Increase of breathing rate mediated by unilateral optogenetic inactivation of inhibitory neurons in the preBötzinger Complex in vivo

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Abstract:

Brainstem neural circuits located in the preBötzinger complex (preBötC) and Bötzinger complex (BötC) play a critical role in the control of breathing. In this study, glycinergic preBötC and BötC neurons were inactivated using optogenetics in vivo using mice with Cre inducible expression of eNpHR3.0-EYFP. Unilateral inhibition of glycinergic neurons in the preBötC, and to a lower extend also in the BötC, led to a higher respiratory rate. It can be concluded that functional inactivation of inhibitory neurons leads to a disinhibition of preBötC excitatory neurons and thus an increase in the respiratory drive of the network.

Introduction

Breathing is controlled by rhythmic neuronal activity produced by brainstem neural circuits (Smith et al, 2013). The most important part, the ventral respiratory column consists of several subregions including the Bötzinger complex (BötC) and the preBötzinger complex (preBötC). The preBötC and BötC interact with each other as well as other compartments of brainstem to regulate inspiration and expiration. The preBötC is known as the critical part that generates inspiratory neuronal activity by a network of interconnected glutamatergic neurons (Smith et al., 1991, McKay et al., 2005; Feldman et al., 2006; Smith et al., 2007; Alheid et al., 2008; Burke et al., 2010; Tan et al., 2010; Ausborn et al., 2018). Post-inspiratory neurons (located mainly in the BötC), are known to inhibit the preBötC inspiratory neurons and this interaction leads to the inspiratory off switch (Fortuna et al. 2016; Ausborn et al., 2018; Hülsmann et al., 2021). It is also known that unilateral optogenetic activation of glycinergic neurons in the preBötC causes a tonic inhibitory effect, leading to a reduction of respiratory rate (Hülsmann et al., 2021). The aim of the present study was to test the effect of inhibitory neurons activity reduction on breathing. Therefore, we used a mouse line with Cre-inducible expression of halorhodopsin (eNpHR3.0-EYFP) to optogenetically inhibit glycinergic neurons in the preBötC and BötC.

Methods:

Ethics and Animal Handling:

Breeding, handling, and all experimental procedures were done in compliance with the welfare of experimental animal guidelines issued by the European Communities Council Directive (2010/63/EU) and the German Protection of Animals Act (Tierschutzgesetz; TierSchG). All the procedures were approved by the Animal Welfare Commission of University Medical Center Göttingen, Germany and Niedersächsische Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES).

Mice expressing eNpHR3.0-EYFP in glycinergic neurons were generated by crossbreeding mice expressing Cre-recombinase under the control of the glycine transporter 2 promoter (Tg(Slc6a5icre)121Veu; Ishihara et al., 2010) with B6;129S-Gt(ROSA)26Sortm39(CAG-hop/EYFP)Hze/J mice (JAX stock #014539), which provide a tool for Cre-dependent eNpHR3.0 EYFP expression (Madisen et al., 2012). For the experiments, we used male and female mice heterozygous and homozygous for the floxed eNpHR3.0-EYFP and positive for Slc6a5icre were used (GlyT2-ai39). For the negative control experiments, littermates from the breading that did not express the Cre-recombinase were used (GlyT2 cre-).

Regarding age and body weight, no significant difference was detected between the experimental groups. The age of the mice ranged from postnatal day P39 to P163 and the body weight was in the range of 26 to 36g.

Stereotactic optical fiber implantation

Prior to surgery, the mice received Carprofen (5 mg/kg) for analgesia 30 minutes (subcutaneous). Afterwards they received (intraperitoneal) a combination by Ketamine (200 mg/kg) / Medetomidine (0.5 mg/kg), and Lidocaine (16 mg/kg) (intraperitoneal) for anesthesia. All drugs were dissolved in NaCl 0.9%. The depth of anesthesia was controlled every 10 min by the absence of the tail and interdigital reflex response during the entire experiment. The animals were placed in a stereotaxic frame (robot stereotaxic; Neurostar, Tübingen, Germany) and received O_2 via a silicon tube placed in front of their nostrils (0.4 l/min). A small skin incision was made above the bregma and lambda. The coordinates used for the preBötC and BötC was Bregma −6.72 mm, 1.30 mm lateral for the preBötC and Bregma −6.60 mm, 1.25 mm lateral for BötC. The

Neurostar automatic stereotaxic software was used to define the correct drilling location.

For photostimulation, we used the 200 µm diameter and 5 mm length fiber optic cannula (CFMLC12L05; Thorlabs, Newton, NJ, USA) connected with a ceramic split mating sleeve (ADAL1; Thorlabs, Newton, NJ, USA) to a 400 µm fiber patch cable (MAF2L1; Thorlabs, Newton, NJ, USA). The tip of the fiber was placed in the preBötC (Bregma −6.72 mm, Lateral 1.30 mm) or BötC (Bregma −6.60 mm, Lateral 1.25 mm). Unilateral illumination to the desired areas was achieved by 545nm (light irradiance of >80 mW/mm² at fiber implant tip) Optogenetics-LED-Lime-Green (Prizmatix Ltd. Southfield, MI, USA). The LED was controlled by the digital output of an Axon Digidata 1440A Digitizer and Axon pCLAMP™ software (Molecular Devices LLC., San Jose, CA, USA).

Monitoring of animals

Breathing was monitored with a piezo ceramic element (FT-31T-1.3A1-472; KEPO, Ningbo, China), which was placed underneath the thorax. Peripheral oxygen saturation and heart rate of the animal was monitored with a Pulse Oximeter (MouseOx plus, Starr Life Sciences Corp., PA, USA). Breathing, heart rate and SpO2 were recorded with Axon™Digidata 1440A digitizer using pCLAMP™/clampex software (Molecular Devices LLC., San Jose, CA, USA). Offline analysis was performed by LabChart 8 software (ADInstruments, Spechbach, Germany).

The mice were perfused by Phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA) at the end of the experiment and the extracted brain was post fixed in 4% PFA overnight. The brain medullary area was sectioned (150 µm thickness) by vibratome (Leica VT1200s) and the correct fiber position was confirmed.

Genotyping

Mice were genotyped by PCR using the primers 5′-ATTGCCTACAACACCCTGCTGC-3′ and 5′-CCACACCATTCTTTCTGACCCG-3′ for GlyT2-cre and 5′-TTCACCTTGATGCCGTTCT-3′ and 5′-GCCGCTACCCCGACCAC for eNpHR3.0-EYFP.

Statistical analysis

Light stimulation preceded and followed by a period of at least 10 s without light stimulation and it was repeated for 2-5 times per animal. The respiratory rate (fR) was determined by counting the breaths during a 10 s period. This data was multiplied by 6 to calculate breaths-per-minute (min^{-1}). The data from the repetitive stimulations was averaged. The cycle-to-cycle variability of the breathing interval (int) was calculated as $IrrScore_{Int} = 100 \cdot [(Int(n)- Int(n-1))/Int(n-1)]$ (Hirrlinger et al., 2019). Data is given as Mean ± SD.

The mean fR and $IrrScore_{Int}$ before and during illumination was compared by paired T-Test using GraphPad 9 (GraphPad Software, CA, USA). The relative increase of the respiratory rate (ΔfR, in % of base line) and fR was compared between preBötC and BötC using unpaired T-tests.

Results

Inhibition of inhibitory neurons in the preBötC

In a first set of experiments, glycinergic neurons in the preBötC were continuously inhibited by constant light application. This led to an increase of the respiratory rate to 93.6 ± 25.0 min^{-1} (Mean ± SD) as compared to baseline 82.0 ± 25.8 min^{-1} (Mean ± SD) (n = 6, p= 0,0005; Fig 1, A & C), which equals to an increase by 16.1 ± 7.5% (Mean ± SD). Since partial inactivation of inhibitory neurons has been shown to increase the irregularity of the breathing rhythm (Hirrlinger et al. 2019), we also analyzed if the irregularity score of the breath-to-breath interval ($IrrScore_{Int}$) was altered by inhibition of preBötC inhibitory neurons. There was no difference between the $IrrScore_{Int}$ for the period before stimulation 0.24 ± 0.08 (Mean ± SD) and during the stimulation 0.29 ± 0.17 (Mean ± SD, p=0.64). To exclude that thermal effects of the optical stimulation on the neuronal circuits are responsible for observed modulation of the respiratory rate, we also tested our stimulation protocol in GlyT2 cre-mice that did not express eNpHR3.0. Here, illumination did not induce alterations in respiratory rate 144.2 ± 22.3 min^{-1} (Mean ± SD) in comparison to control (144.7 ± 21.5 min^{-1} Mean ± SD; n = 3, p= 0.42).

Inhibition of inhibitory neurons in the BötC

Glycinergic neurons in the BötC were continuously inactivated by constant light application (10s). This led to a significant increase of the respiratory rate to 120.6 ± 25.6 min^{-1} (Mean ± SD) compared to baseline 114.2 ± 24.5 min^{-1} (Mean ± SD) (n = 6, p =0.001; fig 1, B & D). The overall increase of only 5.9 ±1.9 % (Mean ± SD) was significantly smaller than the effect we observed in the preBötC (p=0.009; Fig 1, E). The $IrrScore_{Int}$ was 0.23 ± 0.18 (Mean ± SD) before stimulation and 0.22 ± 0.18 (Mean ± SD, p=0.8) during the stimulation. In GlyT2 cre- mice no differences in respiratory rate during optical stimulation 134.9 ± 28.3 min-1 (Mean ± SD) and control (135.4 ± 29.7 min-1; Mean ± SD, n = 3, p=0.61) were detected.

Discussion

There are several studies that showed a decrease of the respiratory rate during optogenetic activation of inhibitory neurons in the ventral respiratory column (VRC) (preBöC and/or BötC; Sherman et al., 2015; Ausborn et al., 2018; Fortuna et al., 2019; Hülsmann et al., 2021). Moreover, repetitive phasic activation of glycinergic neurons can also increase the respiratory rate depending on the stimulation protocol (Hülsmann et al., 2021). Thus, it appears that there is a dual function of the inhibitory input to the rhythm generating respiratory neurons in the preBötC: tonic inhibition and phase resetting.

In the present study, inhibitory neurons of either preBötC or BötC were inhibited unilaterally, leading to a moderate but significant increase of the respiratory rate. Interestingly, the relative increase of the respiratory rate by stimulation of the preBötC was more robust (Fig. 1, E), suggesting that also local inhibitory neurons in the preBötC contribute to the effect. Although we placed the optical fiber unilaterally, we can assume that the contralateral VRC was also affected, thus disinhibited, since it is known that preBötC receive synaptic glycinergic inhibition from the contralateral side (Yang & Feldman 2018; Fortuna et al., 2019).

Mouse models with alterations of inhibition have been shown to increase the overall variability of breathing (Hülsmann et al., 2016; Hirrlinger et al., 2019). In the conditional KO for the vesicular inhibitory amino acid transporter (VIAAT) in GABA/Glycine cotransmitting neurons (GGCN) mice, the only phenotype that was detected was increase of the irregularity of the breath to breath variably, leading to longer pauses and an increase of the Irregularity score of the interval ($IrrScore_{Int}$; Hirrlinger et al., 2019). Here, however, optogenetical inhibition of inhibitory neurons did not increase the irregularity, nor did it induce apneas. Thus, the changes observed in the mouse models may be related to adaptive developmental processes, which are not effective during acute short term (10s) disinhibition of the preBötC in our anesthetized mice. Indeed, anesthesia itself might be a factor, since the regularity of breathing certainly is influenced by the behavioral state of the animal (Ren et al. 2012; Le Gal, et al. 2020, Maric et al. 2020).

Limitation of the study

When interpreting our data, one has to consider that there is a significant number GABA/glycine cotransmitting neurons among halorhodopsin expressing inhibitory neuron population (Hirrlinger et al., 2019; Rahman et al., 2015). Some of the neurons might have be exclusively GABAergic, since it has been shown that some neurons that are GABA/glycine cotransmitting during embryonic development can differentiate into some GABAergic neurons (Hirrlinger et al.,2019). Nevertheless, it can be assumed that the majority of the stimulated neurons released glycine.

Apparently, the baseline respiratory rate (f_R) of the mice of the preBötC group tended to be lower as compared to the BötC group. Although this difference was not significant ($p=0.0592$), we cannot rule out that the lower relative increase observed in the BötC group is due to an already higher respiratory rate.

Conclusion

Tonic inhibition plays a significant role in regulation of the respiratory rate *in vivo*. In this study, we showed that already unilateral optogenetic inhibition of inhibitory, mostly glycinergic neurons in the preBötC and to a lower extend also of the BötC is sufficient to disinhibit the preBötC and thus, the output of the respiratory network**.**

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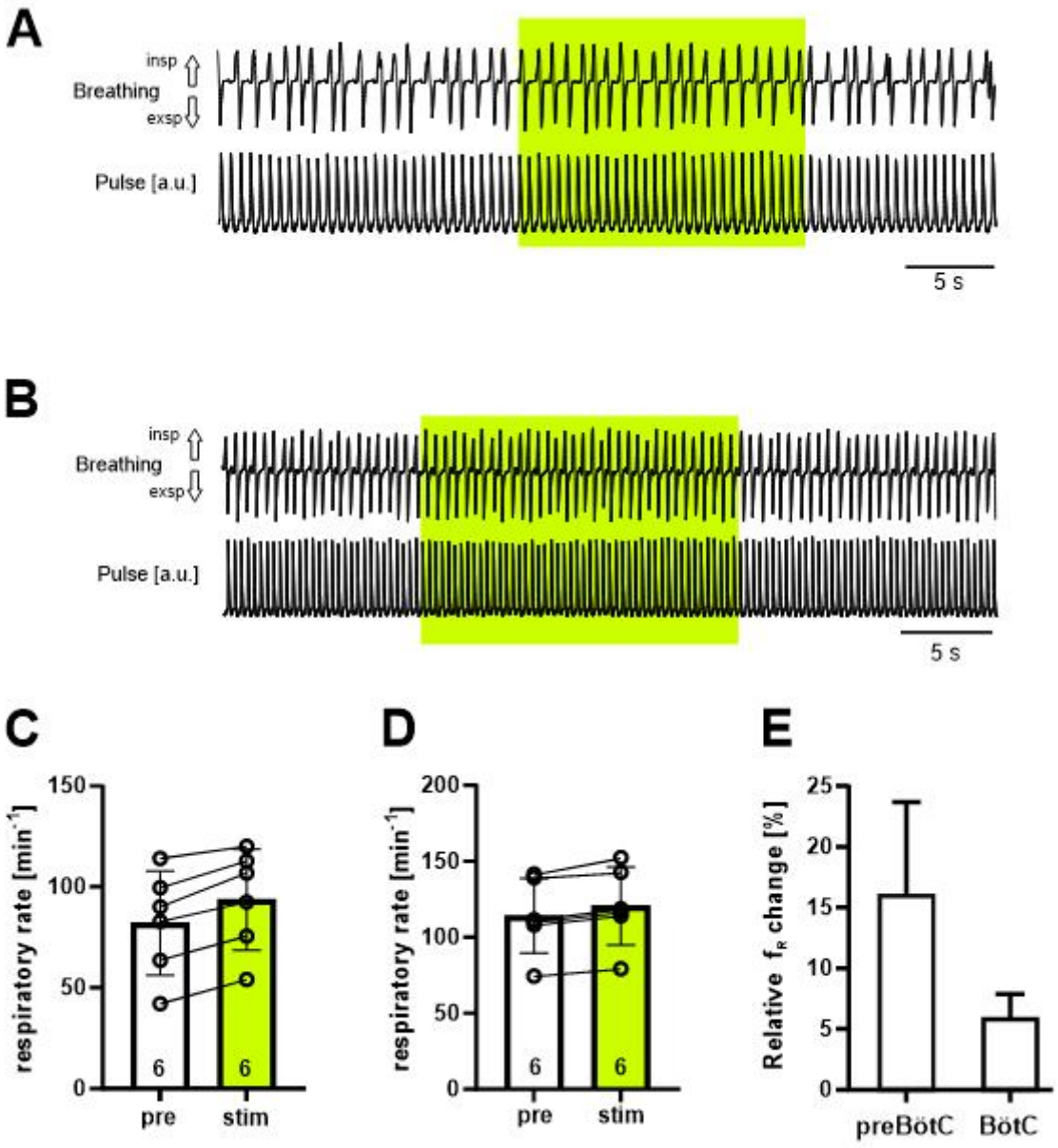
Figure legend

Fig 1. Continuous optogenetic inhibition of inhibitory neurons in preBötC & BötC increases the resipration rate. A, B: Breathing (inspiration upward and expiration downward) recorded by the piezo sensor underneath the thorax of the animal (upper traces) and pulse wave of the pulse oximetry (lower trace). The periode of optogenetic inhibition of inhibitoriy neurons in the preBötC (A) and BötC (B) is marked in green. **C:** Statistical comparisson reveals an increase of the respiration rate during stimulation in preBötC (n = 6, p = 0.0005, Paired T-test, Mean ± SD before stimulation 82.0 ± 25.8 min^{-1}, during stimulation, 93.6 ± 25 min^{-1}). **D:** Statistical comparisson shows a small increase of the respiration rate during stimulation in BötC (n = 6, p = 0.001, Paired T-test Mean ± SD before stimulation 114.2 ± 24.5 min^{-1}, during stimulation 120.6 ± 25.6 min^{-1}). **E**: The relative increase of the respiratory rate induced by optogenetic inhibition of the inhibitory neurons in the preBötC 16.1 ± 7.5% (Mean ± SD) was significantly larger as compared to the BötC 5.9 ±1.9 % (Mean ± SD, p = 0.009).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Relative fR change [%]
25
20
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preBötC
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