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Chronic Intermittent Hypoxia reveals role of the Postinspiratory Complex in the mediation of normal swallow production

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Abstract

Obstructive sleep apnea (OSA) is a prevalent sleep-related breathing disorder that results in multiple bouts of intermittent hypoxia. OSA has many neurologic and systemic comorbidities including dysphagia, or disordered swallow, and discoordination with breathing. However, the mechanism in which chronic intermittent hypoxia (CIH) causes dysphagia is unknown. Recently we showed the Postinspiratory complex (PiCo) acts as an interface between the swallow pattern generator (SPG) and the inspiratory rhythm generator, the preBötzinger Complex, to regulate proper swallow-breathing coordination (Huff et al., 2023). PiCo is characterized by interneurons co-expressing transporters for glutamate (Vglut2) and acetylcholine (ChAT). Here we show that optogenetic stimulation of ChATcre:Ai32, Vglut2cre:Ai32, and ChATcre:Vglut2FlpO:ChR2 mice exposed to CIH does not alter swallow-breathing coordination, but unexpectedly triggers variable swallow motor patterns. This suggests, glutamatergic-cholinergic neurons in PiCo are not only critical for the regulation of swallow-breathing coordination, but also play an important role in the modulation of swallow motor patterning. Our study also suggests that swallow disruption, as seen in OSA, involves central nervous mechanisms interfering with swallow motor patterning and laryngeal activation. These findings are crucial for understanding the mechanisms underlying dysphagia in OSA and other breathing and neurological disorders.

eLife assessment

This **important** study represents a follow-up of previous papers by Huff et al. (2023) in which the authors further investigate a specific medullary region named the Postinspiratory Complex (PiCo) involved in the control of swallow behaviour and its coordination with breathing. In the present work, they tested the impact of chronic intermittent hypoxia on the swallow motor pattern evoked by optogenetic stimulation of the same medullary area in transgenic mice. These **solid** results indicate that in chronic intermittent hypoxia-exposed mice PiCo stimulation triggers atypical swallow motor patterns. The experimental procedures are rigorous and technically remarkable. The work will be of interest in the field of respiratory physiology and pathophysiology since a disruption of swallowing and possibly discoordination with breathing may be involved in diseases characterized by the presence of hypoxic conditions such as obstructive sleep apnea.

Introduction

Obstructive sleep apnea (OSA) is highly prevalent and a major public health concern (Chang et al., 2023; McNicholas et al., 2015; Phillipson, 1993; Ramirez et al., 2013; Roberts et al., 2022; Wright & Sheldon, 1998). It is characterized by frequent bouts of apnea during sleep caused by pharyngeal collapse, resulting in multiple bouts of hypoxia referred to as chronic intermittent hypoxia (CIH). CIH increases the gain of the carotid body response to hypoxia which seems to be a major cause for the multiple comorbidities of OSA (Iturriaga, 2023; Prabhakar et al., 2023). These OSA-related comorbidities (Pack, 2023) include an increase in mortality (Vgontzas et al., 2023) and cancer risk (Sánchez-de-la-Torre et al., 2023), increased arousal and sleep fragmentation (Horner, 2023), increased sympathetic drive leading to cardiovascular disease; metabolic syndromes such as obesity and diabetes (Kurnool et al., 2023), renal disease, asthma, (Bonsignore et al., 2019), and decreased cognition (Brockmann & Gozal, 2022). OSA is also commonly associated with dysphagia, disordered swallow function (Pizzorni et al., 2021; Schindler et al., 2014). Clinical studies have begun to investigate physiologic parameters of OSA-related dysphagia, but little is known about the underlying mechanisms.

CIH and the increased gain in carotid body activity lead to disturbances in multiple neuronal mechanisms originating in the central nervous system (Arias-Cavieres et al., 2021; Arias-Cavieres et al., 2020; da Silva et al., 2021; Domingos-Souza et al., 2021; Jia et al., 2022; Kline, 2010; Kline et al., 2019; Lin et al., 2007; Marciante et al., 2021; Ramirez et al., 2020; Souza et al., 2019). CIH directly affects neuronal network functions within the ventral respiratory column (VRC), in particular the preBötzinger complex (preBötC) (Garcia et al., 2017; Garcia et al., 2016), a critical rhythmogenic network implicated in swallow-breathing coordination (Huff et al., 2022).

Swallows share anatomical structures with breathing and it is critical these two behaviors are coordinated to prevent aspiration of food/liquid into the airway. Dysphagia, or disruption of swallow and discoordination with breathing, is directly linked to altered quality of life and failure to thrive in respiratory related diseases such as OSA (Bhutada et al., 2020; Kato et al., 2016; Pizzorni et al., 2021; Schindler et al., 2014) and chronic obstructive pulmonary disease (COPD) (Garand et al., 2018; Ghannouchi et al., 2016; Nagami et al., 2017), and neurodegenerative diseases such as Parkinson's disease, Alzheimer disease, motor neuron diseases (Walshe, 2014), and aging (Ashley et al., 2006).

Swallow-breathing coordination depends on the precise temporal activation of the pharyngeal and laryngeal muscles, as well as muscles involved in respiratory control. This coordination is controlled by various regions throughout the brainstem. The generation of swallow is thought to be governed by the caudal portion of the nucleus tractus solitaries (cNTS), specifically the interstitial and intermediate portions (Altschuler et al., 1989; Kessler & Jean, 1985a; Kessler & Jean, 1985b), presumably the swallow pattern generator (SPG). In rodents, swallow predominately occurs during a respiratory phase referred to as postinspiration, the transitory phase from inspiration to expiration (Huff et al., 2023). Thus, activity in the cNTS must be coordinated with the inspiratory rhythm generator, the preBötC (Smith et al., 1991), and the postinspiratory rhythm generator, the postinspiratory complex (PiCo) (Anderson et al., 2016), to prevent swallows from occurring during inspiration increasing the risk for aspiration.

Recently published studies have demonstrated that PiCo acts as an interface for swallow and laryngeal postinspiratory behaviors for proper coordination and timing of swallow and breathing (Huff et al., 2023). In the present study we explored the effects of CIH on PiCo and its role in coordinating swallowing and breathing in order to understand how OSA and other disorders associated with intermittent hypoxia (e.g. epilepsy, Rett Syndrome) lead to dysphagia. Continuing on from experiments preformed in control mice exposed to room air (Huff et al., 2023), we studied the impact of CIH using an established mouse model for OSA (Garcia et al., 2017; Garcia et al., 2016; Huff et al., 2022; Peng & Prabhakar, 2004; Peng et al., 2021). Similar to our control model, optogenetic stimulation of PiCo in ChATcre:Ai32, Vglut2cre:Ai32, and ChATcre:Vglut2FlpO:ChR2 mice stimulated both swallow and laryngeal activation. Unexpectedly, we find that PiCo-triggered swallow-breathing coordination itself is not altered, rather the alteration is in the swallow motor pattern. We propose that PiCo is involved in swallow motor patterning and CIH disrupts connections between PiCo and the SPG.

Results

Optogenetic stimulation of neurons in PiCo region

Previously we demonstrated that optogenetic stimulation of PiCo neurons triggers swallow and laryngeal activation when exposed to room air (Huff et al., 2023). However, when exposed to CIH, optogenetic stimulation of ChATcre:Vglut2FlpO:ChR2 neurons triggered a variety of abnormal swallow motor patterns (**Figure 1B**). Only 6% of all PiCo-triggered swallows could be characterized as normal, classic swallows (**Figure 1Bi**), while the vast majority of swallow motor patterns had a-typical shapes and temporal sequences. We characterized these atypical swallow patterns as follows: 1) Non-classic swallow, 28%: submental and laryngeal shape, onset, and offset are similar (**Figure 1Bii**). 2) Tonic pre-swallow, 41%: low amplitude tonic activity of the submental and laryngeal complexes during the laser pulse with a swallow immediately following (**Figure 1Biii**). 3) Laryngeal adductor reflex (LAR) + swallow 4%: quick burst of submental and laryngeal complex followed by a short quiescence in activity then a swallow (**Figure 1Biv**). 4) Non-LAR + swallow, 21%: quick burst of submental and laryngeal complex followed by low amplitude activity merging into a swallow (**Figure 1Bv**). A mixed-effects ANOVA detected a significant effect on swallow duration due to stimulation duration (p-value = 0.03), however Tukey's multiple comparisons test revealed no significant differences in swallow behavior duration across stimulation durations (**Figure 1E**). Regardless of the motor pattern, swallow duration is independent of laser pulse duration, each are considered a swallow and will be grouped as swallows for further analysis.

We also observed differences in laryngeal activation when exposed to CIH. Optogenetic stimulation of ChATcre:Vglut2FlpO:ChR2 neurons did not stimulate laryngeal activation in 3 of the 11 mice. In the 8 mice where laryngeal activation was stimulated, a burst of submental complex activity was present during PiCo-stimulated laryngeal activation in 7 mice, while one mouse had a

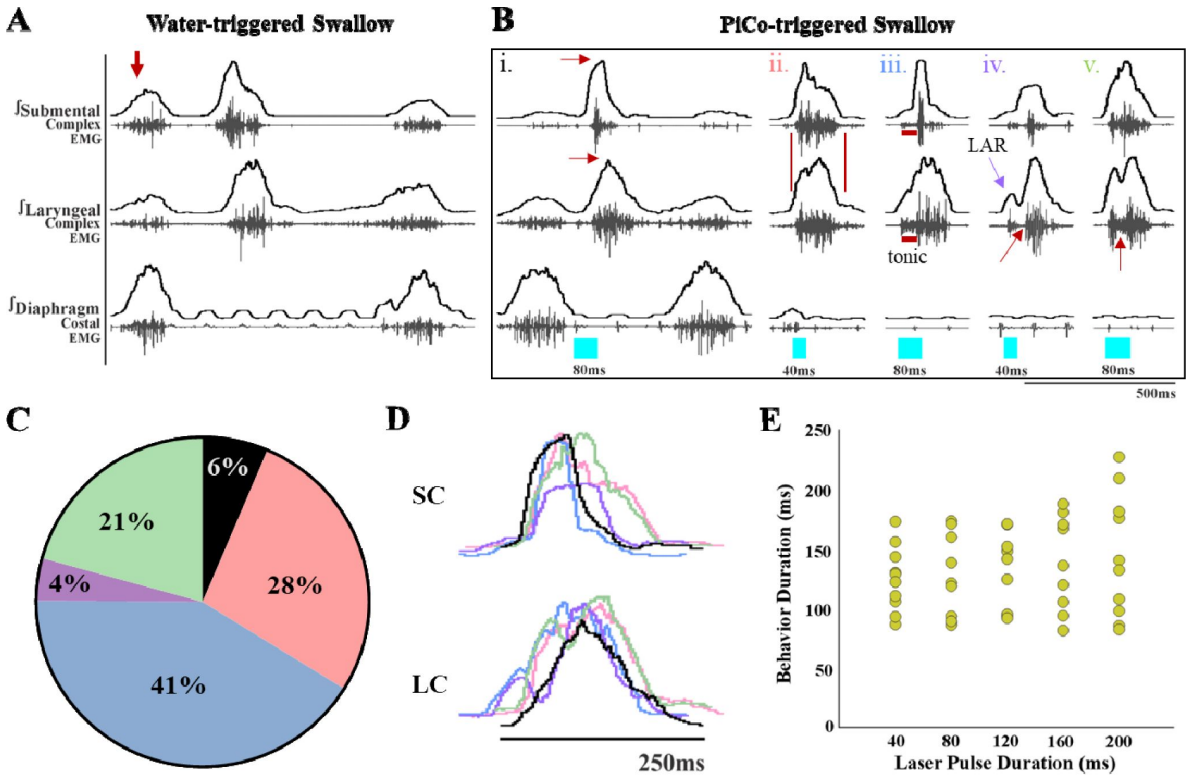

Figure 1

Optogenetic stimulation of PiCo specific ChATcre:Vglut2FlpO:ChR2 neurons triggers variable swallow motor patterns in mice exposed to chronic intermittent hypoxia (CIH). (A) Representative trace of water triggered swallow. (B) Representative traces of PiCo triggered swallows: (Bi) "classic" swallow with the preserved rostrocaudal sequence shown in the red arrows. (Bii) "non-classic" swallow with similar onset, offset and loss of sequence in submental and laryngeal complexes. (Biii) "Tonic" pre-swallow activity with preserved rostrocaudal sequence and low tonic submental and laryngeal activity during the laser pulse, converging into a swallow. (Biv) "Laryngeal Adductor Reflex" (LAR) (blue arrow) followed by a swallow. There is a period of quiescent activity between the LAR and swallow (red arrow). (Bv) "Non-LAR" followed by a swallow. There is an absence of quiescent activity between the laryngeal activity and the swallow (red arrow). (C) Percentage of all PiCo triggered swallows (816 total swallows) in ChATcre:Vglut2FlpO:ChR2 mice. Black is classic, pink is non-classic, blue is tonic, purple is LAR, and green is non-LAR. (D) representative traces of submental complex (SC) and laryngeal complex (LC) from the swallows in panel B with color coding the same as panel C. (E) Scatter plot of behavior duration versus laser pulse duration for swallow in ChATcre:Vglut2FlpO:ChR2 mice (N=11). Each gold dot represents the average duration per mouse.

low amplitude tonic activity (**Figure 2B**). Looking back to the control mice exposed to room air (Huff et al., 2023), only 1 out of 7 mice had a burst of submental complex activity, 4 mice had a low tonic submental complex activity and 2 mice had no submental activity. A mixed-effects ANOVA detected a significant effect on laryngeal activation duration due to stimulation duration (p-value = 0.01) in CIH conditions. Tukey's multiple comparisons test revealed significant differences ($p < 0.05$) between 40ms and 80ms, 120ms, 160ms and 200ms indicating laryngeal activation duration is dependent on laser pulse duration (**Figure 2C**).

Probability of triggering a swallow

We next compared the probability of triggering a swallow between all three genetic types exposed to CIH. There were no PiCo-triggered swallows in 4 out of 14 mice in response to optogenetic stimulation of ChATcre:Ai32. In Vglut2cre:Ai32, no PiCo-triggered swallows in 3 out of 11 mice. However, stimulation of ChATcre:Vglut2FlpO:ChR2 neurons triggered a swallow in all 11 mice.

A two-way ANOVA test revealed a significant difference between temporal characteristics and the genetically defined neuron type (p<0.0001, df= 4, F= 17.37) in ChATcre:Ai32 (N=14), Vglut2cre:Ai32 (N= 11), and ChATcre:Vglut2FlpO:ChR2 (N= 11) when looking at the probability of triggering a swallow (**Figure 3A**). A post-hoc Tukey's multiple comparison test revealed there is no difference in the probability of triggering a swallow between ChATcre:Ai32 and Vglut2cre:Ai32 mice. However, there in an increased probability of triggering a swallow when ChATcre:Vglut2FlpO:ChR2 neurons are activated within 50%, (p= 0.04), 70% (p= 0.03), and 90% (p= 0.02) of the respiratory cycle compared to ChATcre:Ai32. There is also an increased probability of triggering a swallow when ChATcre:Vglut2FlpO:ChR2 neurons are activated at all phases of the respiratory cycle: 10%, (p= 0.03), 30% (p= 0.04), 50% (p= 0.02), 70% (p= 0.02), and 90% (p= 0.01) compared to Vglut2cre:Ai32. Whereas under control conditions, when mice were exposed to room air, we found no significant difference in the probability of triggering a swallow between all three genetic types (Huff et al., 2023). This indicates the importance of further evaluation on all three CIH exposed genetic mouse types.

Probability of triggering laryngeal activation

Optogenetic stimulation of ChATcre:Ai32 and Vglut2:Ai32 neurons stimulated laryngeal activation in all mice exposed to CIH. However, in ChATcre:Vglut2FlpO:ChR2 CIH mice, laryngeal activation was never stimulated in 3 out of 11 mice.

A two-way ANOVA test revealed a significant difference between temporal characteristics and the genetically defined neuron type (p<0.0001, df= 4, F= 31.98) in ChATcre:Ai32, Vglut2cre:Ai32, and ChATcre:Vglut2FlpO:ChR2 CIH-exposed mice with regards to the probability of triggering laryngeal activation (**Figure 3A**). A post-hoc Tukey's multiple comparison test revealed there is no difference in the probability of triggering laryngeal activation between Vglut2cre:Ai32 and ChATcre:Vglut2FlpO:ChR2 CIH-exposed mice. However, there is an increased probability of triggering laryngeal activation when ChATcre:Ai32 neurons are activated within 50%, (p= 0.03), 70% (p= 0.0002), and 90% (p<0.0001) of the respiratory cycle compared to Vglut2cre:Ai32 CIH-exposed mice. There is an increased probability of triggering laryngeal activation when ChATcre:Ai32 neurons are activated at all phases of the respiratory cycle: 10%, (p= 0.03) 30% (p= 0.002), 50% (p= 0.002), 70% (p= 0.01), and 90% (p= 0.05) compared to ChATcre:Vglut2FlpO:ChR2 CIH-exposed mice

PiCo phase-dependent response

Stimulation of PiCo region triggers a swallow or stimulates laryngeal activation in a respiratory phase-dependent manner (Huff et al., 2023) (**Figure 3**). However, this stimulation-evoked phase dependency differs among the three CIH-exposed genetically defined neuron types. A two-way ANOVA test revealed a significant interaction between time and behavior (p<0.0001, df= 4, F=

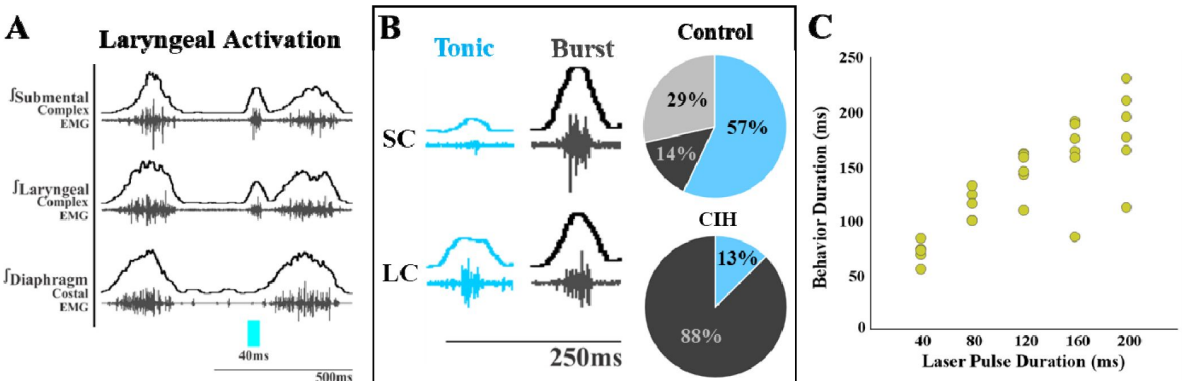

Figure 2.

Optogenetic stimulation of PiCo specific ChATcre:Vglut2FlpO:ChR2 neurons stimulates submental complex burst during laryngeal activation in mice exposed to CIH. (A) Representative traces of PiCo stimulated laryngeal activation with burst pattern submental complex activity. (B) Representative traces of laryngeal activation-related submental complex activity patterns, tonic and burst, and percent of each mouse with the corresponding pattern in control and CIH mice. In control ChATcre:Vglut2FlpO:ChR2 mice, 4 mice had tonic submental complex activity, 1 burst activity and 2 no submental activity (Huff et al., 2023). In CIH exposed 1 mouse had tonic activity and 7 burst submental activity. (C) Scatter plot of behavior duration versus laser pulse duration for laryngeal in ChATcre:Vglut2FlpO:ChR2 CIH exposed mice. Each gold dot represents the average duration per mouse.

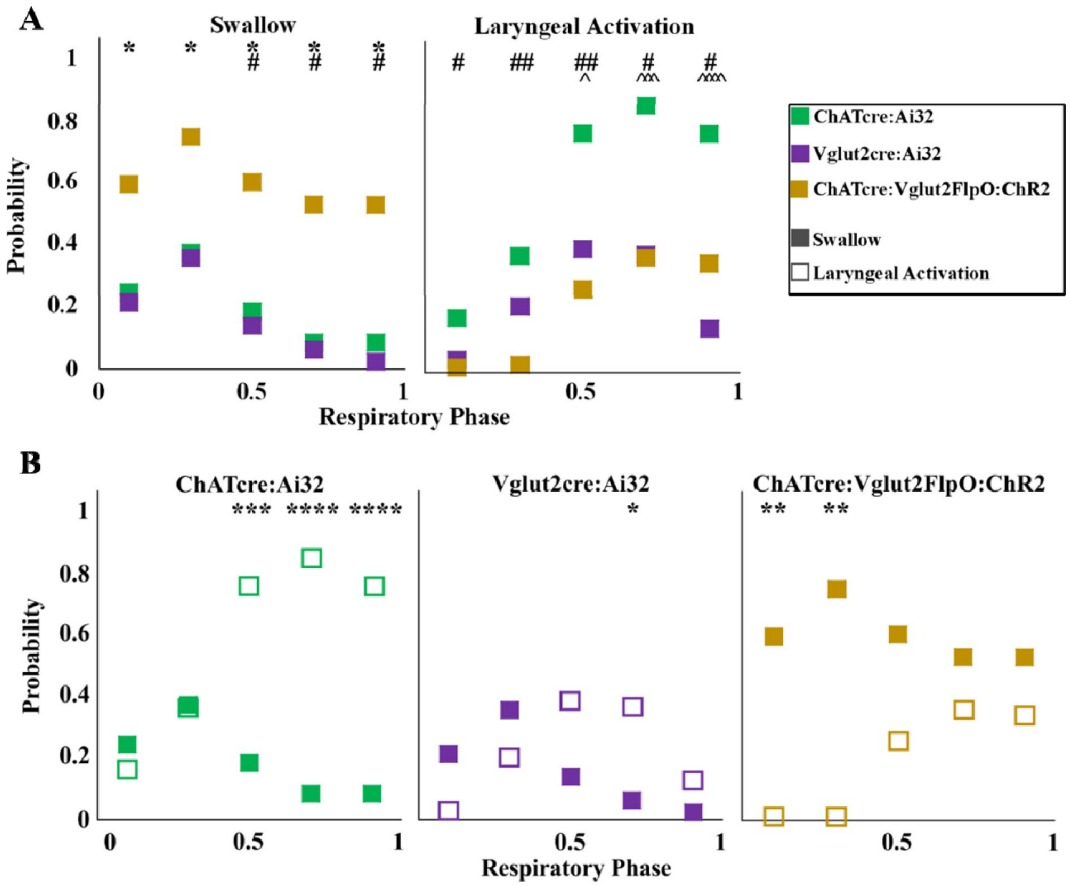

Figure 3.

Optogenetic stimulation of PiCo neurons in mice exposed to CIH regulates swallow and laryngeal activation in a phase-specific manner. A) Scatter plot of the probability of triggering a swallow (left) or laryngeal activation (right) across the respiratory phase (0 start of inspiration, 1 start of next inspiration) in ChATcre:Ai32 mice (green) Vglut2cre:Ai32 mice (purple), and ChATcre:Vglut2FlpO:ChR2 mice exposed to CIH (gold). * Indicates significant difference in probability between Vglut2cre:Ai32 and ChATcre:Vglut2FlpO:ChR2, # Indicates significant difference in probability between ChATcre:Ai32 and ChATcre:Vglut2FlpO:ChR2, and ^ Indicates significant difference in probability between ChATcre:Ai32 and Vglut2cre:Ai32. (B) Scatter plot of the probability of triggering a swallow (closed square) versus laryngeal activation (open square) in all three genetic types exposed to CIH.

10.99) in ChATcre:Ai32, Vglut2cre:Ai32, and ChATcre:Vglut2:ChR2 mice (**Figure 3B**). A post-hoc Tukey's multiple comparison test revealed laryngeal activation is stimulated with a significantly higher probability when CIH-exposed ChATcre:Ai32 neurons are activated at 50% (p= 0.0005), 70% (p<0.0001), and 90% (p<0.0001) of the respiratory cycle. Laryngeal activation is stimulated with a significantly higher probability when CIH-exposed Vglut2cre:Ai32 neurons are activated at 70% (p= 0.03) of the respiratory cycle. However, swallow is triggered with a significantly higher probability when CIH-exposed ChATcre:Vglut2FlpO:ChR2 neurons are activated within the first 10% (p= 0.004) and 30% (p= 0.001) of the respiratory cycle.

Respiratory response to optogenetic stimulation of PiCo

We divided PiCo stimulated responses into either swallow or non-swallow (**Figure 3-figure supplement 1**). Stimulation of PiCo neurons that resulted in either laryngeal activation or in a "no-motor response" were considered non-swallows. Using a Pearson correlation and simple linear regression, the correlation coefficient (r, **Figure 3-figure supplement 1A**) and line of best fit (slope, **Figure 3-figure supplement 1B**), respectively, was calculated for each CIH exposed genetic mouse type and response to determine the degree of correlation between behavior response and reset of the respiratory rhythm. This test reveals that there is a high degree of correlation between shifting or delaying the following inspiratory burst and triggering a swallow when stimulating ChATcre:Ai32 (r = 0.76, p< 0.0001, slope= 0.75), Vglut2cre:Ai32 (r = 0.71, p< 0.0001, slope= 0.82) and, ChATcre:Vglut2FlpO:ChR2 (r = 0.79, p< 0.0001, slope= 0.79) CIH-exposed mice. This suggest that triggering a swallow in each genetic type has a strong effect on resetting the respiratory rhythm in CIH conditions.

We found a moderate degree of correlation between the following inspiratory burst and non-swallows stimulated in ChATcre:Ai32 (r = 0.36, p< 0.0001, slope= 0.16), and a low degree of correlation in Vglut2cre:Ai32 (r = 0.22, p< 0.0001, slope =0.17) and ChATcre:Vglut2FlpO:ChR2 (r = 0.28, p= 0.0001, slope= 0.18) mice. This suggests that triggering a swallow has a stronger effect on resetting the respiratory rhythm than activating non-swallows in all the genetic mouse types exposed to CIH.

Swallow-related characteristics in water-triggered and PiCo-triggered swallows

Figure 1A depicts the swallow motor patterns of a water-evoked swallow and 1B of various swallow motor patterns of PiCo-evoked swallow. A mixed-effect ANOVA revealed no significant difference in swallow onset relative to inspiratory onset (**Figure 1-figure supplement 1A**) and swallow onset relative to inspiratory peak (**Figure 1-figure supplement 1B**) between PiCo-evoked and water-evoked swallows in CIH-exposed mice. We were unable to perform a repeated measures ANOVA due to swallows not being triggered by PiCo stimulation in some mice, as mentioned above. All water- and PiCo-triggered swallow-related characteristics in all three CIH-exposed genetic mouse lines are reported in **Table S1**.

PiCo-triggered swallows are characterized by a significant decrease in duration compared to swallows evoked by water in ChATcre:Ai32 (265 ± 132ms vs 144 ± 101ms; paired t-test: p= 0.0001, t= 5.21, df= 8), Vglut2cre:Ai32 (308 ± 184ms vs 125 ± 44ms; paired t-test: p= 0.0003, t= 6.46, df= 7), and ChATcre:Vglut2FlpO:ChR2 (230 ± 67ms vs 130 ± 35ms; paired t-test: p= 0.0005, t= 5.62, df= 8) mice exposed to CIH (**Table S1**).

PiCo-triggered swallows have a significant decrease in submental amplitude compared to swallows evoked by water in ChATcre:Ai32 (91 ± 7 vs 38 ± 35 % of max; paired t-test: p= 0.002, t= 4.91, df= 7), Vglut2cre:Ai32 (84 ± 10 vs 45 ± 32 % of max; paired t-test: p= 0.006, t= 3.84, df= 7), and ChATcre:Vglut2FlpO:ChR2 (88 ± 10 vs 39 ± 22 % of max; paired t-test: p= 0.001, t= 7.47, df= 8) CIH-exposed mice (**Table S1**).

Sex-specific differences in swallows triggered by optogenetic stimulation of PiCo region

All water- and PiCo-triggered sex-specific swallow-related characteristics in all three CIH-exposed genetic mouse lines are reported in **Table S2-4**. In ChATcre:Ai32 female mice, PiCo-triggered swallow onset relative to inspiratory onset occurs later in the respiratory cycle (0.31 ± 0.04 vs 0.37 ± 0.04; paired t-test: p= 0.04, t= 2.38, df= 8). There are no sex-specific differences in PiCo-triggered swallows in Vglut2cre:Ai32 or ChATcre:Vglut2FlpO:ChR2 CIH-exposed mice.

Neuroanatomy of PiCo transfection

Post-hoc histological analysis was performed in the double conditioned ChATcre:Vglut2FlpO:ChR2 mouse to check the transfection of PiCo neurons after injection of the pAAV-hSyn Con/Fon hChR2(H134R)-EYFP vector (ChATcre:Vglut2FlpO:ChR2) (**Figure 4**). NAmb cholinergic neurons had no transfection and the rostrocaudal distribution of the transgene-expressing neurons was analyzed. Eleven ChATcre:Vglut2FlpO:ChR2 mice were stimulated and triggered a swallow, while laryngeal activation was triggered in only 8 mice. In all 11 CIH-exposed mice we found that 155 ± 9 neurons expressed EYFP (**Figure 4**). There was a high bilateral transfection of ChR2 in PiCo neurons ranging from 121-189 neurons with one animal having 88 neurons. The animal with 88 neurons did not appear to have a different optogenetic response compared to the other animals.

Discussion

In mice, and other mammals including humans, exposed to room air, swallow related muscles follow a stereotypic, rostro-caudal sequential muscle pattern activation (Basmajian & Dutta, 1961; Doty & Bosma, 1956; Ertekin & Aydogdu, 2003; Pitts & Iceman, 2023; Thexton et al., 2007). The activation pattern must be precisely timed and shaped to guarantee that food and liquid particles are guided into the esophagus and ultimately the digestive system, rather than the lungs. Disturbances in any aspect of the swallow patterning could lead to aspiration resulting in the penetration of food or liquid through the vocal folds, entering the lungs. In the long-term, aspiration may lead to aspiration pneumonia. We found that after exposure to CIH only 6% of swallows triggered by optogenetic stimulation of PiCo, specifically the ChATcre:Vglut2FlpO:ChR2 neurons, followed this classic swallow motor pattern (**Figure 1Bi**), while 28% of PiCo-triggered swallows lost important characteristics of the sequential motor activation crucial for proper bolus transport (Pitts & Iceman, 2023). In these “non-classic swallows” the submental and laryngeal muscles were activated simultaneously, which could impair the effective transport of food/liquid from the oral cavity into the esophagus and digestive system. Stimulation of glutamatergic/cholinergic neurons also induced “tonic pre-swallow” activity of both the submental and laryngeal complexes prior to triggering a swallow in 41% of PiCo-triggered swallows. While fine wire EMG studies are an excellent evaluation tool to observe temporal motor pattern of sequential swallow related muscles; it must be combined with tools such as videofluoroscopic swallow study (VFSS) and/or high resolution manometry (HRM) in order to characterize the functional significance of these alterations to the swallow motor pattern shown in this study (Park et al., 2017). Since the preparation in this study utilizes only fine wire EMGs we are not able to evaluate or comment on the functional significance of the variable swallow motor patterns.

While we do not intend to make direct quantitative comparisons between the previously published PiCo-triggered swallows in control mice exposed to room air (Huff et al., 2023) and the data presented here for mice exposed to CIH, we believe it is important to compare the conclusions made in these two studies. There is a higher probability of triggering a swallow when PiCo is activated during inspiration or immediately after during postinspiration. Whereas laryngeal activation is more probable when PiCo is activated further into expiration. This remains

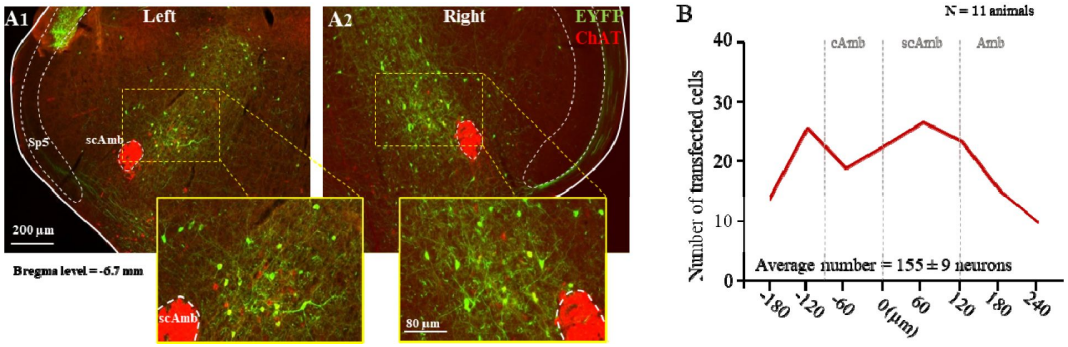

Figure 4.

Transfection of cholinergic/glutamatergic neurons in PiCo in ChATcre:Vglut2FlpO:ChR2 CIH exposed mice. (A) Transverse hemisection through Bregma level (−6.7 mm) of the transfected neurons into PiCo bilaterally, left (A1) and right (A2), with the pAAV-hSyn Con/Fon hChR2(H134R)-EYFP vector. (B) Rostrocaudal distribution of the total number of transfected neurons counted 1:2 series of 25 µm sections into PiCo of 11 animals with an average of 155 ± 9 SEM neurons. Abbreviations: Amb, nucleus ambiguus; cAmb, nucleus ambiguus pars compacta; scAmb, nucleus ambiguus pars semi-compacta.

true for both ChATcre:Vglut2FlpO:ChR2 control mice exposed to room air (Huff et al., 2023) and mice exposed to CIH (**Figure 3**). However, there is a decreased probability of PiCo-triggered swallows in ChATcre:Ai32 and Vglut2:Ai32 exposed to CIH, unlike the mice exposed to room air. We observed a high degree of correlation between shifting or delaying the following inspiratory burst and triggering a swallow in both control mice (Huff et al., 2023) and mice exposed to CIH (**Figure 3-figure supplement 1**). We also observed no differences between the water-triggered swallow onset and PiCo-triggered swallow onset in relation to inspiration onset and peak inspiration (**Figure 1-figure supplement 1**). Thus, CIH does not alter PiCo's ability to coordinate swallow and breathing. Rather, our data reveal CIH disrupts the swallow motor sequence which is likely due to changes in the interaction between PiCo and the SPG, presumably located in the cNTS.

While it has previously been demonstrated that PiCo is an important region in swallow-breathing coordination (Huff et al., 2023), previous studies did not demonstrate that PiCo is involved in swallow motor patterning itself. Here we show for the first time that CIH leads to disturbances in the generation of the swallow motor pattern that is activated by stimulating PiCo. This suggests that PiCo is not only important for coordinating swallow and breathing, but also modulating swallow motor patterning. Further studies are necessary to directly evaluate the presumed interactions between PiCo and the cNTS.

Variability to swallow motor pattern

Previously we suggested that the PiCo-evoked laryngeal activation could be a central and integral component to the laryngeal adductor reflex (LAR) (Huff et al., 2023). However, there had been no reports of centrally evoked LAR or an LAR independent of mechanical, electrical, or chemical peripheral stimulation or swallow. Here we show, for the first time, optogenetic stimulation of PiCo, in mice exposed to CIH, triggers swallow-related LAR (**Figure 1Biv**). The afferent limb of the LAR is governed by the internal branch of the superior laryngeal nerve (SLN) where sensory impulses travel through the nodose ganglion and terminate on the cNTS (Ambalavanar et al., 2004; Sessle, 1973). It is likely that both swallow and LAR involves interneurons of the solitarius-ambiguus pathway, possibly activated by PiCo stimulation (Ambalavanar et al., 2004; Mifflin, 1993).

In addition to the classic LAR response, PiCo stimulation also triggered non-LAR swallows (**Figure 1Bv**). To be considered an LAR there must be a quiescence period of laryngeal activity between the reflex and the swallow (Ambalavanar et al., 2004; Ludlow et al., 1992). In these swallows there was a convergence of the initial laryngeal peak and augmenting swallow-related laryngeal activity. The functional merging of these two activity patterns is not understood. It has been shown that excessive or inappropriate laryngeal activity could lead to functional disorders or life-threatening conditions (Sun et al., 2011), such as obstructive apnea, laryngospasm, spasmodic dysphonia, asphyxia, sudden infant death syndrome (SIDS) and aspiration pneumonia (Ikari & Sasaki, 1980; Ludlow et al., 1995; Thompson et al., 2005; Wang et al., 2016). While we are unable to evaluate the functional significance of the variable swallow motor patterns triggered by PiCo in this study, we previously reported CIH causes a disruption in swallow motor pattern with a delay in swallow-related laryngeal activation. This same delay was also generated when preBötC Dbx1 neurons were stimulated (Huff et al., 2022).

The known rostrocaudal swallow motor sequence can be modulated due to changes in sensory feedback (King et al., 2020). However, here we are stimulating a central microcircuit, believed to not activate sensory components of the SPG (Huff et al., 2023), which induces great variability to this motor sequence when exposed to CIH. Our study indicates that PiCo neurons are highly integrated with the overall swallow motor pattern. Variability in response to CIH on swallow motor pattern is reminiscent to the increased variability also seen in the generation of the respiratory motor pattern (Garcia et al., 2017; Garcia et al., 2016). To understand the complex disruption of CIH on the swallow motor pattern, it is important to note that we are measuring changes in two muscle complexes which spread among three motor neuron pools: hypoglossal

nucleus, trigeminal nucleus, and nucleus ambiguus (Badran et al., 2005; Bieger & Neuhuber, 2006; Kemplay & Cavanagh, 1983; Razlan et al., 2018). There was no statistical difference in the probability of triggering a swallow during optogenetic stimulation of ChATcre:Ai32, Vglut2cre:Ai32 and ChATcre:Vglut2FlpO:ChR2 neurons in mice exposed to room air (Huff et al., 2023). However, when exposed to CIH, ChATcre:Ai32 and Vglut2:Ai32 mice have a lower probability of triggering a swallow -- in some mice swallow was never triggered via PiCo activation, while water-triggered swallows remained – compared to the ChATcre:Vglut2FlpO:ChR2 mice. While it is possible that portions of the presumed SPG remain less affected by CIH, which could offset these instabilities to produce functional swallows, our data suggest that PiCo targets microcircuits within the SPG that are highly affected by CIH. The NTS is a primary first site for upper airway and swallow-related sensory termination in the brainstem (Jean, 1984). CIH induces changes to the cardio-respiratory Vglut2 neurons, resulting in an increase in cNTS neuronal activity (Kline, 2010; Kline et al., 2007), as well as changes to preBötzinger complex neurons (Garcia et al., 2017; Garcia et al., 2016) and ChAT neurons in the basal forebrain (Tang et al., 2020). It is reasonable to suggests that CIH has differential effects on neurons that only express ChATcre and Vglut2cre versus the PiCo-specific interneurons that co-express ChATcre and Vglut2FlpO, emphasizing the importance of targeting and manipulating these PiCo-specific interneurons.

Variability to laryngeal activation motor pattern

Acute bouts of extreme hypoxia reduce the excitability of laryngeal adductor neurons, suggesting a “fail-safe” mechanism that in the presence of hypoxia, LAR is prevented (Ikari & Sasaki, 1980). However, acute bouts of intermittent hypoxia elicits long-term facilitation of the recurrent laryngeal nerve, but does not recruit additional postinspiratory laryngeal muscle activity or augmented LAR (Bautista et al., 2012). After exposure of CIH, we observed a shift in the dominance of “laryngeal activation” submental complex motor activity pattern. Under control conditions optogenetic stimulation of ChATcre:Vglut2FlpO:ChR2 neurons resulted in laryngeal activation with 3 types of submental activation: tonic, burst, and no activation; with the majority of mice having tonic submental activation (**Figure 2B**). However, when exposed to CIH, 7 out of 8 mice had burst submental activation. This is consistent with the known increased activity of pharyngeal dilator muscles in OSA patients (Mezzanotte et al., 1992; Saboisky et al., 2012) and in CIH animal models (Kubin, 2019). This could constitute as a compensatory mechanism to keep the airway patent (Dempsey et al., 2010).

Limitations

The use of anesthesia continues to be an unavoidable limitation for this preparation. There were 2 mice that did not swallow in response to water stimulation, most likely due to anesthetic depth. In this preparation we are unable to directly determine the functionality of the variable swallow motor patterns seen after CIH. Different experimental techniques, such as videofluoroscopy would need to be used to directly evaluate functional significance. This technique is beyond the scope of this study and not possible to perform in this preparation. We acknowledge this limits our ability to make direct comparisons between dysphagic swallows in OSA patients. In addition, this preparation does not allow for recording of PiCo neurons to evaluate the direct effects of CIH in PiCo neuronal activity. These limitations are a trade-off for the unique advantages of manipulating specific genetically defined neuron types under *in vivo* conditions to assess neuronal alterations in OSA related dysphagia.

Conclusion

Clinical dysphagia is typically seen in OSA, and other disorders associated with CIH. OSA related dysphagia has been characterized as a delayed swallow reflex, decreased time between swallow and initiation of the next inspiration, and disruption of the pharyngeal phase of swallow (Levring Jaghagen et al., 2003; Teramoto et al., 1999; Valbuza et al., 2011). Using an *in vivo* mouse model we recently described that CIH delays swallow-related laryngeal activation which increases the risk for foreign materials to enter into the airway (aspiration) instead of the esophagus (Huff et al., 2022). Here we show that PiCo, a neuronal network which is critical for the generation of postinspiratory activity (Andersen et al. 2016) and implicated in the coordination of swallowing and breathing (Huff, 2023), is severely affected by CIH. Stimulating PiCo-specific interneurons after CIH exposure evoked swallows characterized by abnormal swallow motor patterns, while the swallow-breathing coordination was relatively unaffected. The severe disruption in the precise temporal sequences of swallows evoked by PiCo stimulation after CIH exposure has important implications for understanding the mechanisms underlying dysphagia. Our data suggest that PiCo is not only a neuronal structure critical for regulating postinspiratory and swallow activity, but also critical for the patterning of swallow itself. To the best of our knowledge, no microcircuit has previously been identified that can directly impact the swallow motor pattern itself. Swallows are characterized by a very robust, yet complex stereotypic motor sequence that is typically triggered in an all-or-none manner. The strict temporal sequence of this motor pattern is critical for allowing the physiological transport of food to reach the digestive system. Any disturbance in this sequence has detrimental consequences including aspiration, the leading cause of death in many neurodegenerative diseases. Our study also suggests that OSA related dysphagia, may not primarily be due to swallow-breathing discoordination, but rather involve a central nervous dysfunction of the swallow pattern and laryngeal activation.

Methods

Animals

Adult (P51-148, average P76) male and female mice were bred at Seattle Children's Research Institute (SCRI) and used for all experiments. Vglut2-IRES-cre and ChAT-IRES-cre homozygous breeder lines were obtained from Jackson laboratories (stock numbers 028863 and 031661, respectively). Cre mice were crossed with homozygous mice containing a floxed STOP channelrhodopsin-2 fused to an EYFP (Ai32) reporter sequence from Jackson laboratories (stock number 024109). Vglut2-IRES-cre crossed with Ai32 will be reported as Vglut2:Ai32 and the ChAT-IRES-cre crossed with Ai32 as ChAT:Ai32. ChAT-IRES-cre, and Vglut2-IRES2-FlpO-D, *technically known as* 129S-Slc17a6$^{tm1.1(flpo)Hze}$/J were obtained from Jackson Laboratories (#031661 and #030212, respectively). To generate double-transgenic mice, the ChATcre and Vglut2FlpO strains were interbred to generate compound homozygotes expressing both ChATcre$^{(+/+)}$ and Vglut2FlpO$^{(+/+)}$ and will be reported as ChATcre:Vglut2FlpO. Mice were randomly selected from the resulting litters by the investigators. Offspring were group housed with ad libitum access to food and water in a temperature controlled (22 $\pm$ 1°C) facility with a 12h light/dark cycle. All experiments and animal procedures were approved by the Seattle Children's Research Institute's Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health guidelines.

Brainstem injection of AAV

For the AAV injections, we target the PiCo neurons, as previously described (Anderson et al., 2016; Huff et al., 2023), and also confirmed by the present results (**Figure 4**). We restricted ChR2 expression to the PiCo region in order to transfect and photo-stimulate the region with the highest density of ChATcre:Vglut2FlpO neurons in PiCo region (Anderson et al., 2016). For AVV

injection, the mice were anesthetized with isoflurane (2%). The correct plane of anesthesia was assessed by the absence of the corneal and hind-paw withdrawal reflexes. Mice received postoperative ketoprofen [7 mg/kg, subcutaneous (s.c.)] for two consecutive days. All surgical procedures were performed under aseptic conditions. The hair over the skull and neck were removed and skin disinfected. The mice were then placed prone on a stereotaxic apparatus (bite bar set at −3.5 mm for flat skull; David Kopf Instruments Tujunga, CA, USA). A 0.5 mm diameter hole was drilled into the occipital plate on both sides caudal to the parieto-occipital suture. Viral solutions were loaded into a 1.2 mm internal diameter glass pipette broken to a 20 µm tip (external diameter). To target the PiCo region with ChR2-AAV, the pipette was inserted in the brainstem in the following coordinates: 4.8 mm below the dorsal surface of the cerebellum, 1.1 mm lateral to the midline and 1.6 mm caudal to the lambda and bilateral injections of 150 nL were made slowly at 50 nL/min, using a glass micropipette and an automatic nanoliter injector (NanoinjectII, Drummond Scientific Co. Broomall, PA). The mouse was allowed to recover for 3 days before beginning the chronic intermittent hypoxia protocol (see below).

The mouse strain containing ires-cre and ires-FlpO in $ChAT^+$ and $Vglut2^+$ respectively, had successful transfection of PiCo neurons by using a pAAV-hSyn Con/Fon hChR2(H134R)-EYFP adenovirus vector (cat# 55645-AAV8; AddGene, USA; abbreviated as AAV8-ConFon-ChR2-EYFP) herein named ChATcre:Vglut2FlpO:ChR2 in this study. This AAV is a cre-on/FlpO-on ChR2-EYFP under the synapsin promoter and encoded the photoactivatable cation channel channelrhodopsin-2 (ChR2, H134R) fused to EYFP. The vector was diluted to a final titer of 1 x 10^{13} viral particles/ml with sterile phosphate-buffered saline.

Chronic Intermittent Hypoxia

Mice of the ChATcre:Ai32, Vglut2cre:Ai32 and ChATcre:Vglut2FlpO:ChR2 were kept in collective cages with food and water ad libitum placed inside custom built chambers (volume: 185 liters) equipped with gas injectors as well as oxygen (O_2) sensors (Oxycycler, Huff Technologies Inc.). One chamber was used for CIH and the other for control. The CIH group was exposed to intermittent episodes of hypoxia, continuous injection of nitrogen (N_2) for 60 seconds, in order to reduce the percentage of inspired O_2 inside the chamber from 21% to 4.5-5%. Then continuous injection of compressed air for 5 minutes into the chamber to return the percentage of O_2 to 21% before the start of a new hypoxia cycle. Compressed air and N_2 injection into the chambers were regulated by a valve system, automatically operated by customized software (Oxycycler, Huff Technologies Inc.). This protocol was repeated with 80 bouts per day (8 hours) during the light cycle in a 12hr light/dark cycle room, for an average of 21 days. Of note the range was 10-29 days, but internal analysis of pilot 10 day protocol showed no difference from the 21 day protocol and were combined in this study. In the remaining 16 hours, the mice were kept under normoxia condition (21% O_2). Control mice were kept in a replicated chamber under normoxic conditions (21% O_2), 24 hours a day during the same duration as the CIH protocol (**Figure 1-figure supplement 2**). The mice under control conditions has been published (Huff et al., 2023).

In Vivo Experiments

The same experimental protocol was performed for all Vglut2cre:Ai32, ChATcre:Ai32, ChATcre:Vglut2FlpO:ChR2 mice. Adult mice were initially anesthetized with 100% O_2 and 1.5% Isoflurane (Aspen Veterinary Resources Ltd, Liberty, MO, USA) for 2-3 minutes in an induction chamber. Once the breathing slowed, they were injected with Urethane (1.5 g/kg, i.p. Sigma Aldrich, St. Louis, MO, USA) and secured supine on a custom surgical table. Core temperature was maintained through a water heating system (PolyScience, Niles, IL, USA) built into the surgical table. Mice were then allowed to spontaneously breathe 100% O_2 for the remainder of the surgery and experimental protocol. Adequate depth of anesthesia was determined via heart and breathing rate, as well as lack of toe pinch response every 15 minutes. A supplemental dose of 0.01mL of Urethane was given to maintain adequate anesthetic depth, when necessary. Bipolar electromyograms (EMG) electrodes were placed in the costal diaphragm to monitor respiratory

rate and heart rate throughout the experiment. The trachea was exposed through a midline incision and cannulated caudal to the larynx with a curved (180 degree) tracheal tube (PTFE 24 G, Component Supply, Sparta, TN, USA). The hypoglossal (XII) and vagus (X) nerves were then dissected followed by cannulation of the trachea. The recurrent laryngeal nerve (RLN) was carefully dissected away from each side of the trachea before the cannula was tied in and sealed with super glue to ensure no damage to the RLN. The trachea and esophagus were then cut to detach the rostral end of the trachea just caudal to the cricoid cartilage, preserving the arytenoids and bilateral RLN. A tube filled with 100% O_2 was attached to the cannulated trachea to provide supplemental oxygen throughout the experiment. Continuing in the supine position, the occipital bone was removed, followed by continuous perfusion of the ventral medullary surface with warmed (~36°C) artificial cerebral spinal fluid (aCSF; in mM: 118 NaCl, 3 KCl, 25 $NaHCO_3$, 1 NaH_2PO_4, 1 $MgCl_2$, 1.5 $CaCl_2$, 30 D-glucose) equilibrated with carbogen (95% O_2, 5% CO_2) by a peristaltic pump (Dynamax RP-1, Rainin Instrument Co; Emeryville CA, USA). As previously published (Figure. 6a (Huff et al., 2022)), the XII and X nerves were isolated unilaterally, cut distally, and their activity was recorded from a fire-polished pulled borosilicate glass tip (B150-86-15, Sutter Instrument; Novato, CA, USA) filled with aCSF connected to the monopolar suction electrode (A-M Systems, Sequim, WA, USA) and held in a 3D micromanipulator (Narishige, Tokyo, Japan). Multiple bipolar EMGs, using 0.002" and 0.003" coated stainless steel wires (A-M Systems, Sequim, WA, USA, part no.790600 and 791000 respectively), simultaneously recorded activity from several swallow and respiratory-related muscle sites. According to the techniques of Basmajian and Stecko (Basmajian & Stecko, 1962), the electrodes were placed using hypodermic needles 30G (part no 305106, BD Precision Glide ™, Franklin Lakes, NJ, USA) in the *submental complex*, which consists of the geniohyoid, mylohyoid and anterior digastric muscles, to determine swallow activity. The *laryngeal complex,* consisting of the posterior cricoarytenoid, lateral, transverse and oblique arytenoid, cricothyroid and thyroarytenoid muscles, to determine laryngeal activity during swallow, as well as postinspiratory activity (**Figure 1-figure supplement 2C**). The *costal diaphragm*, used to measure the multifunctional activity for both inspiration, as well as *Schluckatmung*, a less common diaphragmatic activation during swallow activity. Glass fiber optic (200 um diameter) connected to a blue (447 nm) laser and DPSS driver (Opto Engine LLC, Salt Lake City, Utah, USA) was placed bilaterally in light contact with the ventral surface of the brainstem overtop of the predetermined PiCo (Anderson et al., 2016) (**Figure 1-figure supplement 2**). At the end of the experiment mice were euthanized by an overdose of anesthetic followed by rapid decapitation or trans-cardial perfusion (see histology section below).

Stimulation protocols

First, swallow was stimulated by injecting 0.1cc of water into the mouth using a 1.0 cc syringe connected to a polyethylene tube. Second, 25 pulses of each 40ms, 80ms, 120ms, 160ms and 200ms continuous TTL laser stimulation at PiCo was repeated, at random, throughout the respiratory cycle. The lasers were each set to 0.75mW and triggered using Spike2 software (Cambridge Electronic Design, Cambridge, UK). These stimulation protocols were performed in all ChATcre:Ai32, Vglut2cre:Ai32, and ChATcre:Vglut2FlpO:ChR2.

Analysis

All electroneurogram (ENG) and EMG activity were amplified and band-pass filtered (0.03 – 1 KHz) by a differential AC Amplifier (A-M System model 1700, Sequim, WA, USA), acquired in an A/D converter (CED 1401; Cambridge Electronic Design, Cambridge, UK) and stored using Spike2 software (Cambridge Electronic Design, Cambridge, UK). Using the Spike2 software, data was further processed using a band pass filtered (200-700Hz, 40Hz transition gap) then rectified and smoothed (20ms). Using the Spike2 software, the ECGdelete 02.s2s script is used to remove heart artifact, when present, from the ENG and EMG recordings.

We evaluated swallows that were trigged by injection of water into the mouth as well as behaviors in response to laser stimulation applied to the PiCo region: swallow, laryngeal activation and no motor response. Swallow was characterized as a delayed response to the laser outlasting and independent of the laser duration, activation of XII, X, submental and laryngeal complex. Diaphragm activity during PiCo-triggered swallows (*schluckatmung*) was present in some animals but not all. Laryngeal activation was characterized as activity of the XII, X, and laryngeal complex dependent on laser pulse duration, and absence of the diaphragm EMG activity. The submental complex was active in a tonic or burst pattern during laryngeal activation. No response was characterized as lack of motor response to the laser and was grouped with laryngeal activation for the non-swallow analysis in respiratory phase shift plots (**Figure 3-figure supplement 1**). Previously published swallow-related parameters were used to look at swallow-breathing characteristics (Fig. 6 in Huff et al. (2022)) *Swallow duration* was determined by the onset to the termination of the submental complex EMG activity. In the case the submental complex muscles were not available then it was determined by the onset to the termination of the XII ENG activity. *Swallow sequence* was calculated as the time difference between the peak activation of the laryngeal and submental complex EMG activity. *Schluckatmung* duration was determined by the onset to the termination of the diaphragm EMG activity during a swallow. *Laryngeal activation duration* was determined by the onset to the termination of the laryngeal complex EMG activity. *Diaphragm inter-burst interval* was calculated as the offset of the diaphragm EMG activity to the onset of the subsequent breath. *Inspiratory delay* was calculated as the offset of the swallow-related laryngeal complex EMG activity to the onset of the subsequent breath. Duration and amplitude of each nerve and muscle was determined by the onset to the termination of that respective nerve/muscle activity during swallow. All durations are reported in milliseconds (ms) and all amplitudes are reported as a "% of max" calculated as the % of the maximum baseline (water swallow) amplitude.

As previously reported (Fig. 6d Huff et al. (2022)), respiratory phase reset curves calculated by defining the respiratory cycle as the onset of the diaphragm to the onset of the subsequent diaphragm activity. The *phase shift* elicited by each stimulation of water was calculated as the duration of the respiratory cycle containing the stimulus, divided by the preceding respiratory cycle. The phase of the swallow stimulation *(respiratory phase)* was calculated as the time between the onset of the inspiration (diaphragm) and the stimulus onset, divided by the expected phase. The average phase shift was then plotted against the respiratory phase in bins containing 1/10 of the expected phase (Baertsch et al., 2018). Line graphs of swallow frequency in relation to inspiration were created by the phase of breathing in which swallow occurred in, calculated as the onset of inspiration to the onset of swallow divided by the respiratory cycle duration and plotted against the number of swallows that occurred within the 1/10 binned respiratory phase, (*swallow onset: insp onset)*. Swallow was also plotted in relation to the peak activation of the diaphragm as a duration with zero equaling the peak of the inspiratory related diaphragm activity, (*swallow onset: insp peak)*.

Probability plots were calculated by assigning a "0" to the no response behavior or a "0 or 1" to the laryngeal activation or swallow behavior. These numbers were then averaged and plotted against the *respiratory phase* and binned to 1/10 of the respiratory phase.

All data are expressed as mean ± standard deviation (SD), unless otherwise noted. Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software®, Inc. La Jolla, USA). Differences were considered significant at $p \leq 0.05$. Investigators were not blinded during analysis. Sample sizes were chosen on the basis of previous studies.

Histology

At the end of experiments, animals were deeply anesthetized with 5% isoflurane in 100% oxygen and perfused through the ascending aorta with 20 ml of phosphate buffered saline (PB; pH 7.4) followed by 4% phosphate-buffered (0.1 M; pH 7.4; 20 ml) paraformaldehyde (Electron Microscopy

Sciences, Fort Washington, PA). The brains were removed and stored in the perfusion fixative for 4 h at 4 °C, followed by 20% sucrose for 8h. Series of coronal sections (25 µm) from the brains were cut using a cryostat and stored in cryoprotectant solution at −20°C (20% glycerol plus 30% ethylene glycol in 50 ml phosphate buffer, pH 7.4) prior to histological processing. All histochemical procedures were done using free-floating sections.

Choline acetyltransferase (*ChAT*) was detected using a polyclonal goat anti-ChAT antibody (AB144P; Millipore; 1:100) and EYFP was detected using a polyclonal mouse anti-GFP (06-896, Millipore; 1:1000) diluted in PB containing 2% normal donkey serum (017-000-121, Jackson Immuno Research Laboratories) and 0.3% Triton X-100, and incubated for 24 h. Sections were subsequently rinsed in PB and incubated for 2 h in an Alexa 594 donkey anti-goat (705-585-003; 1:250; Jackson Immuno Research Laboratories) and Alexa 488 donkey anti-mouse (715-545-150; 1:250; Jackson Immuno Research Laboratories). For all secondary antibodies used, control experiments confirmed that no labeling was observed when primary antibodies were omitted. The sections were mounted on slides in a rostrocaudal sequential order, dried, and covered with fluoromount (00-4958-02; Thermo Fisher). Coverslips were affixed with nail polish.

Sections were also examined to confirm the transfected cells. Section alignment between specimens was done relative to a reference section. The rostral segment of PiCo was identified by the last section with the caudal end of the facial motor neurons and the first section with the rostral portion of the inferior olives. To distinguish PiCo in each section, we used the nucleus ambiguus (Amb), the inferior olives (IO) and the ventral spinocerebellar tract (vsc) as the main anatomic structures. The section that contains the rostral portion of Amb (more densely packed, i.e. cAmb) is the section that contains the rostral portion of PiCo, in a caudal direction, the compacta portion of Amb turns into a semi-compacta portion (scAmb), being aligned as the zero point in the rostral-caudal graphs. Further caudal, the scAmb turns in the non-compacta portion of Amb (Akins et al., 2017; Baertsch et al., 2018; Kottick et al., 2017; Vann et al., 2018), characterizing the caudal edge of PiCo. PiCo was also anatomically characterized by immunohistological labeling, revealing ChAT-positive neurons located dorsomedial to c-scAmb and caudal to the facial nucleus as previously described (Ain Summan Toor et al., 2019; Anderson et al., 2016). As shown in **figure 4**, according to the Paxinos and Franklin mouse atlas (Paxinos & Franklin, 2019), the transfected cells were located slightly dorsal to the NAmb near Bregma level −6.84 mm, ~1100 µm from the midline, and ~700 µm above the marginal layer.

Cell counting, Imaging and Data analysis

A VS120-S6-W Virtual Slide Scanner (Olympus) was used to scan all the sections. Images were taken with a color camera (Nikon DS-Fi3). To restrict any influences on our counted results, the photomicrography and counting were performed by one blind researcher. Image J (version 1.41; National Institutes of Health, Bethesda, MD) was used for cell counting and Canvas software (ACD Systems, Victoria, Canada, v. 9.0) was used for line drawings. A one-in-two series of 25-µm brain sections was used per mouse, which means that each section analyzed was 50 µm apart. The area analyzed was delimited based on previously reports (Anderson et al., 2016) (mean of 5,423 µm²). The sections were counted bilaterally, averaged and the numbers reported as mean ± standard error of the mean (SEM). Section alignment were relative to a reference section, as previously described (Anderson et al., 2016) and based on Paxinos and Franklin (Paxinos & Franklin, 2019).

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Competing Interest Statement

The authors declare no conflict of interest.

Data Availability

All data is publicly available at 10.6084/m9.figshare.24777798

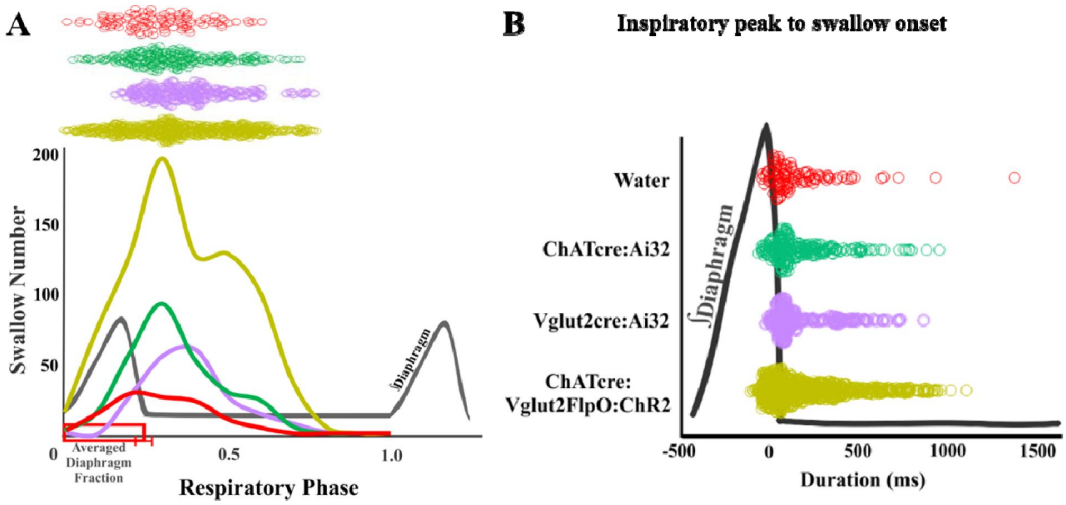

Figure 1-figure supplement 1

No significant differences in swallow-breathing characteristics between water-triggered swallows and PiCo-triggered swallows in mice exposed to CIH. (A) Line graph of swallow frequency in relation to the onset of inspiration for water swallows (red), ChATcre:Ai32 (green), Vglut2cre:Ai32 (purple) and ChATcre:Vglut2FlpO:ChR2 (gold). (B) Dot plot of each swallow in relation to the inspiratory peak. Data for each genetic type is located in **supplement table 1**.

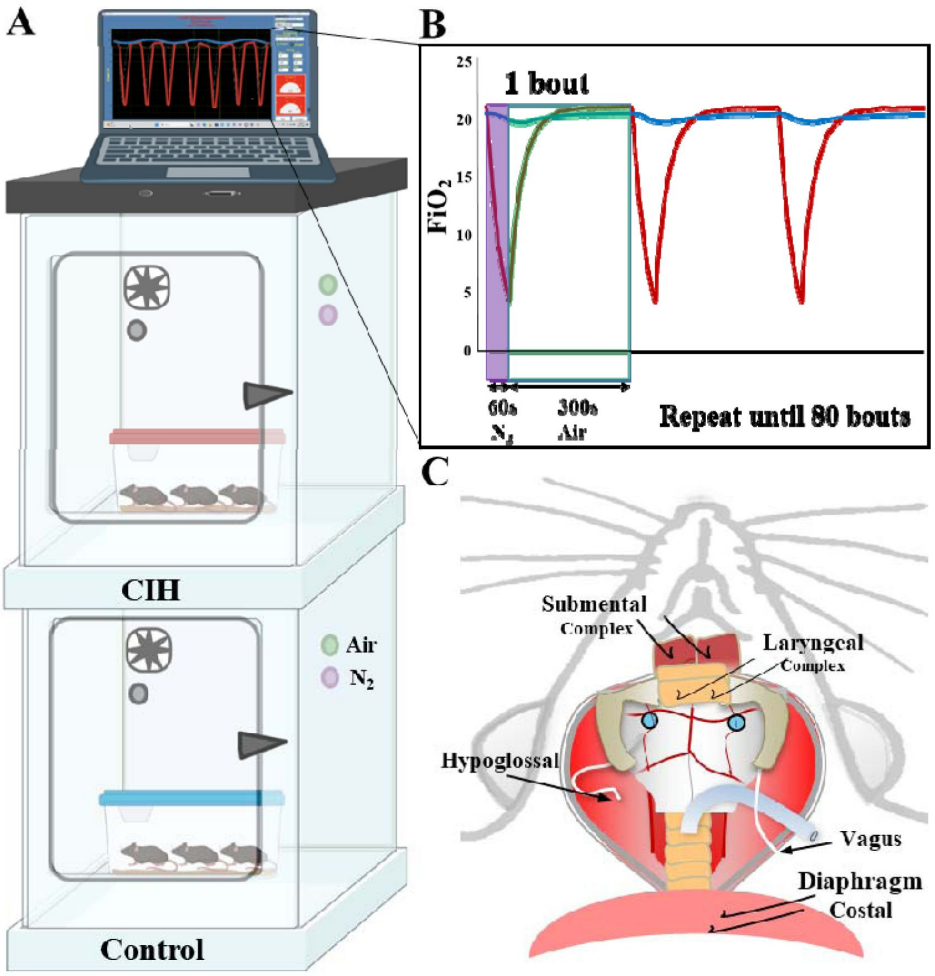

Figure 1-figure supplement 2

Chronic intermittent experimental protocol. (A) Equipment set up for control and CIH protocols. Two plexiglass chambers supplied with a fan, oxygen sensor, and valves for compressed air and Nitrogen. The top chamber serves as the CIH chamber where the oxygen level is automatically controlled by the Oxycycler computer software on top of the chambers. The bottom chamber serves as the control chamber where only air flows through the chamber and the oxygen level is kept at 21% O_2. The mice are kept in their home cage and placed in either chamber for 21 days. (B) In the CIH chamber nitrogen (N_2, purple dot) flows into the chamber for 60s, or until the O_2 level reaches 5%. In the event the O_2 level reaches 5% before 60s, no gas flows into the chamber. After 60s, compress air (green dot) flows into the chamber for 5 minutes. These 6 minutes create one bout and is repeated 80 times a day. (C) Graphical depiction of all muscles: submental complex, laryngeal complex and diaphragm; and nerves: hypoglossal and vagus, recorded to measure swallow and breathing activity. The blue circles depict optrode placement over PiCo.

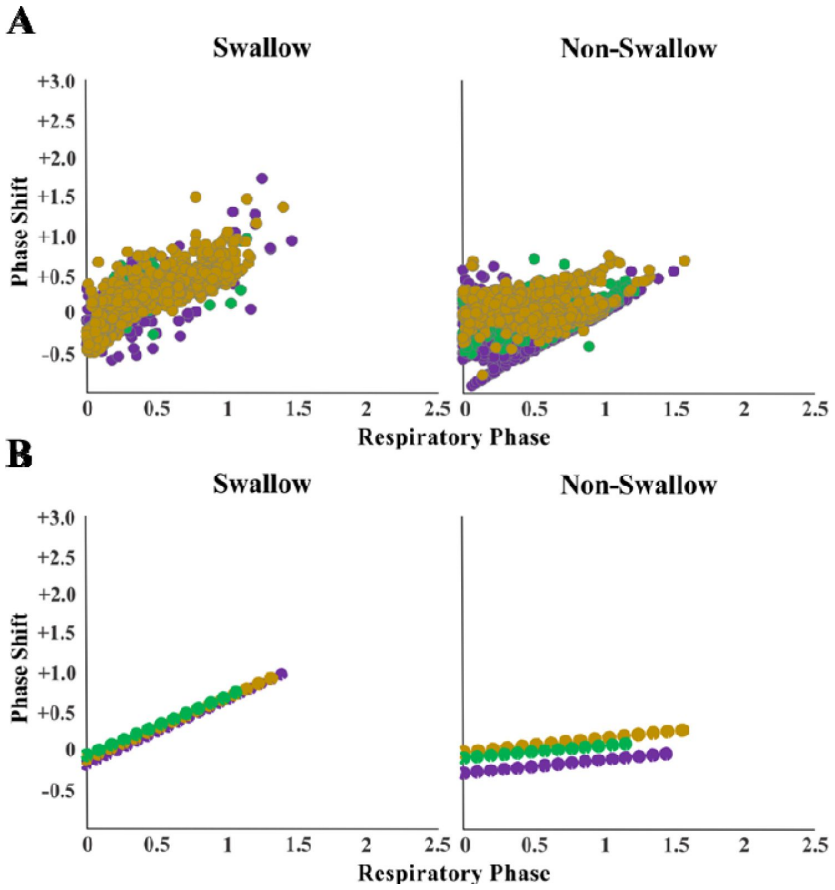

Figure 3-figure supplement 1

PiCo-triggered swallows reset the respiratory rhythm, while non-swallows have minimal effect, a concept not altered by CIH. Respiratory phase shifts plots were divided into two groups: swallow, PiCo activation that triggered a swallow, or non-swallow, PiCo activation that resulted in laryngeal activation or no motor response. A) Individual responses in ChATcre:Vglut2FlpO:ChR2 (gold), ChATcre:Ai32 (green), and Vglut2cre:Ai32 (purple) mice exposed to CIH and B) line of best fit from the above graphs.

A	Water Swallow		+ ChATcre:Ai32 Stimulation					
	mean	(SD)	mean	(SD)	*p*-value	t	df	Change
(*n*=14)								
Swallow Duration (ms)	265	(132)	144	(101)	**0.001**	5.21	8	↓
Hypoglossal Duration (ms)	299	(120)	164	(95)	**0.004**	4.07	8	↓
Vagus Duration (ms)	291	(90)	175	(90)	0.05	2.27	8	↓
Laryngeal Complex Duration (ms)	277	(104)	139	(36)	**0.003**	4.42	7	↓
Schluckatmung Duration (ms)	198	(148)	-	(-)	-	-	-	-
Diaphragm Inter-Burst Interval (ms)	637	(184)	588	(179)	0.75	0.34	8	-
SR Inspiratory Delay (ms)	286	(118)	351	(102)	**0.01**	3.14	8	↑
Swallow Sequence (ms)	69	(79)	30	(20)	0.40	0.90	7	-
Swallow Onset: Insp Peak (ms)	122	(90)	144	(88)	0.41	0.87	8	-
Swallow Onset: Insp Onset (0-1 of RC)	0.27	(0.07)	0.32	(0.05)	0.06	2.14	8	-
Hypoglossal Amplitude (% max)	91	(6)	64	(42)	0.06	2.18	8	-
Vagus Amplitude (% max)	95	(5)	62	(35)	**0.03**	2.74	7	↓
Submental Complex Amplitude (% max)	91	(7)	38	(35)	**0.002**	4.91	7	↓
Laryngeal Complex Amplitude (% max)	83	(11)	71	(45)	0.43	0.83	7	-
Schluckatmung Amplitude (% max)	88	(14)	-	(-)	-	-	-	-

B	Water Swallow		+ Vglut2cre:Ai32 Stimulation					
	mean	(SD)	mean	(SD)	*p*-value	t	df	Change
(*n*=11)								
Swallow Duration (ms)	308	(184)	125	(44)	**0.0003**	6.46	7	↓
Hypoglossal Duration (ms)	335	(187)	144	(41)	**0.003**	4.59	7	↓
Vagus Duration (ms)	330	(188)	146	(44)	**0.005**	4.09	7	↓
Laryngeal Complex Duration (ms)	380	(238)	155	(46)	**0.004**	4.17	7	↓
Schluckatmung Duration (ms)	119	(75)	87	(12)	0.63	0.66	1	-
Diaphragm Inter-Burst Interval (ms)	827	(526)	459	(246)	**0.01**	3.35	7	↓
SR Inspiratory Delay (ms)	361	(310)	258	(150)	0.26	1.22	7	-
Swallow Sequence (ms)	55	(72)	28	(9)	0.08	2.02	7	-
Swallow Onset: Insp Peak (ms)	171	(113)	131	(91)	0.82	0.24	7	-
Swallow Onset: Insp Onset (0-1 of RC)	0.28	(0.09)	0.38	(0.06)	0.10	1.91	7	-
Hypoglossal Amplitude (% max)	82	(11)	44	(7)	**0.0001**	9.45	7	↓
Vagus Amplitude (% max)	79	(17)	50	(19)	**0.001**	5.13	7	↓
Submental Complex Amplitude (% max)	84	(10)	45	(32)	**0.006**	3.84	7	↓
Laryngeal Complex Amplitude (% max)	86	(11)	64	(34)	0.10	1.87	7	-
Schluckatmung Amplitude (% max)	95	(6)	160	(147)	0.62	0.69	1	-

C	Water Swallow		+ ChATcre:Vglut2FlpO:ChR2 Stimulation					
	mean	(SD)	mean	(SD)	*p*-value	t	df	Change
(*n*=11)								
Swallow Duration (ms)	230	(67)	130	(35)	**0.0005**	5.62	8	↓
Hypoglossal Duration (ms)	239	(45)	133	(27)	**0.0003**	6.68	7	↓
Vagus Duration (ms)	256	(62)	140	(37)	**0.0001**	7.11	8	↓
Laryngeal Complex Duration (ms)	260	(58)	144	(34)	**0.0002**	6.66	8	↓
Schluckatmung Duration (ms)	263	(141)	114	(-)	-	-	-	-
Diaphragm Inter-Burst Interval (ms)	786	(327)	711	(345)	0.11	1.79	8	-
SR Inspiratory Delay (ms)	429	(264)	458	(278)	0.55	0.62	8	-
Swallow Sequence (ms)	34	(26)	21	(13)	0.08	1.98	8	-
Swallow Onset: Insp Peak (ms)	175	(95)	188	(151)	0.36	0.98	8	-
Swallow Onset: Insp Onset (0-1 of RC)	0.29	(0.06)	0.34	(0.10)	**0.003**	4.16	8	↑
Hypoglossal Amplitude (% max)	87	(9)	76	(43)	0.47	0.76	7	-
Vagus Amplitude (% max)	87	(7)	58	(32)	**0.01**	3.18	8	↓
Submental Complex Amplitude (% max)	88	(10)	39	(22)	**0.001**	7.47	8	↓
Laryngeal Complex Amplitude (% max)	83	(13)	83	(48)	0.98	0.02	8	-
Schluckatmung Amplitude (% max)	95	(7)	77	(-)	-	-	-	-

Supplement Table 1.

Provides means, standard deviations (SD), *p*-values, t-statistic (t), degrees of freedom (df), from a paired t-test; and the direction of change for swallow-related parameters when evoked by water (water swallows) and optogenetic stimulation of PiCo in A) ChATcre:Ai32, B) Vglut2cre:Ai32 and C) ChATcre:Vglut2FlpO:ChR2 mice.

Water Swallow	Male (n=8)		Female (n=4)					
	mean	(SD)	mean	(SD)	p-value	t	df	Change
Swallow Duration (ms)	307	(142)	181	(55)	0.13	1.67	10	-
XII Duration (ms)	336	(133)	224	(22)	0.13	1.64	10	-
Vagus Duration (ms)	312	(99)	249	(57)	0.27	1.16	10	-
Laryngeal Complex Duration (ms)	312	(122)	225	(43)	0.21	1.36	8	-
Schluckatmung Duration (ms)	198	(148)	-	(-)	-	-	-	-
Diaphragm Inter-Burst Interval (ms)	655	(181)	601	(213)	0.66	0.46	10	-
SR Inspiratory Delay (ms)	261	(94)	335	(160)	0.33	1.02	10	-
Swallow Sequence (ms)	93	(95)	33	(26)	0.26	1.21	8	-
Swallow Onset: Insp Peak (ms)	135	(86)	94	(105)	0.48	0.74	10	-
Swallow Onset: Insp Onset (0-1 of RC)	0.27	(0.06)	0.27	(0.08)	0.93	0.09	10	-
+ ChATcre:Ai32	**Male (n=7)**		**Female (n=3)**					
Stimulation	**mean**	**(SD)**	**mean**	**(SD)**	**p-value**	**t**	**df**	**Change**
Swallow Duration (ms)	154	(118)	122	(55)	0.67	0.44	8	-
XII Duration (ms)	178	(107)	129	(60)	0.48	0.73	8	-
Vagus Duration (ms)	187	(103)	148	(59)	0.57	0.60	8	-
Laryngeal Complex Duration (ms)	135	(30)	147	(54)	0.67	0.44	7	-
Schluckatmung Duration (ms)	-	(-)	-	(-)	-	-	-	-
Diaphragm Inter-Burst Interval (ms)	573	(107)	622	(327)	0.72	0.37	8	-
SR Inspiratory Delay (ms)	340	(59)	377	(187)	0.62	0.51	8	-
Swallow Sequence (ms)	29	(18)	32	(28)	0.83	0.22	7	-
Swallow Onset: Insp Peak (ms)	129	(23)	182	(183)	0.43	0.83	8	-
Swallow Onset: Insp Onset (0-1 of RC)	0.31	(0.04)	0.37	(0.04)	**0.04**	2.38	8	↑

Supplement Table 2.

Provides means, standard deviations (SD), *p*-values, F value, t-statistic (t), degrees of freedom (df), from a unpaired t-test; and the direction of change for swallow-related parameters between male and female mice during water swallows and PiCo-stimulated swallows in ChATcre:Ai32 mice.

Water Swallow	Male (n=5)		Female (n=6)					
	mean	(SD)	mean	(SD)	p-value	t	df	Change
Swallow Duration (ms)	346	(238)	277	(139)	0.56	0.61	9	-
XII Duration (ms)	365	(242)	306	(132)	0.65	0.48	8	-
Vagus Duration (ms)	365	(239)	294	(138)	0.58	0.58	8	-
Laryngeal Complex Duration (ms)	410	(326)	356	(162)	0.73	0.36	9	-
Schluckatmung Duration (ms)	85	(39)	219	(-)	0.10	2.95	2	-
Diaphragm Inter-Burst Interval (ms)	1097	(687)	602	(208)	0.13	1.69	9	-
SR Inspiratory Delay (ms)	542	(399)	211	(72)	0.07	2.02	9	-
Swallow Sequence (ms)	66	(80)	45	(70)	0.66	0.46	9	-
Swallow Onset: Insp Peak (ms)	225	(134)	125	(75)	0.15	1.57	9	-
Swallow Onset: Insp Onset (0-1 of RC)	0.29	(0.13)	0.28	(0.06)	0.97	0.03	9	-
+ Vglut2cre:Ai32	Male (n=3)		Female (n=5)					
Stimulation	mean	(SD)	mean	(SD)	p-value	t	df	Change
Swallow Duration (ms)	145	(52)	113	(39)	0.36	0.99	6	-
XII Duration (ms)	168	(45)	129	(35)	0.22	1.38	6	-
Vagus Duration (ms)	172	(51)	131	(37)	0.24	1.32	6	-
Laryngeal Complex Duration (ms)	170	(61)	145	(40)	0.49	0.73	6	-
Schluckatmung Duration (ms)	95	(-)	79	(-)	-	-	-	-
Diaphragm Inter-Burst Interval (ms)	584	(281)	384	(218)	0.30	1.14	6	-
SR Inspiratory Delay (ms)	358	(173)	197	(109)	0.15	1.65	6	-
Swallow Sequence (ms)	28	(10)	28	(10)	0.97	0.03	6	-
Swallow Onset: Insp Peak (ms)	141	(99)	125	(97)	0.84	0.22	6	-
Swallow Onset: Insp Onset (0-1 of RC)	0.35	(0.10)	0.40	(0.02)	0.33	1.06	6	-

Supplement Table 3.

Provides means, standard deviations (SD), *p*-values, F value, t-statistic (t), degrees of freedom (df), from a unpaired t-test; and the direction of change for swallow-related parameters between male and female mice during water swallows and PiCo-stimulated swallows in Vglut2cre:Ai32 mice.

Water Swallow	Male (n=2)		Female (n=7)					
	mean	(SD)	mean	(SD)	p-value	t	df	Change
Swallow Duration (ms)	194	(65)	240	(68)	0.43	0.85	7	-
XII Duration (ms)	245	(11)	237	(52)	0.84	0.21	6	-
Vagus Duration (ms)	234	(51)	262	(68)	0.61	0.53	7	-
Laryngeal Complex Duration (ms)	221	(56)	271	(58)	0.33	1.06	7	-
Schluckatmung Duration (ms)	-	(-)	263	(141)	-	-	-	-
Diaphragm Inter-Burst Interval (ms)	1086	(13)	701	(322)	0.15	1.61	7	-
SR Inspiratory Delay (ms)	730	(51)	343	(232)	0.06	2.24	7	-
Swallow Sequence (ms)	8	(7)	41	(25)	0.12	1.79	7	-
Swallow Onset: Insp Peak (ms)	202	(113)	167	(98)	0.68	0.43	7	-
Swallow Onset: Insp Onset (0-1 of RC)	0.26	(0.09)	0.38	(0.05)	**0.04**	2.51	7	↑
+ ChATcre:Vglut2FlpO:ChR2	Male (n=4)		Female (n=7)					
Stimulation	mean	(SD)	mean	(SD)	p-value	t	df	Change
Swallow Duration (ms)	130	(27)	127	(38)	0.89	0.14	9	-
XII Duration (ms)	128	(33)	137	(26)	0.63	0.50	8	-
Vagus Duration (ms)	138	(42)	139	(36)	0.96	0.05	9	-
Laryngeal Complex Duration (ms)	122	(32)	158	(30)	0.10	1.82	9	-
Schluckatmung Duration (ms)	-	(-)	114	(-)	-	-	-	-
Diaphragm Inter-Burst Interval (ms)	847	(290)	623	(367)	0.32	1.04	9	-
SR Inspiratory Delay (ms)	625	(294)	357	(238)	0.13	1.66	9	-
Swallow Sequence (ms)	13	(6)	22	(12)	0.21	1.34	9	-
Swallow Onset: Insp Peak (ms)	159	(188)	191	(139)	0.75	0.33	9	-
Swallow Onset: Insp Onset (0-1 of RC)	0.26	(0.14)	0.30	(0.05)	0.48	0.74	9	-

Supplement Table 4.

Provides means, standard deviations (SD), *p*-values, F value, t-statistic (t), degrees of freedom (df), from a unpaired t-test; and the direction of change for swallow-related parameters between male and female mice during water swallows and PiCo-stimulated swallows in ChATcre:Vglut2FlpO:ChR2 mice.

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Reviewer #1 (Public Review):

Summary:

Authors were attempting to determine the extent that CIH altered swallowing motor function; specifically, the timing and probability of the activation of the laryngeal and submental motor pools. The paper describes a variety of different motor patterns elicited by optogenetic activation of individual neuronal phenotypes within PiCo in a group of mice exposed to CIH. They show that there are a variety of motor patterns that emerge in CIH mice; this is apparently different than the more consistent motor patterns elicited by PiCo activation in normoxic mice (previously published)

Strengths:

The preparation is technically challenging and gives valuable information related to the role of PiCo in the pattern of motor activation involved in swallowing and its timing with phrenic activity. Genetic manipulations allow for the independent activation of the individual neuronal phenotypes of PiCo (glutamatergic, cholinergic) which is a strength.

Weaknesses:

(1) Comparisons made between experimental data acquired currently with those previously published are extremely problematic, with the potential confounding influence of changing environments, genetics and litter effects. For example, were the current mice tested at the same time as those exposed to normoxia? Are they littermates (or at least from the same colony) as those previously examined? If they were tested at the same time and age, then the authors should explicitly state this in the methods. The authors have provided no statistical analyses to determine whether there is an effect of CIH on the motor patterns. In short, how can they be sure that the phenomena they observe with respect to motor patterns is due to CIH?

(2) The data are descriptive in nature, reporting only differences (diversity) of motor patterns in this cohort of animals exposed to CIH. There is limited mechanistic insight into how PiCo manipulation alters the pattern and probability of motor activation. Can they utilize Fos or marker of activation within the nTS or other regions to provide initial insight? Or in another nucleus that contributes as part of the circuit.

(3) The differences between the genotypes (ChaTcre; Vglut2Cre; ChatCre:Vglut2FlpO) with regard to the probability of generating a swallow are not sufficiently discussed, in my view. If, as the authors state, it is "reasonable to suggest that CIH differentially affects" these populations, then what are some viable reasons? What are the known differences in these populations of neurons that could lead to variable responses? Do they project to different places?

(4) The Results section is difficult to follow and interpret. It would be beneficial to have a couple of sentences after each sub-section stating what the data actually mean. As of now it reads like a statistical report of the data with little "basic" interpretation of the data.

(5) I have a hard time understanding the functional significance of calculating and plotting the degree of correlation between shifting/delaying the following inspiratory burst and triggering a swallow.

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Reviewer #2 (Public Review):

The manuscript has been revised according to Reviewer's suggestions. Recommendations for the Authors have been almost entirely followed. However, there are some points where the authors state that they have made changes, but the text does not show this. The revised version would have gained in clarity if it was with track changes and numbered rows. In particular, I cannot see the following changes:

Lines 104-105: Did you mean: "We confirmed that optogenetic stimulation of PiCo neurons in ChATcre:Vglut2FlpO:ChR2 mice exposed to CIH triggers swallow and laryngeal activation similar to the control mice exposed to room air (Huff et al., 2023)." Otherwise, the sentence is not clear.
Thank you, this has been changed

Lines 228-232: "PiCo-triggered swallows are characterized by a significant decrease in duration compared to swallows evoked by water in ChATcre:Ai32 mice (265 {plus minus} 132ms vs 144 {plus minus} 101ms; paired t-test: p= 0.0001, t= 5.21, df= 8), Vglut2cre:Ai32 mice (308 {plus minus} 184ms vs 125 {plus minus} 44ms; paired t-test: p= 0.0003, t= 6.46, df= 7), and ChATcre:Vglut2FlpO:ChR2 mice (230 {plus minus} 67ms vs 130 {plus minus} 35ms; paired t-

test: p= 0.0005, t= 5.62, df= 8) exposed to CIH (Table S1).".
Thank you, this has been changed

Lines 283-290: "Thus, CIH does not alter PiCo's ability to coordinate the timing for swallowing and breathing. Rather, our data reveals that CIH disrupts the swallow motor sequence likely due to changes in the interaction between PiCo and the SPG, presumably the cNTS.

While it has previously been demonstrated that PiCo is an important region in swallow-breathing coordination (Huff et al., 2023), previous studies did not demonstrate that PiCo is involved in swallow pattern generation itself. Thus, here we show for the first time that CIH resulted in the instability of the swallow motor pattern activated by stimulating PiCo, suggesting PiCo plays a role in its modulation.".
Thank you, this has been changed

Line 437: Mice of the ChATcre:Ai32, Vglut2cre:Ai32 and ChATcre:Vglut2FlpO:ChR2 lines were kept in collective cages with food and water ad libitum placed inside custom-built chambers.
Thank you, this has been changed.

Overall, the manuscript has been improved.

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Author Response

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *The authors were attempting to determine the extent that CIH altered swallowing motor function; specifically, the timing and probability of the activation of the laryngeal and submental motor pools. The paper describes a variety of different motor patterns elicited by optogenetic activation of individual neuronal phenotypes within PiCo in a group of mice exposed to CIH. They show that there are a variety of motor patterns that emerge in CIH mice; this is apparently different than the more consistent motor patterns elicited by PiCo activation in normoxic mice (previously published).*
>
> *Strengths:*
>
> *The preparation is technically challenging and gives valuable information related to the role of PiCo in the pattern of motor activation involved in swallowing and its timing with phrenic activity. Genetic manipulations allow for the independent activation of the individual neuronal phenotypes of PiCo (glutamatergic, cholinergic) which is a strength.*

We thank the reviewers for acknowledging and summarizing the strengths of this study.

> *Weaknesses:*
>
> *(1) The data presented are largely descriptive in terms of the effect of PiCo activation on the probability of swallowing and the pattern of motor activation changes following CIH. Comparisons made between experimental data acquired currently and those obtained in a previous cohort of animals (possibly years before) are extremely problematic, with the potential confounding influence of changing environments, genetics, and litter effects.*

> *The statistical analyses (i.e. comparing CIH with normoxic) appear insufficiently robust. Exactly how the data were compared is not described.*

Yes, we agree the data are descriptive in terms of characterizing the effect of CIH on PiCo activation. However, we would like to emphasize that the data are also mechanistic because they characterize the effects of specifically, optogenetically manipulating PiCo neurons after being exposed to CIH.

Thank you for this comment and for pointing out our misleading description in the paper. This manuscript is meant to independently characterize the effects of CIH to the response of PiCo stimulation. We are not making direct comparisons between the previously published manuscript where mice were exposed to room air. There has been no statistical analysis made between previously published control and current CIH data, since we are not making a direct comparison, only an observational comparison.

To make this clearer, and to address the reviewers concern, we have removed the room air data from figures 1E, 2C and 3A. However, we believe it is important to keep the data from mice exposed to room air in Figure 2B since we did not include this information in the previously published manuscript. It is important to point out that all mice exposed to CIH have some form of submental activity during laryngeal activation in response to PiCo stimulation. This is not the case when mice are exposed to room air only. In this figure, only descriptive analysis are presented. We adjusted our wording throughout the text, particularly in the discussion, to eliminate any confusion that we are making direct comparisons between the two studies. The following sentence has been added to the discussion "While we do not intend to make direct quantitative comparisons between the previously published PiCo-triggered swallows in control mice exposed to room air (Huff et al 2023) and the data presented here for mice exposed to CIH, we believe it is important to compare the conclusions made in these two studies." This was the motivation for using the eLife Advance format. Since the present study demonstrates that PiCo affects swallow patterning which was not observed in the control data.

> *(2) There is limited mechanistic insight into how PiCo manipulation alters the pattern and probability of motor activation. For example, does CIH alter PiCo directly, or some other component of the circuit (NTS)? Techniques that silence or activation projections to/from PiCo should be interrogated. This is required to further delineate and define the swallowing circuit, which remains enigmatic.*

We agree with the reviewer that our study raises many more questions than we are able to answer at the moment. This however applies to most scientific studies. Even though swallowing has been studied for many decades, the underlying circuitry remains largely enigmatic. We will continue to investigate the role of PiCo and its interaction with the NTS, in healthy and diseased states. These investigations require many different techniques, and approaches, some of which are still in development. For example, we are currently conducting experiments that silence portions of the NTS related to swallow and PiCo: ChAT/Vglut2 neurons using novel unpublished viral approaches. However, these are separate and ongoing studies beyond the scope of the current one.

To address the reviewer's comment, we have added to the following to the limitation section: "In addition, this preparation does not allow for recording of PiCo neurons to evaluate the direct effects of CIH in PiCo neuronal activity". The following has also been added to the discussion: "Rather, our data reveal CIH disrupts the swallow motor sequence which is likely due to changes in the interaction between PiCo and the SPG, presumably located in the cNTS. While it has previously been demonstrated that PiCo is an important region in swallow-breathing coordination (Huff et al., 2023), previous studies did not demonstrate that PiCo is involved in swallow motor patterning itself. Here we show for the first time that CIH leads to

disturbances in the generation of the swallow motor pattern that is activated by stimulating PiCo. This suggests that PiCo is not only important for coordinating swallow and breathing, but also modulating swallow motor patterning. Further studies are necessary to directly evaluate the presumed interactions between PiCo and the cNTS."

> *(3) The functional significance of the altered (non-classic) patterns is unclear.*

Like in our original study, the preparation used to stimulate PiCo does not allow to simultaneously characterize the functional significance of swallowing. Therefore, we have included this as a limitation in the limitation section: "In this preparation we are unable to directly determine the functionality of the variable swallow motor pattern seen after CIH. Different experimental techniques, such as videofluoroscopy would need to be used to directly evaluate functional significance. This technique is beyond the scope of this study and not possible to perform in this preparation. We acknowledge this limits our ability to make direct comparisons between dysphagic swallows in OSA patients."

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *(1) A more rigorous experimental approach is required. Littermates should be separated and exposed to either room air or CIH at the same (or close to the same) time.*

As stated above, we did not directly compare mice exposed to room air with mice exposed to CIH. Hence, we believe this is not necessary, and it would have meant repeating all the experiments already published in the original eLife paper.

> *(2) Robust statistical analyses are required to determine whether the effects of CIH on the pattern/probability of motor activation are required.*

Since control and CIH group were not compared in this study, statistical hypothesis testing is not appropriate or applicable.

> *(3) Use a combination of retrograde, Cre- AAVs and Cre-dependent approaches to interrogate the circuitry to/from PiCO that forms the swallowing network. This is what is needed to push this area forward, in my view.*

Thank you for this suggestion, we will consider this suggestion as we plan for future experiments. Indeed, we are in the process of developing novel approaches. However, in this context we would like to emphasize that further network investigations are exponentially more complicated given that we need to use a Flpo/Cre approach to specifically characterize the glutamatergic-cholinergic PiCo neurons. Most other laboratories that have studied PiCo have avoided this experimental complication and used only a "cre-dependent" approach. This approach is much simpler, but the data are much less specific and the conclusions sometimes misleading. Stimulating for example cholinergic neurons in the PiCo area will also activate Nucleus ambiguus neurons, stimulating glutamatergic neurons will also activate glutamatergic neurons that are not necessarily the glutamatergic/cholinergic neurons that we use to define PiCo specifically. Readers that are unfamiliar with these different approaches often miss this important difference. Hence, compared to stimulating other areas, stimulating the cholinergic-glutamatergic neurons in PiCo is much more specific than e.g. stimulating preBötzinger complex neurons. There are no markers that will specifically stimulate only preBötzinger complex neurons or neurons in the parafacial Nucleus. Unfortunately, this difference is often overlooked.

> *(4) It should be made more clear how each of the "non-classic" swallowing patterns could cause dysfunction - especially to the reader who is not completely familiar with the*

> *neural control of swallowing.*

We agree that it would be helpful to understand the functional implications of these alterations in swallow-related motor activation, however since our approach does not allow us to use any tools to measure or evaluate functional activity it would be inappropriate to make suggestions of this type without any data to back up our conclusion. This is why we have not speculated on the functional implications. We have added the following to the discussion section of this manuscript. “While fine wire EMG studies are an excellent evaluation tool to observe temporal motor pattern of sequential swallow related muscles; it must be combined with tools such as videofluoroscopic swallow study (VFSS) and/or high resolution manometry (HRM) in order to characterize the functional significance of these alterations to the swallow motor pattern shown in this study (Park et al., 2017). Since the preparation in this study utilizes only fine wire EMGs we are not able to evaluate or comment on the functional significance of the variable swallow motor patterns. ”

> *Minor:*
>
> *The Results should be written in a way that better conveys the neurophysiological effects of the manipulations. As it stands, it reads like a statistical report on how activation of each neuronal phenotype is statistically different from each other. As such it is difficult to read and understand the salient findings.*

Thank you for this insight. We have adjusted the language in the results section.

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *In this study, the authors investigated the role of a medullary region, named Postinspiratory Complex (PiCo), in the mediation of swallow/laryngeal behaviours, their coordination with breathing, and the possible impact on the reflex exerted by chronic intermittent hypoxia (CIH). This region is characterized by the presence of glutamatergic/cholinergic interneurons. Thus, experiments have been performed in single allelic and intersectional allelic recombinase transgenic mice to specifically excite cholinergic/glutamatergic neurons using optogenetic techniques, while recording from relevant muscles involved in swallowing and laryngeal activation. The data indicate that in anaesthetized transgenic mice exposed to CIH, the optogenetic activation of PiCo neurons triggers swallow activity characterized by variable motor patterns. In addition, these animals show an increased probability of triggering a swallow when stimulation is applied during the first part of the respiratory cycle. They conclude that the PiCo region may be involved in the occurrence of swallow and other laryngeal behaviours. These data interestingly improve the ongoing discussion on neural pathways involved in swallow-breathing coordination, with specific attention to factors leading to disruption that may contribute to dysphagia under some pathological conditions.*
>
> *The Authors' conclusions are partially justified by their data. However, it should be acknowledged that the impact of the study is to a certain extent limited by the lack of knowledge on the source of excitatory inputs to PiCo during swallowing under physiological conditions, i.e. during water-evoked swallowing. Also the connectivity between this region and the swallowing CPG, a structure not well defined, or other brain regions involved in the reflex is not known.*

We thank the reviewer for the comments and the strength of the paper. However, with regards to the “lack of knowledge”, we would like to emphasize that PiCo was first described in 2016, while e.g. the preBötzinger complex was described in 1991. Thus, it is not fair to

assume the same level of anatomical and physiological understanding for PiCo as we became accustomed to for the preBötzinger complex. We are fairly confident that in 25 years from now, our knowledge of the in- and outputs of PiCo will be much less limited than it currently is.

> *Strengths:*
>
> *Major strengths of the manuscript:*
>
> - *The methodological approach is refined and well-suited for the experimental question. The in vivo mouse preparation developed for this study takes advantage of selective optogenetic stimulation of specific cell types with the simultaneous EMG recordings from upper airway muscles involved in respiration and swallowing to assess their motor patterns. The animal model and the chronic intermittent hypoxia protocol have already been published in previous papers (Huff et al. 2022, 2023).*
>
> - *The choice of the topic. Swallow disruption may contribute to the dysphagia under some pathological conditions, such as obstructive sleep apnea. Investigations aimed at exploring and clarifying neural structures involved in this behaviour as well as the connectivity underpinning muscle coordination are needed.*
>
> - *This study fits in with previous works. This work is a logical extension of previous studies from this group on swallowing-breathing coordination with further advances using a mouse model for obstructive sleep apnea.*

We thank the reviewers for acknowledging and summarizing the strengths of this study.

> *Weaknesses:*
>
> *Major weaknesses of the manuscript:*
>
> - *The Authors should be more cautious in concluding that the PiCo is critical for the generation of swallowing itself. It remains to demonstrate that PiCo is necessary for swallowing and laryngeal function in a more physiological situation, i.e. swallow of a bolus of water or food. It should be interesting to investigate the effects of silencing PiCo cholinergic/glutamatergic neurons on normal swallowing. In this perspective, the title should be slightly modified to avoid "swallow pattern generation" (e.g. Chronic Intermittent Hypoxia reveals the role of the Postinspiratory Complex in the mediation of normal swallow production).*

Thank you for pointing out that this manuscript suggest PiCo is necessary for swallow generation. We agree further interventions to silence specifically PiCo ChAt/Vglut2 neurons will be necessary to investigate this claim. Which we have begun to evaluate for a future study by developing a novel as yet unpublished approach. We have altered language throughout the text to limit the perception that PiCo is the swallow pattern generator. We have also changed the title to say: Chronic Intermittent Hypoxia reveals the role of the Postinspiratory Complex in the mediation of normal swallow production

> - *The duration of swallows evoked by optogenetic stimulation of PiCo is considerably shorter in comparison with the duration of swallows evoked by a physiological stimulus (water). This makes it hard to compare the timing and the pattern of motor response in CIH-exposed mice. In Figure 1, the trace time scale*

> *should be the same for water-triggered and PiCo-triggered swallows. In addition, it is not clear if exposure to CIH alters the ongoing respiratory activity. Is the respiratory rhythm altered by hypoxia? If a disturbed or irregular pattern of breathing is already present in CIH-exposed mice, could this alteration interfere with the swallowing behaviour?*

Thank you. We have changed the time scale so that all representative traces are on the same time scale.

We explained in the original paper (Huff et al 2023) that the significant decrease in PiCo-evoked swallow duration compared to water evoked is likely due to the absence of oral/upper airway feedback. We are not making comparisons of the effects of CIH on swallow motor pattern between water-evoked and PiCo-evoked. Rather, we are only characterizing the effects of CIH on the swallow motor pattern in PiCo-evoked swallows. The purpose of Figure 1A is to show that the rostocaudal submental-laryngeal sequence in water-evoked swallows is preserved in “canonical” PiCo-evoked swallow like is shown in the original study. While we did not measure the effects of CIH on breathing and the respiratory pattern in this study, it has been established, by others, that CIH causes respiratory muscle weakness, impaired motor control of the upper airway and variable respiratory rhythm and rhythm generation. However, when characterizing the timing of swallow in relation to inspiration (Figure 1 Figure Supplement 1) and the reset of the respiratory rhythm (Figure 3 figure supplement 1) and by observationally comparing these results with mice exposed to room air (Huff et al 2023) we do not observe any obvious differences in swallow-breathing coordination. However, a separate study in wild-type mice focusing on a characterization of swallowing via water after CIH would be better suited to achieve a better understanding of the physiological changes of swallowing after CIH. We would like to point out that this has shown in Huff et al 2022 that altering respiratory rate/pattern via activation of various preBötzinger Complex neurons does not change swallow behavior. Except in the case of Dbx1 PreBötC neuron activation, which was independent of CIH. Increasing or decreasing respiratory rate via activation of PreBötC Vgat and SST neurons did not change the swallow pattern rather it changed the timing of when swallows occurred. It has been reported before by others that swallow has a hierarchical control over breathing and has the ability to shut breathing down. We believe that the swallowing behavior is independent of respiratory pattern and alterations in breathing pattern does not necessarily affect the swallow motor pattern rather could affect the swallow timing.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *Abstract*
>
> *Lines 37-41 "Here we show that optogenetic stimulation of ChATcre:Ai32, Vglut2cre:Ai32, and ChATcre:Vglut2FlpO:ChR2 mice exposed to CIH does not alter swallow-breathing coordination, but unexpectedly the generation of swallow motor pattern was significantly disturbed."*
>
> *It should be better:*
>
> *"Here we show that optogenetic stimulation of ChATcre:Ai32, Vglut2cre:Ai32, and ChATcre:Vglut2FlpO:ChR2 mice exposed to CIH does not alter swallow-breathing coordination, but unexpectedly triggers variable swallow motor patterns".*

Thank you, this has been changed

> *Lines 41-43 "This suggests, glutamatergic-cholinergic neurons in PiCo are not only critical for the gating of postinspiratory and swallow activity but also play important*

> *roles in the generation of swallow motor pattern." I suggest removing any language claiming PiCo is swallow gating and change "generation" in "modulation"*
>
> *"This suggests that glutamatergic-cholinergic neurons in PiCo are not only critical in regulating swallow-breathing coordination but also play important roles in the modulation of swallow motor pattern."*

Thank you, this has been changed

> *Introduction:*
>
> *Line 88-90: Actually, in Huff et al. 2023 it is said "PiCo acts as an interface between the swallow pattern generator and the preBötzinger complex to coordinate swallow and breathing". Please, change accordingly. Please, remove Toor et al., 2019 since their conclusions are quite different.*
>
> *Line 100-101: Please, change the sentence according to the comments reported above.*

Thank you, this has been changed

> *Results:*
>
> *Lines 104-105: Did you mean: "We confirmed that optogenetic stimulation of PiCo neurons in ChATcre:Vglut2FlpO:ChR2 mice exposed to CIH triggers swallow and laryngeal activation similar to the control mice exposed to room air (Huff et al., 2023)." Otherwise, the sentence is not clear.*

Thank you, this has been changed

> *Lines 129-130: This finding is not surprising since similar results have been reported in Huff et al. 2023.*

Thank you, we wanted to confirm that CIH did not alter this characteristic, which it did not. We believe that it is important to include this as it is a criterion for characterizing laryngeal activation.

> *Lines 219: The number of water swallows is considerably lower than stimulation-evoked swallows. Why?*

We inject water into the mouth three times. Typically, there is one swallow in response to each water injection. Pico is stimulated 25 times at each duration. If we were to stimulate swallow with water as many times as optogenetic stimulation there would be an adaptive response to the water stimulation and the mouse would not respond. This does not seem to be the case with PiCo stimulation. Simple answer is, there are many more PiCo stimulations than water stimulation.

> *Lines 228-232: "PiCo-triggered swallows are characterized by a significant decrease in duration compared to swallows evoked by water in ChATcre:Ai32 mice (265 {plus minus} 132ms vs 144 {plus minus} 101ms; paired t-test: p= 0.0001, t= 5.21, df= 8), Vglut2cre:Ai32 mice (308 {plus minus} 184ms vs 125 {plus minus} 44ms; paired t-test: p= 0.0003, t= 6.46, df= 7), and ChATcre:Vglut2FlpO:ChR2 mice (230 {plus minus} 67ms vs 130 {plus minus} 35ms; paired t-test: p= 0.0005, t= 5.62, df= 8) exposed to CIH (Table S1).".*

Thank you, this has been changed

> *Line 252 and 254: remove SEM.*

Thank you, this has been changed

> *Discussion*
>
> *Line 267: ...(Figure 1Bi), while 28% of PiCo-triggered swallows...*

Thank you, this has been changed

> *Lines 283-290: "Thus, CIH does not alter PiCo's ability to coordinate the timing for swallowing and breathing. Rather, our data reveals that CIH disrupts the swallow motor sequence likely due to changes in the interaction between PiCo and the SPG, presumably the cNTS.*
>
> *While it has previously been demonstrated that PiCo is an important region in swallow-breathing coordination (Huff et al., 2023), previous studies did not demonstrate that PiCo is involved in swallow pattern generation itself. Thus, here we show for the first time that CIH resulted in the instability of the swallow motor pattern activated by stimulating PiCo, suggesting PiCo plays a role in its modulation.".*

Thank you, this has been changed

> *Could the observed effects be due to a non-specific effect of hypoxia on neuronal excitability? In addition, it should be considered that PiCo-triggered swallows lack the behavioural setting of water-evoked swallows and do not activate the sensory component of the SPG to the same extent as the water-evoked swallows.*

Yes, this is very possible. We stated in our first manuscript that the decrease in PiCo-triggered swallow duration, as compared to water-triggered swallow duration, is likely because oral sensory components are not being activated to the same extent (Huff et al. 2023). Since we do not directly measure neuronal excitability, it is not known (in this study) whether CIH causes changes in the excitability to swallow related areas. However, others have shown increased excitability and activity of Vglut2 neurons after CIH exposure (Kline et al 2007,2010), and we have shown e.g. changes in the excitability of preBötC neurons (Garcia et al. 2016, 2017).

> *Lines 293-300: The sentence is not clear. Is there any evidence indicating that glutamatergic neurons are differently affected by hypoxia than cholinergic neurons?*

Thank you, these sentences have been changed to increase clarity. The section now reads: There was no statistical difference in the probability of triggering a swallow during optogenetic stimulation of ChATcre:Ai32, Vglut2cre:Ai32 and ChATcre:Vglut2FlpO:ChR2 neurons in mice exposed to room air (Huff et al 2023). However, when exposed to CIH, ChATcre:Ai32 and Vglut2:Ai32 mice have a lower probability of triggering a swallow -- in some mice swallow was never triggered via PiCo activation, while water-triggered swallows remained – compared to the ChATcre:Vglut2FlpO:ChR2 mice. While it is possible that portions of the presumed SPG remain less affected by CIH, which could offset these instabilities to produce functional swallows, our data suggest that PiCo targets microcircuits within the SPG that are highly affected by CIH. The NTS is a primary first site for upper airway and swallow-related sensory termination in the brainstem (Jean, 1984). CIH induces changes to the cardio-respiratory Vglut2 neurons, resulting in an increase in cNTS neuronal activity (Kline, 2010; Kline et al., 2007), as well as changes to preBötzinger neurons (Garcia et al., 2017; Garcia et al., 2016) and ChAT neurons in the basal forebrain (Tang et al., 2020). It is reasonable to suggests that CIH has differential effects on neurons that only express ChATcre and Vglut2cre

versus the PiCo-specific interneurons that co-express ChATcre and Vglut2FlpO, emphasizing the importance of targeting and manipulating these PiCo-specific interneurons."

> *Lines 372-374: "Here we show that PiCo, a neuronal network which is critical for the generation of postinspiratory activity (Andersen et al. 2016) and implicated in the coordination of swallowing and breathing (Huff et al., 2023), is severely affected by CIH.".*

Thank you, this has been changed.

> *Methods*
>
> *Line 398: Did you mean Slc17a6-IRES2-FlpO-D?*

Thank you, this has been changed.

> *Line 399: were.*

Thank you, this has been changed.

> *Line 403: ... expressing both ChAT and Vglut2 and will be reported as ChATcre:Vglut2FlpO.*

Thank you, this has been changed.

> *Line 437: Mice of the ChATcre:Ai32, Vglut2cre:Ai32 and ChATcre:Vglut2FlpO:ChR2 lines were kept in collective cages with food and water ad libitum placed inside custom-built chambers.*

Thank you, this has been changed.

> *Line 479: (Figure 6a in Huff et al., 2022).*
>
> *Line 497: What does Fig 7 refer to?*

This should say Figure 1- figure supplement 2, This has been changed

> *Lines 501-506: "First, swallow was stimulated by injecting 0.1cc of water into the mouth using a 1.0 cc syringe connected to a polyethylene tube. Second, 25 pulses of each 40ms, 80ms, 120ms, 160ms and 200ms continuous TTL laser stimulation at PiCo was repeated, at random, throughout the respiratory cycle. The lasers were each set to 0.75mW and triggered using Spike2 software (Cambridge Electronic Design, Cambridge, UK). These stimulation protocols were performed in all ChATcre:Ai32, Vglut2cre:Ai32, and ChATcre:Vglut2FlpO:ChR2." .*

Thank you, this has been changed.

> *Line 526 and 540: (Fig.6 in Huff et al., 2022) and (Fig.6d in Huff et al., 2022).*

Thank you, this has been fixed

> *Line 594: Figure 5 doesn't exist. Please, change the sentence.*

Thank you, this has been fixed

> *Line 595 and 609: The reference Kirkcaldie et al. 2012 is referred to the neocortex and doesn't seem appropriate. Please, quote the atlas of Paxinos and Franklin.*

Thank you, this has been changed.

> *Reference:*
>
> *Please, correct throughout the text editing of references by removing e.g J.M. or A. or David D. and so on. Only surnames should be mentioned.*

Thank you, this has been changed.

> *Figures:*
>
> *Figure 1. A and B as well as the purple arrow are lacking. In addition, optogenetic stimulation is applied during different periods of inspiratory activity and this could impact the swallow motor pattern. In Bv, Non-LAR seems very similar to LAR. In panel E, please add the number of animals.*

Thank you, this has been fixed.

> *We used the same optogenetic protocols in the original paper (Huff et al. 2023) and did not observe any changes to the swallow motor patter in relation to the time PiCo was stimulated. The only phase dependent response seen in both control and CIH is when PiCo Is stimulated during inspiration and a swallow is triggered, inspiration will be inhibited. Therefore, we do not believe variability in swallow motor pattern is dependent on the phase of breathing in which PiCo is stimulated.*

Biv LAR has a pause in EMG activity before the swallow begins (red arrow pointing to the pause). While Bv Non-LAR does not have this pause, rather the two behaviors converge (red arrow). In order for something to be considered an LAR the pause must be present which is why we separated these two motor patterns.

> *Figure 1 - Figure Supplement 1. Why do the Authors call the lines "histograms"?*

Thank you, this has been fixed. This is a line graph of swallow frequency in relation to inspiration.

> *Tables:*
>
> *In tables, data are provided as means and standard deviation. Please, specify this in the Method section.*

Thank you, the following is listed in the methods section: “All data are expressed as mean ± standard deviation (SD), unless otherwise noted.”

> ***Reviewer #3 (Public Review):***
>
> *In the present study, the authors investigated the effects of CIH on the swallowing and breathing responses to PICO stimulation. Their conclusion is that glutamatergic-cholinergic neurons from PICO are not only critical for the gating of post-inspiratory and swallow activity, but also play important roles in the generation of swallow motor patterns. There are several aspects that deserve the authors' attention and comments, mainly related to the studyś conclusions.*
>
> - *The authors refer to PICO as the generator of post-inspiratory rhythm. However, evidence points to this region as a modulator of post-inspiratory activity rather*

> *than a rhythmogenic site (Toor et al., 2019 - 10.1523/JNEUROSCI.0502-19.2019; Oliveira et al., 2021 - 10.1016/j.neuroscience.2021.09.015). For example, sustained activation of PICO for 10 s barely affected the vagus or laryngeal post-inspiratory activity (Huff et al., 2023 - 10.7554/eLife.86103).*

Yes, we did refer to PiCo as the postinspiratory rhythm generator as defined as Anderson et al. 2016. We base this statement on the following criteria and experiments: In Anderson et al. 2016, we demonstrate that PiCo can be isolated in vitro, that PiCo neurons are activated in phase with postinspiration, and that they are inhibited during inspiration by preBötC neurons via GABAergic mechanisms and not glycinergic mechanisms. We also demonstrate that optogenetically stimulating cholinergic neurons in the PiCo area resets the inspiratory rhythm both in vivo and in vitro. We also show that PiCo when isolated in transverse slices is autorhythmic and that PiCo, like the preBötC in transverse slices can generate respiratory rhythmic activity in vitro and independent of the preBötC. We also demonstrate that PiCo neurons are an order of magnitude more sensitive to opioids (DAMGO) than the preBötC and that local injections of DAMGO into the PiCo area in vivo abolishes postinspiration, and also abolishes the phase delay of the respiratory rhythm. None of these specific rhythmogenic properties have been studied by the Toor study or the Oliveira et al study. Hence, we do not understand why the reviewer cites these studies as evidence for modulation as opposed to rhythmogenic properties. The fact that PiCo is rhythmogenic should not be considered as an "exclusive property". Specifically, this does not mean that PiCo is also "modulating" the swallow-breathing coordination as we have demonstrated more specifically in the Huff et al study. In the same sentence we also referred to the PreBötzinger complex as the inspiratory rhythm generator as defined by Smith et al 1991, and it seems that the reviewer did not object to this reference. But we would like to point out that the same criteria were used to define the preBötzinger complex as we used for PiCo, except that PiCo neurons are better defined than preBötzinger complex neurons. Dbx1 neurons are often used to characterize the PreBötC, but these neurons form a rostrocaudal and ventrodorsal column which involves also glia cells and transcends the preBötC. Glutamatergic neurons are everywhere, and so are Somatostatin or Neurokinin neurons. Moreover, the 1991 study was only performed in vitro, and did not include a histochemical analysis. We would also like to point out that the present manuscript is investigating the role of PiCo in swallow and laryngeal behaviors, and not specifically postinspiration. Thus, we are not entirely sure how this comment relates to this manuscript.

> - *The optogenetic activation of glutamatergic and cholinergic neurons from PICO evoked submental and laryngeal responses, and CIH changed these motor responses. Therefore, the authors proposed that PICO is directly involved in swallow pattern generation and that CIH disrupts the connection between PICO and SPG (swallow pattern generator). However, the experiments of the present study did not provide evidence about connections between these two regions nor their possible disruption after CIH, or even whether PICO is part of SPG.*

We have edited the text to suggest PiCo modulates swallow motor sequence in addition to the coordination of swallow and breathing. We have also added that further experiments will be necessary to further investigate the connections between PiCo and SPG. But, unfortunately, compared to PiCo, the SPG is much less defined. As already stated above, it cannot be expected that a single study can address all possible open questions. Clearly, more work needs to be done outside of this study to answer all of these questions, which makes this an exciting area of research.

> - *CIH affects several brainstem regions which might contribute to generating abnormal motor responses to PICO stimulation. For example, Bautista et al. (1995 - 10.1152/japplphysiol.01356.2011) documented that intermittent hypoxia*

> *induces changes in the activity of laryngeal motoneurons by neural plasticity mechanisms involving serotonin.*

Yes, we thank the reviewer for this comment and we agree that CIH effects multiple brainstem regions. We stated in the manuscript that we are measuring changes in two muscle complexes which spread among three motor neuron pools: hypoglossal nucleus, trigeminal nucleus, and nucleus ambiguus. We have added a discussion on laryngeal activity in the presence of acute bouts of extreme hypoxia, acute intermittent hypoxia, as well as chronic intermittent hypoxia.

- *To support the hypothesis that PICO is directly involved in swallow pattern generation the authors should perform the inhibition of Vglut2-ChAT neurons from PICO and then evoke swallow motor responses. If swallow is abolished when the neurons from this region are inhibited, it would indicate that PICO is crucial to generate this behavior.*

Thank you. We would like to clarify: “involvement” does not mean “necessary for”. Confusing this difference has caused much confusion and debate in the field. Just as an example: We can argue in great length whether inhibition is necessary for respiratory rhythmogenesis in vivo, but I think there is no question that inhibition is involved in respiratory rhythmogenesis in vivo. But to avoid any confusion, we have changed the text to suggest PiCo is involved in the modulation of swallow motor sequence. We agree various additional inhibition experiments are necessary to explain if PiCo is also a necessary component of the SPG, but this is not the question we have set out to address in this study. To specifically target PiCo we must not only inhibit Vglut2 neurons but neurons that express both ChAT and Vglut2. To our knowledge there are no inhibitory DREADD or opsin techniques for cre/FlpO to specifically target these neurons. As stated above, non-experts in the field do not appreciate this technical nuance. However, we have begun to develop novel techniques necessary to inhibit these specific neurons which will be published in the future.

- *In almost all the data presented, the authors observed different patterns of changes in the motor submental and laryngeal responses to PICO activation, including that animals submitted to CIH (6%) presented a "normal" motor response. However, the authors did not discuss the possible explanations and functional implications of this variability.*

We agree that it would be helpful to understand the functional implications of these alterations in swallow-related motor activation, however since we are not using any tools to measure or evaluate functional activity it would be inappropriate to make suggestions of this type without any data to back up our conclusion. This is why we have not included any functional implications. We have added the following to the manuscript. “While fine wire EMG studies are an excellent evaluation tool to observe temporal motor pattern of sequential swallow related muscles; it must be combined with tools such as videofluoroscopic swallow study (VFSS) and/or high resolution manometry (HRM) in order to characterize the functional significance of these alterations to the swallow motor pattern shown in this study (Park et al., 2017). Since the preparation in this study utilizes only fine wire EMGs we are not able to evaluate or comment on the functional significance of the variable swallow motor patterns.”

- *In Figure 4, the authors need to present low magnification sections showing the PICO transfected neurons as well as the absence of transfection in the ventral respiratory column. The authors could also check the scale since the cAmb seems very small.*

Thank you, added different histology images to have a more comparable cAmb. As well as added lower magnification to show absence of transfection in the VRC.

> - *Finally, the title does not reflect the study. The present study did not demonstrate that PICO is a swallow pattern generator.*

We have also changed the title to say: Chronic Intermittent Hypoxia reveals the role of the Postinspiratory Complex in the mediation of normal swallow production