing adult mice.

We utilized conditional gene targeting for restricting protein expression to inhibitory preBötC neurons. Since the *GlyT2* promoter is specific to glycinergic neurons¹⁵, by locally injecting cre recombinase-dependent viruses into the preBötC of transgenic mice expressing Cre driven by the *GlyT2* promoter^{12–14}, we could selectively activate and silence these inhibitory neurons specifically in the preBötC of intact, awake or anesthetized, spontaneously breathing mice. To verify this, we examined the overlap of transfected neurons with known markers of excitatory or inhibitory neurons. We found that transfected neurons rarely colocalized with *Sst*, which demarcates a subset of excitatory preBötC

neurons, and colocalized with glycine immunoreactivity, which identifies glycinergic neurons.

Our injection sites were centered on the preBötC with some *GlyT2*⁺ neuronal somas transfected rostrally including in the BötC, which also contains substantial numbers of respiratory-modulated inhibitory, including glycinergic, neurons^{19–21}, caudally in the rostral ventral respiratory group that contains bulbospinal inspiratory neurons projecting to phrenic and intercostal motoneurons, and dorsally in the adjacent reticular formation. Peak densities of transfected neurons substantially overlapped with the distribution of *Sst*⁺ neurons relative to the caudal boundary of the facial nucleus, indicating that we successfully hit the preBötC. Optical cannulae were placed 200 – 400 μm dorsal to the caudal end of the preBötC to limit the light path to mainly target the somas of preBötC neurons and avoid the somas of transfected neurons outside the preBötC. However, opsins are not localized to any one neuronal domain (including expression in synaptic terminals), so there is the possibility that light affected synaptic terminals in the preBötC that originated from transfected somas adjacent to its boundaries. Insofar as these axons and presynaptic terminals arose from somas in the preBötC (including from the contralateral side), the results reflect the effects of perturbing preBötC *GlyT2* neuron excitability. Of concern are effects that may have resulted from stimulating axons and presynaptic terminals of *GlyT2* neurons with somas outside but synapsing within the preBötC. At the rostrocaudal level of the preBötC, neurons adjacent to the VRC do not have any respiratory-modulated activity²², and have no established monosynaptic projections to the preBötC^{23, 24}. Thus, we consider the likelihood that glycinergic neurons at this level outside the preBötC could have contributed to the observed responses to be small. Caudal to the preBötC in the VRC there are inspiratory neurons, mostly bulbospinal, with no known monosynaptic rostral projections to the preBötC^{23, 24}. Rostral to the preBötC in the VRC is the BötC, which contains considerable numbers of *GlyT2*⁺ neurons that appear to project to the preBötC; if photoactivation (ChR2) or photoinhibition (ARCH) affected their terminals, then they could contribute to responses evoked when light was confined to the preBötC. By aiming at the caudal preBötC with our injection and cannulae placement, we avoided most, but not all, BötC neuronal somas, but may have affected some of their putative transfected terminals in the preBötC. If BötC and preBötC *GlyT2* neurons constitute a functionally homogeneous population (which is presently unknown) we have no concern here. If they represent two distinct populations with different effects, then our interpretation of the source of the observed effects, i.e., preBötC and/or BötC, would need to be parceled out. The most parsimonious explanation for the results we report is that light activated responses are predominately from perturbations affecting preBötC *GlyT2* neurons including their terminals, and we frame the subsequent discussion accordingly.

Brief photoinhibition of Arch-expressing preBötC *GlyT2* neurons during the preinspiratory or inspiratory phases produced significantly larger inspirations with no change in inspiratory duration. Thus, at least a subset of transfected *GlyT2* neurons is endogenously active during (pre)inspiration and is affecting the amplitude of inspiratory outflow without an obligatory effect on its termination. If these neurons were essential to rhythmogenesis, one would predict that their photoinhibition during inspiration would also increase inspiratory duration.

This was not the case when silencing a subset of these neurons sufficient to substantially increase inspiratory amplitude (Fig 4b–d). We suggest that these data further support previous observations that rhythmogenesis *per se* is not particularly sensitive to changes in inhibition¹⁰. When photoinhibition occurred during mid-expiration, expiratory duration was shortened. Thus, withdrawal of inhibition during mid-expiration allows preBötC (pre)inspiratory activity to initiate earlier and/or spread through the network more rapidly²⁵, resulting in inspiration being triggered sooner than expected. These data are consistent with the interpretation that these neurons can modulate respiratory timing.

Brief photostimulation of ChR2-expressing preBötC *GlyT2* neurons when delivered during the inspiratory phase prematurely terminated inspiration, i.e., shorter inspiratory durations and smaller peak inspiratory airflows. Photostimulation during expiration, including the preinspiratory phase, lengthened that phase, significantly delaying the subsequent inspiration. In response to longer stimuli, this effect was sufficiently strong to produce apnea in anesthetized or awake mice at rest, as well as when ventilation was increased by hypoxic or hypercapnic challenges. We found that respiration resumed with a consistent latency after the laser shut off regardless of the phase of photostimulation, which indicates that activation of preBötC *GlyT2* neurons can reset the respiratory cycle.

Activation of preBötC *GlyT2* neurons inhibits other respiratory-modulated preBötC neurons. We found 7 (pre)inspiratory-modulated neurons in total and all were silenced by ChR2 activation, at any point in the respiratory cycle. Silencing preinspiratory neurons prior to the start of inspiration coincided with typical preinspiratory spiking activity that was not followed by an inspiratory burst. That initial preinspiratory activity can occur without a subsequent burst is consistent with our hypothesis that inspiratory burst initiation and patterning are separate components of a two-stage process that can be experimentally decoupled²⁵. We conclude that preBötC *GlyT2* neurons can modulate the activity of rhythmogenic preBötC neurons and, when activated, can prematurely terminate an inspiratory burst, but that rhythmogenesis is not particularly sensitive to changes in inhibition.

We suggest that one essential role of preBötC *GlyT2* inhibitory neurons is to modulate basic breathing parameters such as inspiratory flow, tidal volume, inspiratory and expiratory duration, and respiratory frequency, irrespective of any role in rhythmogenesis *per se*. By modulating the activity of preBötC *GlyT2* neurons, we were able to, in effect, tune the dynamics of the preBötC network.

We did not isolate any neurons that were activated by photostimulation. There are several possibilities: i) Our sample size was too small. ii) Photostimulation activated a substantial number of preBötC *GlyT2* neurons that were silent under control conditions and therefore we did not isolate them prior to stimulation. At present, we do not know the fraction of preBötC *GlyT2* neurons that are respiratory-modulated or tonically active in anesthetized spontaneously breathing mice, and we have to allow the possibility that this fraction is small. iii) Laser light caused rapid, potent, and as long as the light was on, continual release of glycine from synaptic terminals of the transfected neurons. This would rapidly terminate the activity of all preBötC, including glycinergic, neurons. Thus, glycine could continue to

be released during the entire duration of stimulation while all neurons were silent. The multi-unit field potential observed after laser onset may, instead, be the signature of transient activation of nearby *GlyT2* neurons (Fig. 6c, inset).

Numerous models of central pattern generation focus on inhibition as a key component in the generation of rhythmic behaviors^{26–29}; for breathing, inspiratory burst termination and/or phase transitions are often postulated to be mediated by postsynaptic actions of inhibitory neurons^{9, 30, 31}. One recent model postulates that interactions between excitatory preBötC neurons and inhibitory subpopulations within the preBötC and BötC are essential to generate a normal respiratory rhythm¹⁷. However, while profound changes in breathing result from slow or relatively rapid onset perturbations to excitatory preBötC subpopulations^{1, 5}, a normal respiratory rhythm persists in anesthetized adult rats after substantial blockade of inhibitory neurotransmission in preBötC and BötC by local injection of GABA_A and glycine antagonists¹⁰. Our data are consistent with this result. We did not observe any breakdown in eupneic breathing when silencing a subset of preBötC *GlyT2* neurons that was nonetheless sufficient to substantially increase inspiratory amplitude (Fig. 4b–d). Whether silencing a larger percentage of the preBötC *GlyT2* neurons affect rhythmogenesis *per se* remains to be determined. Thus, we consider it unlikely that preBötC inhibitory neurons are playing an obligatory role in terminating inspiration.

Photoinhibition of preBötC *GlyT2* neurons increased respiratory drive, producing larger breaths at a higher frequency. Thus, the preBötC *in vivo* with diminished preBötC *GlyT2* neuronal activity but otherwise intact continued to generate a eupneic breathing rhythm¹⁰. Photoinhibition did not change the firing of (pre)inspiratory neurons, suggestive of weak or absent Arch expression in these neurons. We do not know from our experiments whether these neurons were excitatory, i.e., non-*GlyT2*, or simply failed to express sufficient protein. Since we did not observe increased activity in (pre)inspiratory neurons, we suggest either unilateral photoinhibition of preBötC *GlyT2* is not sufficient to induce changes in individual (pre)inspiratory neurons or the increased respiratory drive results from an increase in the total number of active neurons, not the number of spikes *per se*.

Precisely timed apneas are an essential component of many vital, complex oropharyngeal movements, e.g., swallowing, vocalization, suckling, and chewing, and there are many powerful reflex apneas, such as the BHIR or following superior laryngeal nerve activation^{32–34} that can be triggered by tracheal obstruction. We hypothesize that inhibitory neurons play an important role in the generation of central apneas¹⁰. In support of this hypothesis and refining the experimental protocol to target the preBötC, we found that prolonged photostimulation of preBötC *GlyT2* neurons initiated at any point in the respiratory cycle can produce apnea. Moreover, photoinhibition of preBötC *GlyT2* neurons can counteract a reflex-induced apnea generated by the BHIR (Fig. 5a).

In summary, we employed a virus-based strategy to selectively introduce ChR2 or Arch into inhibitory preBötC neurons. By delivering light into the preBötC via chronically implanted optical cannulae in transfected anesthetized or awake mice, we have shown that preBötC *GlyT2* neuronal activity modulates the respiratory output, may be critical in the manifestation and control of apneas essential for normal behavior, but does not appear to be

an essential component underlying respiratory rhythmogenesis. Finally, the phase-response curves for photostimulation and photoinhibition represent a new class of data whose reproduction represents an essential test for any model purporting to explain respiratory rhythmogenesis.

Methods

Animals

Animal use was in accordance with the guidelines approved by the UCLA Institutional Animal Care and Use Committee. Animals were housed in a vivarium under a 12 hr light cycle with free access to food and water. All experiments were done on adult male *GlyT2-Cre* mice (24–30 g; C57Bl6 background; average post-surgery duration of ~30 days) with peripheral nerves, e.g., vagus, carotid sinus nerve intact, that were 10–14 weeks of age at the time of AAV injection. Prior to surgery, animals were housed in groups of variable size up to 5 mice per cage; after surgery, animals were housed individually as required by our study protocols. Mice breathed spontaneously. The *GlyT2-Cre* mice were kindly provided by H.U. Zeilhofer, University of Zurich, Switzerland.

Viral vector design

The viral constructs *AAV2/1-Efl α -DIO-ChR2-eYFP* (Addgene plasmid 20298; provided by K. Deisseroth, Stanford University, Palo Alto, CA)¹⁶ and *AAV2/1-flex-CBA-Arch-GFP* (Penn Vector P2432; provided by E. Boyden, Massachusetts Institute of Technology, Cambridge, MA)¹³ were produced by the University of Pennsylvania Gene Therapy Program Vector Core. We used a ChR2 variant with the H134R mutation, which produces a twofold increase in the steady-state current as compared to the wildtype variant of ChR2^{35, 36}.

Microinjection

Mice were anesthetized with isoflurane (4% for induction and 2% for maintenance) and placed in a stereotaxic apparatus (David Kopf Instruments) with Bregma and Lambda skull landmarks level. Two holes were drilled in the skull at predefined coordinates relative to Bregma to allow the insertion of glass pipettes containing a virus solution (ChR2: 5 – 10 \times 10¹² GC/ml; Arch: 6 – 10 \times 10¹² GC/ml) connected to a pressure ejection system (Picospritzer II; Parker Hannafin) targeted to the preBötC. Once situated, 100–200 nl of per side was injected into the preBötC. Injections were made 6.80 mm caudal to Bregma, 1.20 mm lateral to the midline, and 4.65 mm ventral from the dorsal surface of the brain. Coordinates were determined from microinjections (n = 7) of red fluorescent microspheres (1.0 μ m diameter, 5% solution; Invitrogen) into calculated coordinates based on a mouse brain atlas³⁷ and adjusted based on the distance from preBötC, as determined by the location of *Sst* immunoreactive neurons⁴. The pipette was left in place for 5 min after injection to minimize backflow. The wound was closed with 5–0 non-absorbable sutures. The mice were returned to their home cage and allowed 2–3 weeks to recover, allowing time for sufficient levels of protein to express. For controls, negative littermates (those not expressing Cre recombinase) received virus injections as described above; as expected, there was no virus

expression. Microinjections of cre-dependent virus were performed with the investigator blind to whether the animal was Cre⁺ or Cre⁻.

Implantation of fiber optics

Mice were placed into the stereotaxic frame and fiber-optic cannulae with a 1.25 mm stainless steel ferrule (200 μ m fiber; Doric Lenses), held by a stereotaxic adaptor (Doric Lenses), were implanted bilaterally to a depth 200–400 μ m dorsal to the preBötC, based on predetermined coordinates. The cannulae were glued to the skull using Metabond™ (Parkell) and the wound was sealed by applying a layer of clear dental acrylic (Lang Dental).

Photostimulation and photoinhibition

Branching optical fibers (200 μ m fiber; Doric Lenses) with 1.25 mm stainless steel ferrules as ends were connected to the implanted cannulae via plastic sleeves. The back end of the fiber was connected to a 473nm/593nm dual wavelength laser (OptoDuet Laser; IkeCool) via an optical rotary joint (Doric Lenses). Light pulses were controlled by a pulse generator (Pulsemaster A300 Generator; WPI). Anesthetized mice were placed on a heating pad and in front of a nose cone connected to a flowmeter (GM Instruments) to record airflow. Awake mice were placed in a plethysmograph chamber (Buxco) connected to a pressure transducer (F.W. Kirk Company) with the optical fibers exiting the chamber through a small hole.

Breuer-Hering inflation reflex (BHIR)

The BHIR was induced in anesthetized Arch transfected mice (FLEX-Arch) by inflating the lungs with continuous positive airway pressure (CPAP). The pressure was adjusted to yield an apnea that lasted for ~10 – 15s (~4 cm H₂O) before being “broken” by breaths reappearing despite the CPAP. We applied one control period of CPAP, waited for 30–60s, and then applied a second period of CPAP combined with a 1s pulse of light (593nm) delivered bilaterally into the preBötC. We measured both the amount of time it took for breathing to resume relative to the start of the reflex and the onset of the laser pulse.

Single-unit recording

To record neuronal activity, transfected mice were placed in the stereotaxic frame as described above. An optical cannula was implanted at a 27° angle from vertical, tilted across the midline from right to left, down to preBötC and glued to the skull with Metabond (Parkell). A window was drilled through the skull of sufficient size to allow the recording electrode free range of movement to record on the left side of the brain. Respiratory-modulated units were found 4.6 to 4.9 mm below the cerebellar surface and 1.1 to 1.3 mm lateral to the midline. A glass electrode (~3 μ m inner tip diameter; filled with aCSF containing (in mM) 124 NaCl, 3 KCl, 1.5 CaCl₂, 1 MgSO₄, 25 NaHCO₃, 0.5 NaH₂PO₄, and 30 d-glucose) was connected to a headstage (Siskiyou) and the signal amplified (Grass Model P511; Grass Instruments) and sampled at 20 kHz (PowerLab 16SP; ADInstruments). Due to a laser artifact apparent at the start and end of laser pulses, we used a single 1 s pulse for photostimulation to reduce noise. Most neural recordings were stable for 5 – 10 min.

Histology

After the completion of the experiment, mice were deeply anesthetized with pentobarbital and perfused transcardially with saline followed by 4% paraformaldehyde in phosphate-buffered saline (PBS). Brains were removed, postfixed overnight at 4°C, and cryoprotected in 30% sucrose in PBS for 24–48 h before sectioning. Free-floating coronal or sagittal sections (40 µm) were cut using a cryostat (CryoStar NX70, Leica Microsystems) in two series and stored at 4°C until further processing. Sections were incubated with primary antibodies in PBS containing 0.3% Triton X-100 (PBT) overnight at room temperature. After 3 washes, sections were incubated in species-appropriate secondary antibodies in PBS for 4h at room temperature. After 3 washes, sections were mounted onto gelatin-coated glass slides, dehydrated in graded alcohols, cleared in Xylenes, and coverslipped. Fluorescence was visualized with a confocal laser scanning microscope (LSM710; Carl Zeiss). Images were acquired with Zen software (Carl Zeiss), exported as TIFF files, and arranged to prepare figures in Photoshop and Illustrator (Adobe).

We used the following primary antibodies: rabbit polyclonal anti-somatostatin-14 (1:500; Peninsula Laboratories; T4103), mouse monoclonal anti-NeuN (1:500; Millipore; MAB377), chicken polyclonal anti-GFP (1:500; Aves Labs; GFP-1020), and rabbit anti-glycine (1:500; Millipore; AB5020). Rhodamine Red-X donkey anti-rabbit, DyLight488 or Cy2 donkey anti-chicken, and Cy5 donkey anti-mouse conjugated secondary antibodies (1:250; Jackson ImmunoResearch) were used to detect primary antibodies. These four antibodies are frequently used in mice and have been well validated. The validity of the SST-14 antibody has been shown in both rat^{1, 38} and mice^{5, 25}. The GFP antibody has been used successfully in mice as well⁵. In addition, the glycine antibody has been extensively used and validated as a marker of glycinergic neurons in mice and rats^{39, 40}. The NeuN antibody has been extensively used in mice throughout the field of neuroscience research.

Data analysis and statistics

Traces were recorded on a 64-bit computer using LabChart 7 Pro (ADInstruments). The airflow signal was high-pass filtered (>0.1 Hz) to eliminate DC shifts and slow drifts, and used to calculate respiratory frequency, period, inspiratory (T_I) and expiratory (T_E) durations. The airflow signal was integrated to compute tidal volume (V_T).

Cell counts were performed on 3 sequential sagittal sections as defined by the localization of *Sst*-immunoreactive neurons and soma distances relative to the caudal boundary of the facial motor nucleus were calculated. Counts were performed with ImageJ (<http://rsb.info.nih.gov/ij/>) and exported to Igor (Wavemetrics) for analysis with custom software.

All statistics were performed in Igor (Wavemetrics). Statistical significance was set at $p < 0.05$. One way ANOVAs and pairwise comparisons using Tukey's HSD test were performed for all experiments except in Figure 5b, where an unpaired t-test was used. All values are presented as mean \pm s.e.m. No statistical methods were used *a priori* to pre-determine sample sizes but our sample sizes are similar to those reported in previous publications^{1, 41}. Data distribution was assumed to be normal but this was not formally tested.

Phase response analysis

The photostimulation-evoked reset of the respiratory rhythm was studied by applying a single 100 ms light pulse at various times during the respiratory cycle. The respiratory phase in each photostimulation or photoinhibition breath was based on the prior (control) respiratory period and defined as spanning $0^\circ - 360^\circ$. Across experiments, inspiration (defined as the period of inward airflow) lasted from onset at 0° until $71.8^\circ \pm 11.6^\circ$ ($n = 5$) in ChR2 experiments and until $77.9^\circ \pm 7.1^\circ$ ($n = 7$) for Arch experiments; expiration was defined as the remainder of the cycle. Thus, the phase of any event could be calculated based on the time at which it occurred relative to the preceding inspiratory onset and referenced to the preceding control period. More specifically, phase values were obtained by calculating the ratio of the event onset time to the respiratory period and multiplying by 360° .

Stimulus phase (ϕ_{stim}), induced phase (ϕ_i), and expected phase (ϕ_e) of the respiratory cycle were computed (see Fig. 2a)^{38, 42} and plotted using software written in Igor (Wavemetrics). ϕ_{stim} , i.e., the time of laser onset relative to the ongoing respiratory cycle, was defined as the delay from the beginning of the previous inspiration to the time of laser onset. ϕ_e represents the total respiratory period, defined using the previous cycle as a control. ϕ_i is the respiratory period during the light-affected cycle. The difference between ϕ_i and ϕ_e was the net phase shift (ϕ_{shift}). If the light had no effect, $\phi_e = \phi_i$, and $\phi_{shift} = 0$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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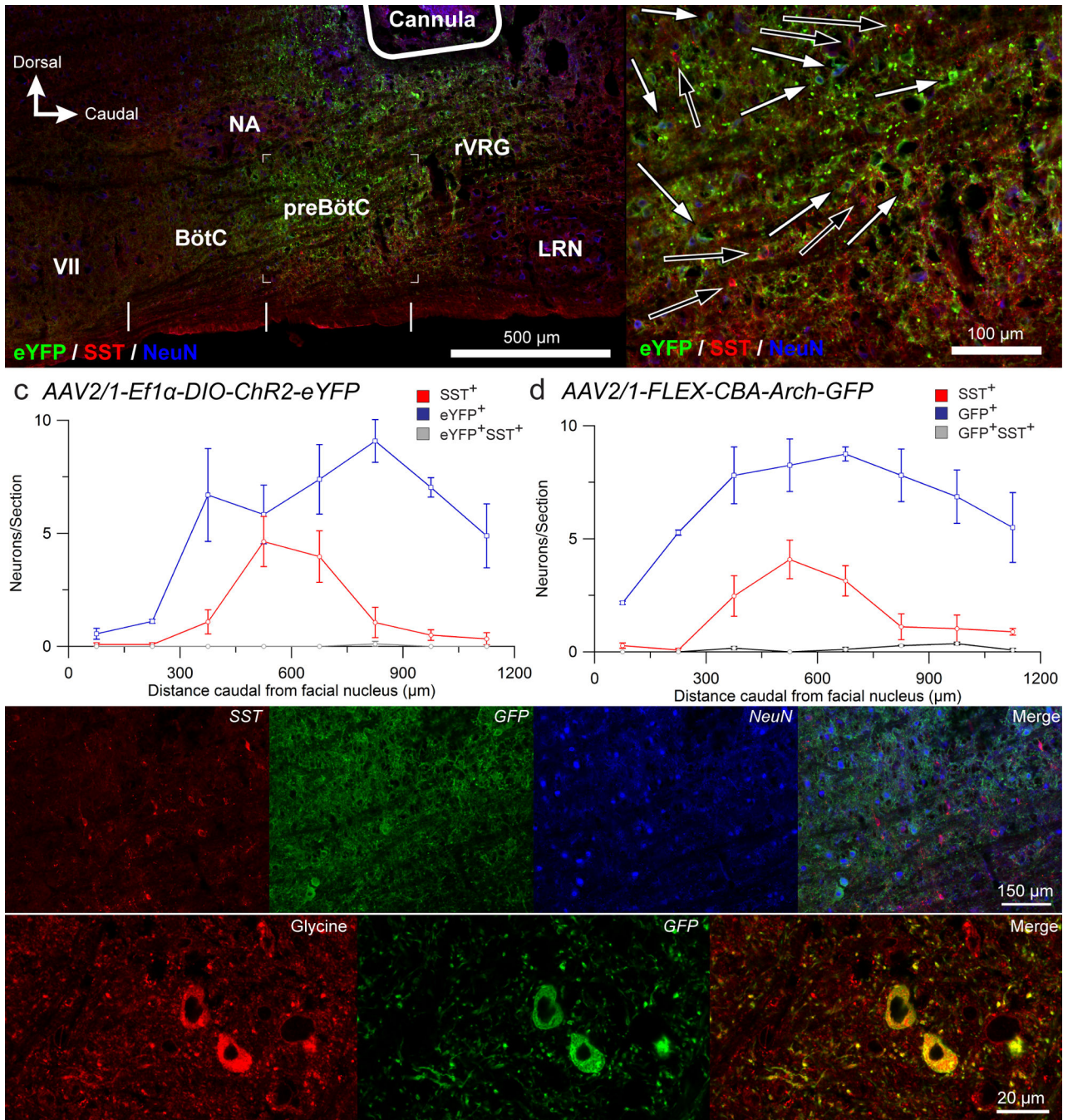


Figure 1. Cre-dependent ChR2 or Arch expression targeted to preBötC *GlyT2* neurons
(a) Representative confocal mosaic micrograph of sagittal brainstem section of *GlyT2-cre*⁺ mouse showing the extent of *eYFP*⁺ and *Sst*⁺ neurons after injection of *AAV2/1-Ef1α-DIO-ChR2-eYFP* into preBötC (n = 5). Vertical white lines at bottom mark approximate rostral-caudal boundaries of Bötzing Complex (BötC) and preBötzing Complex (preBötC). Outline of cannula tip placement at top right. No labeled somas were found in the brainstem outside the boundaries of this micrograph. Abbreviations: VII: facial nucleus; NA: nucleus ambiguus; LRN: lateral reticular nucleus. **(b)** High magnification micrograph of bracketed

segment in (a) showing *eYFP*⁺ preBötC neurons (white arrows) intermingled with *Sst*⁺ neurons (blue arrows). (c, d) Distribution of *eYFP/GFP*⁺ (blue line), marking location of Chr2- (c; n = 3) or Arch- (d; n = 3) expressing neurons and *Sst*⁺ (red line) neurons relative to caudal boundary of facial nucleus. Error bars: mean ± s.e.m. (e) Representative single channel and overlay confocal micrographs showing *eYFP*⁺ preBötC neurons (green) after *AAV2/1-Efl1α-DIO-ChR2-eYFP* injection. Section also labeled for *Sst* (red) and *NeuN* (blue) immunoreactivity. (f) Single channel and overlay confocal micrographs (n = 3) showing *eYFP*⁺ preBötC neurons (green) with glycine immunoreactivity (red).

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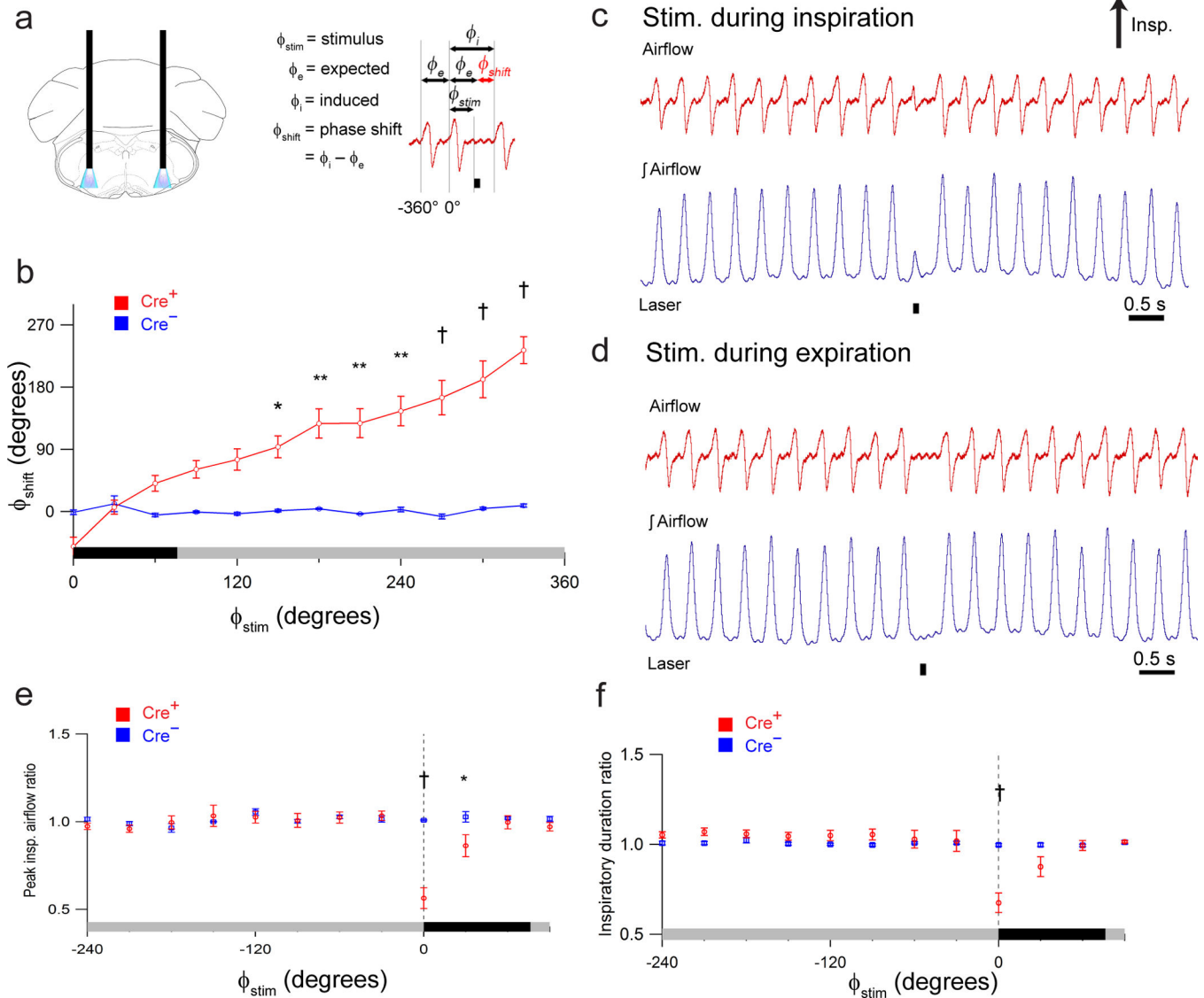


Figure 2. Photostimulation of preBötC *GlyT2* neurons depresses breathing

(a) Left: Schematic depicting bilateral placement of optical cannulae targeting preBötC. Right: schematic airflow trace depicting definitions of stimulus phase (ϕ_{stim}), expected phase (ϕ_e), induced phase (ϕ_i), and phase shift (ϕ_{shift}), demarcated with horizontal arrows, relative to the reference cycle that spans $0^\circ - 360^\circ$; the control cycle preceding reference cycle spans phase $360^\circ - 0^\circ$. Black bar below trace is laser-on period that defines the start of ϕ_{stim} . ϕ_e is the period of the previous control respiratory cycle, and ϕ_{shift} is the difference between ϕ_e and ϕ_i . (b) Shift in respiratory phase (ϕ_{shift}) resulting from bilateral photostimulation (100 ms pulse) of preBötC *GlyT2* neurons in *GlyT2-cre⁺* (Cre⁺, red; n = 5) and *GlyT2-cre⁻* (Cre⁻, blue; n = 5) mice. Stimulus phase (ϕ_{stim}) is depicted on x-axis (b, e, f) with inspiration (black bar) defined from $0^\circ - 72^\circ$, with gray bar: (b) the subsequent expiration defined from $72^\circ - 360^\circ$ or (e, f) the preceding expiration defined from $-288^\circ - 0^\circ$. P values: 150 – 180°: P = 0.007; 180 – 210°: P = 2×10^{-5} ; 210 – 240°: P = 4×10^{-6} ; 240 – 270°: P = 4×10^{-7} ; 270 – 300°: P = 1×10^{-9} ; 300 – 330°: P = 5×10^{-10} ; 330 – 360°: P =

4×10^{-10} . **(c, d)** Representative airflow and tidal volume (airflow) traces illustrating effect of photostimulation (100 ms pulse; black bar beneath trace) during inspiration (c) and expiration (d). **(e, f)** Comparison of ratio of peak inspiratory airflow (**e**; $0 - 30^\circ$: $P = 4 \times 10^{-10}$; $30 - 60^\circ$: $P = 0.02$) or inspiratory duration (**f**; $0 - 30^\circ$: $P = 4 \times 10^{-10}$) in *GlyT2-cre*⁺ (Cre⁺, red) and *GlyT2-cre*⁻ (Cre⁻, blue) anesthetized mice. Dotted vertical line (**e, f**) at 0° indicates onset of inspiration. Error bars, mean \pm s.e.m. Statistical significance was determined with a one-way ANOVA and pair-wise comparisons were made with Tukey's HSD test. * $P < 0.05$; ** $P < 0.001$; † $P < 10^{-8}$.

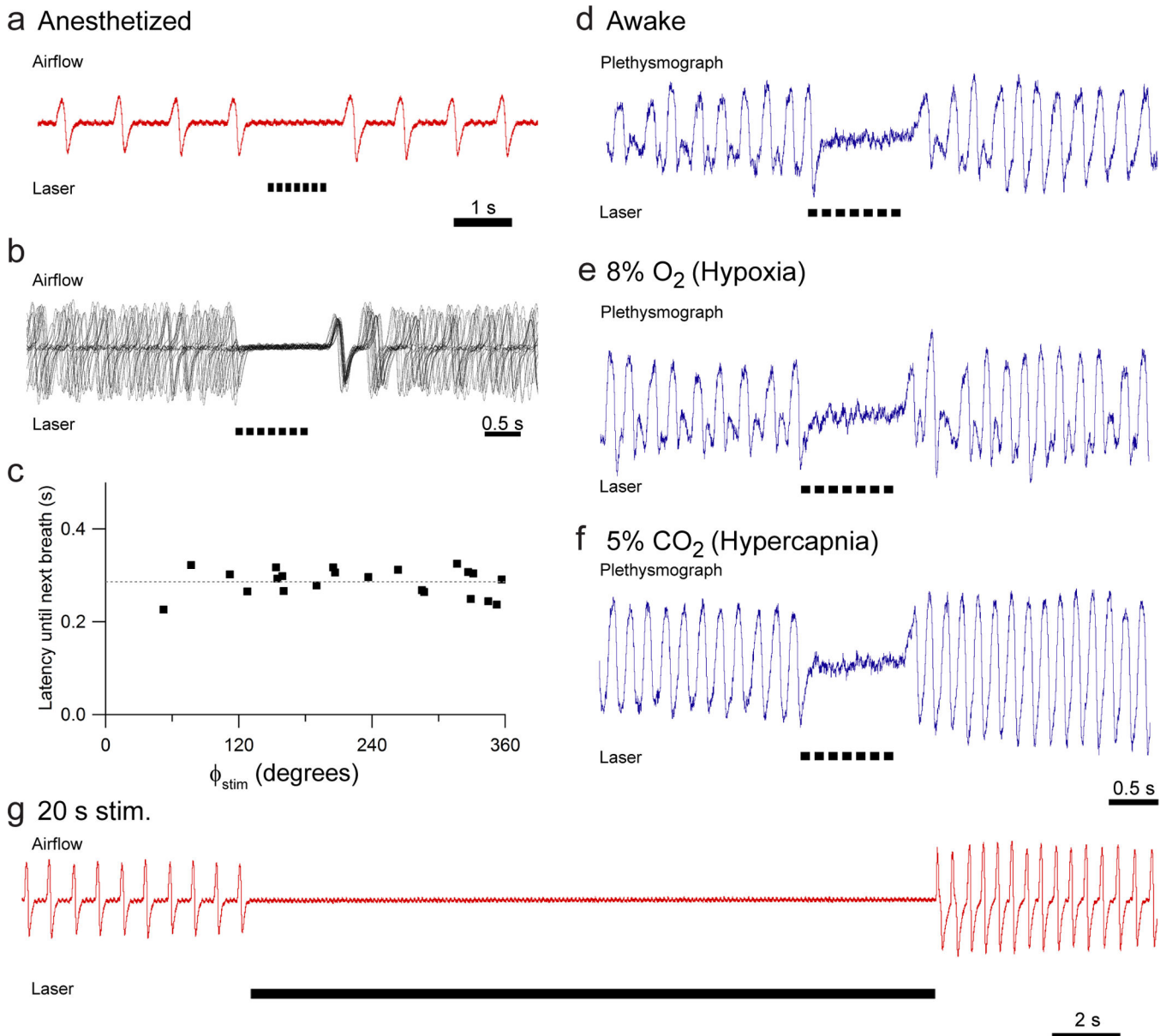


Figure 3. Prolonged photostimulation of preBötC *GlyT2* neurons results in apnea
(a) Representative airflow trace illustrating effect of 1 s pulse train of photostimulation (black bars beneath trace; 7× 100ms pulses with a 50 ms interpulse interval; n = 5). **(b)** Overlay of airflow traces aligned to the laser onset at various phases of respiratory cycle (7× 100 ms pulses with a 50 ms interpulse interval) reveals that the next breath occurred at a fairly constant delay after the laser shuts off. **(c)** Representative graph of latency to next breath as a function of initial phase of photostimulation. Dotted line indicates mean latency. **(d, e, f)** Photostimulation with a 1 s pulse train (7× 100 ms pulses with 50 ms interpulse interval) of awake, behaving mice in a plethysmograph under eupneic (**d**; n = 5), hypoxic (**e**; n = 5), or hypercapnic (**f**; n = 5) states. **(g)** Airflow during bilateral 20 s pulse train of light (100 ms pulses with 50 ms interpulse interval; n = 3) in anesthetized mouse.

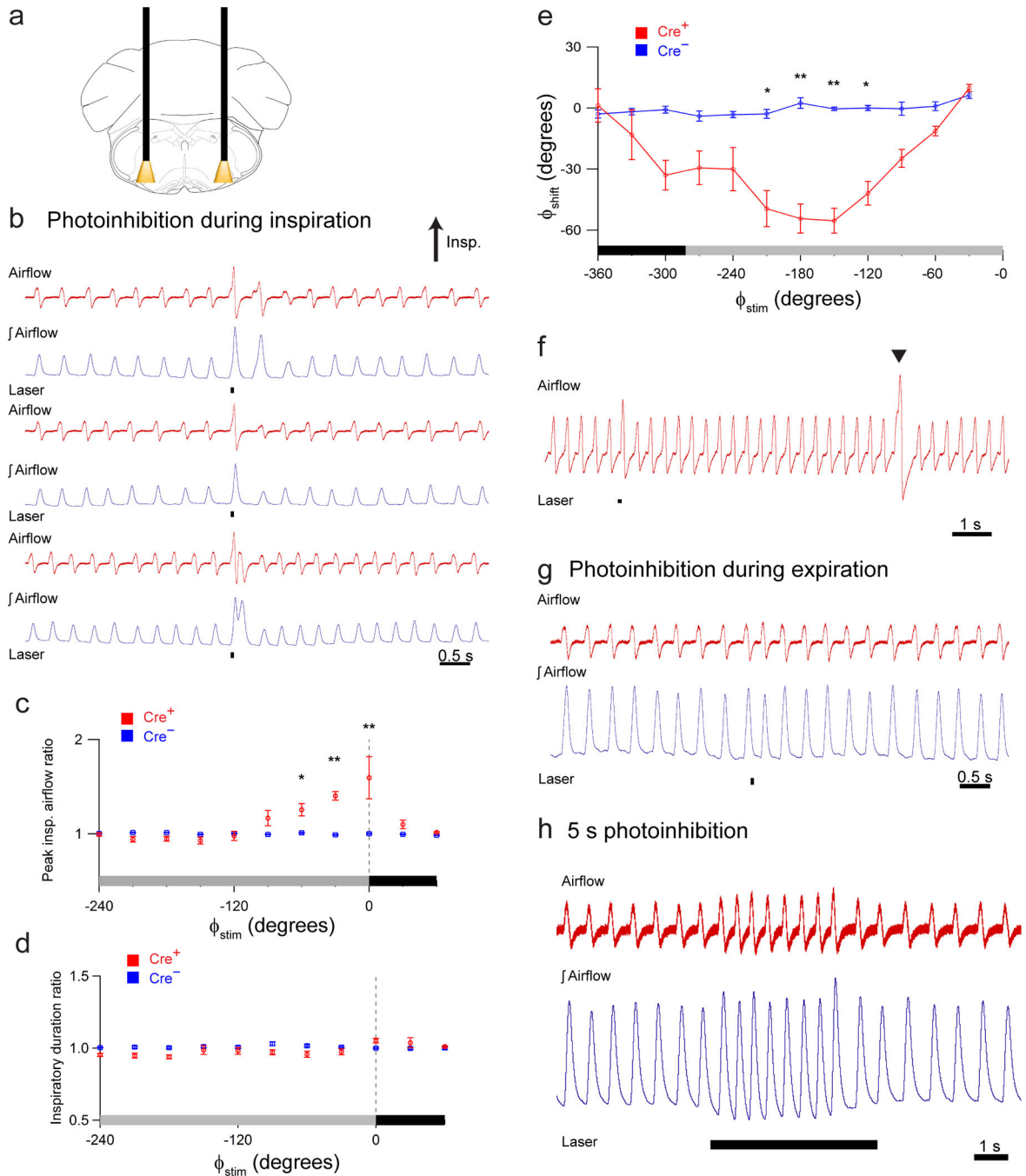


Figure 4. Photoinhibition of preBötC *GlyT2* neurons augments breathing

(a) Schematic depicting bilateral placement of optical cannulae targeting preBötC. (b) Representative airflow and tidal volume (\int airflow) traces (3 pairs) illustrating effect of brief photoinhibition (100 ms pulse; black bar) during inspiration. (c, d) Comparison of photoinhibited cycle to prior control cycle as a function of stimulus phase (ϕ_{stim}), for ratio of peak inspiratory airflow (c; $n = 7$; $0 - 30^\circ$: $P = 2 \times 10^{-7}$; $30 - 330^\circ$: $P = 0.01$; $330 - 360^\circ$: $P = 5 \times 10^{-7}$) or inspiratory duration (d; $n = 7$; all $P > 0.05$) in Cre^+ (red) and Cre^- (blue) anesthetized mice. Respiratory stimulus phase (ϕ_{stim}) on x-axis (c, d, e) with inspiration

(black) defined from 0° to 78° (-360° to -282°) and expiration (gray) defined from 78° to 360° (-282° to 0°). The dotted vertical line (**c**, **d**) at 0° indicates onset of inspiration. (**e**) Shift in respiratory phase (ϕ_{shift}) resulting from preBötC-targeted laser pulses (100 ms pulse) in *GlyT2-cre*⁺ (Cre⁺, red; n = 7) and *GlyT2-cre*⁻ (Cre⁻, blue; n = 5) mice. P values: 150 – 180°: P = 0.002; 180 – 210°: P = 2×10^{-5} ; 210 – 240°: P = 8×10^{-6} ; 240 – 270°: P = 0.01. (**f**) Representative light response in response to photoinhibition (black bar) compared to endogenous sigh (arrow). (**g**) Representative airflow and tidal volume (\int airflow) traces illustrating effect of brief photoinhibition (100 ms pulse; black bar) during expiration. (**h**) Representative airflow and tidal volume traces illustrating that longer photostimulation (5 s continuous pulse) increased peak inspiratory airflow and frequency (n = 3). Error bars, mean \pm s.e.m. Statistical significance was determined with a one-way ANOVA and pair-wise comparisons were made with Tukey's HSD test. * P < 0.05; ** P < 0.001; † P < 10^{-8} .

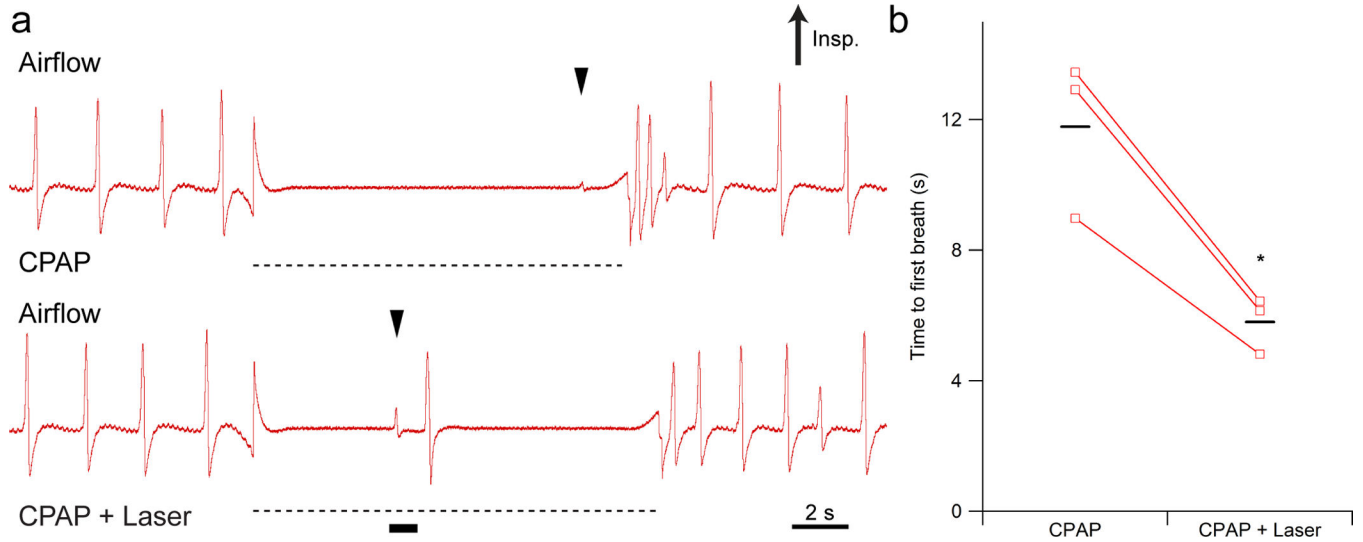


Figure 5. Photoinhibition of preBötC *GlyT2* neurons during a reflex apnea rescues breathing (a) Breuer-Hering lung inflation reflex (BHIR) triggered by continuous positive airway pressure (CPAP; ~4 cm H₂O; dotted black line) induced apnea in a tracheotomized, anesthetized Arch-transfected mouse in control (top) and bilateral photoinhibition (bottom, black bar) conditions. Top: After onset of BHIR, first breathe was at ~11 sec (black arrow). Bottom: 1s pulse applied 5.5 ± 0.5 s from onset of BHIR produced a first breath (indicated with a black arrow) 5.8 ± 0.5 s after the start of reflex, i.e., a 300 ms delay from the laser onset, versus an expected first breath at 11.8 ± 1.4 s, as seen during control (CPAP only) periods. (b) Duration of induced apnea, measured from onset of BHIR to onset of the next inspiration, in CPAP only and CPAP + laser conditions. Means are indicated with black horizontal lines (n = 3; P = 3×10⁻⁶). Statistical significance was determined with an unpaired t-test. * P < 10⁻⁵.

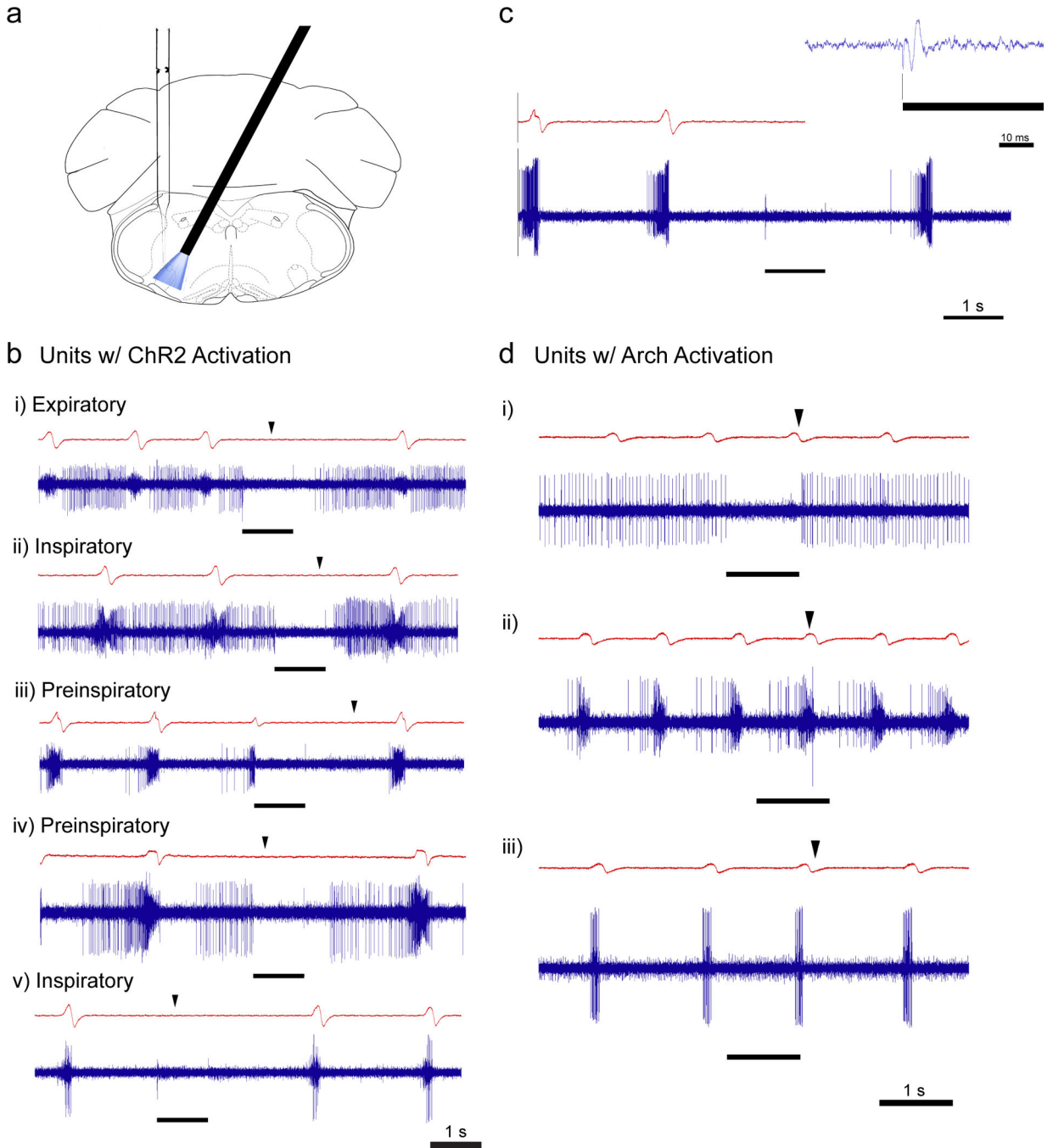


Figure 6. Unit recording with concurrent ChR2 or Arch activation

(a) Schematic showing cannulae implantation at a 27° angle and vertical electrode placement to record from units within the cone of light (473 nm or 593 nm) in opsin-transfected mice. (b) Representative airflow traces (red) and respiratory-modulated units (blue; i) expiratory, ii) inspiratory, iii) pre-inspiratory, iv) pre-inspiratory, v) inspiratory) showing strong inhibition during 1s continuous laser pulse (black bar). Black arrows indicate the expected onset of inspiration following photostimulation. In total, 18 neurons were recorded from 3 mice. (c) Representative recording of multiple units following

photostimulation of ChR2-expressing preBötC *GlyT2* neurons with airflow trace. Inset: Shorter time-scale at onset of continuous laser pulse (black line) showing large field potential within ~2 msec. Vertical black line shows alignment of laser onset and initial artifact on trace. **(d)** Response of preBötC neurons to photoinhibition of Arch-expressing preBötC *GlyT2* neurons with 1 s continuous laser pulse (black line). Black arrows indicate the expected onset of inspiration following photoinhibition as measured on the airflow trace. In total, 12 neurons were recorded from 2 mice.