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Distinct parafacial regions in control of breathing in adult rats

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Abstract

Recently, based on functional differences, we subdivided neurons juxtaposed to the facial nucleus into two distinct populations, the parafacial ventral and lateral regions, i.e., pFV and pFL. Little is known about the composition of these regions, i.e., are they homogenous or heterogeneous populations? Here, we manipulated their excitability in spontaneously breathing vagotomized urethane anesthetized adult rats to further characterize their role in breathing. In the pFL, disinhibition or excitation decreased breathing frequency (f) with a concomitant increase of tidal volume (VT), and induced active expiration; in contrast, reducing excitation had no effect. This result is congruent with pFL neurons constituting a conditional expiratory oscillator comprised of a functionally homogeneous set of excitatory neurons that are tonically suppressed at rest. In the pFV, disinhibition increased f with a presumptive reflexive decrease in VT; excitation increased f, VT and sigh rate; reducing excitation decreased VT with a presumptive reflexive increase in f. Therefore, the pFV, has multiple functional roles that require further parcellation. Interestingly, while hyperpolarization of the pFV reduces ongoing expiratory activity, no perturbation of pFV excitability induced active expiration. Thus, while the pFV can affect ongoing expiratory activity, presumably generated by the pFL, it does not appear capable of directly inducing active expiration. We conclude that the pFL contains neurons that can initiate, modulate, and sustain active expiration, whereas the pFV contains subpopulations of neurons that differentially affect various aspects of breathing pattern, including but not limited to modulation of ongoing expiratory activity.

Introduction

Several brainstem motor nuclei are surrounded by respiratory-related neurons [1, 2]. In the case of the facial nucleus, parafacial neurons are essential components of the breathing central pattern generator (bCPG). In particular, parafacial neurons that express the neurokinin-1 receptor (NK1R), the homeobox gene Phox2b, and the glutamate transporter VGlut2, are essential to CO₂ chemoreception [3–6]; notably, a subpopulation of these neurons have rhythmic respiratory-related activity, both in vitro and in vivo [7–9], leading us to postulate that...
Fig 1. Histological analysis of parafacial regions. A) Localization of injections into pF\textsubscript{V} and pF\textsubscript{L}. Transverse view of medulla at Bregma -11.25 mm. Red circles show locations of injection sites for pF\textsubscript{V} and pF\textsubscript{L}. Green dashed box is magnified in C. B) Ventral view of medullary surface with location of pF\textsubscript{V} and pF\textsubscript{L} injection sites, marked by white circles, superimposed. C) Micrographs of injection sites. Green marks staining for choline acetyl transferase (ChAT), highlighting the cholinergic neurons of the facial (VII) nucleus, and red marks fluorescent beads coinjected with micropipette solutions in to the pF\textsubscript{V} and pF\textsubscript{L}. Py—Pyramidal tract, SP-5—Spinal trigeminal tract, 7n—Facial nucleus.
breathing is driven by a dual oscillator system [10]. We identified two neighboring parafacial regions, lateral (pF_L) and ventral (pF_V) that appear to be functionally distinct components of the bCPG [11]. We hypothesized that the pF_L is a conditional expiratory oscillator that is inhibited at rest [8, 11, 12], whereas the pF_V provides a generic source of excitatory drive for both inspiration and expiration whose activity depends, at least in part, on CO₂-related signals [11, 13–15]. Furthermore, two parafacial subpopulations, containing Gastrin-Releasing Peptide and Neuromedin B (GRP and NMB, respectively) modulate sighing [16]. Therefore, further subdivision of the parafacial region into functionally distinct nuclei may be warranted, as is the case for other subcortical brain regions, such as the nucleus tractus solitarius, periaqueductal gray, and paraventricular nucleus [17–19]. To further investigate the functional contributions of the pF_L and pF_V, we selectively modulated their excitability and measured the effects on ventilation in spontaneously breathing vagotomized urethane anesthetized adult rats.

We conclude that the: i) pF_L contains a functionally homogenous population of excitatory neurons that are tonically inhibited at rest, which following an increase in excitability can initiate and maintain active expiration; ii) pF_V contains at least four functionally distinct subpopulations of neurons: three subpopulations that are tonically inhibited at rest, which can separately affect f, modulate active expiration, and modulate basal sigh rate, and one tonically active subpopulation that predominately affects V_T. Interestingly no subpopulation of pF_V neurons appears capable of directly inducing active expiration; instead the pF_V modulates active expiration generated elsewhere, presumably by effects in the pF_L and/or (pre)motoneuron pools.

**Methods**

All protocols were approved by the University of California Los Angeles Chancellor’s Animal Research Committee. All experiments were performed in spontaneously breathing vagotomized urethane anesthetized adult Male Sprague-Dawley rats (350–450 g) rats.

**Ventral approach**

Anesthesia was induced with isofluorane and maintained with urethane (1.2–1.7 g/kg; Sigma) in sterile saline via a femoral catheter. Rats were placed supine in a stereotaxic apparatus on a heating pad to maintain body temperature at 37±0.5°C. The trachea was cannulated. Respiratory flow was monitored via a flow head (GM Instruments), and CO₂ via a capnograph (Type 340: Harvard Apparatus) connected to the tracheal tube. Paired electromyographic (EMG) wires (Cooner Wire Co.) were inserted into genioglossal (GG), diaphragmatic (Dia), and oblique abdominal muscles (Abd). Anterior neck muscles were removed, a basiooccipital craniotomy exposed the ventral medullary surface, and the dura was resected. After bilateral
vagotomy, exposed tissue around the neck and mylohyoid muscle were covered with dental putty (Reprosil; Dentsply Caulk) to prevent drying. Rats were left for 30 minutes for breathing to stabilize. At rest, ventilation consisted of alternating active inspiration and passive expiration. Once stabilized, solutions of drugs in micropipettes were pressure injected (100–200 nL) bilaterally using a Picospritzer II (General Valve Corp.) controlled by a Master 8 pulse generator (AMPI) into the pFL bilaterally. At rest, the pFL was defined as the area ventral to the lateral edge of the facial nucleus, juxtaposed to the spinal trigeminal tract [11]. The pFV was defined as the area ventral to the caudal half of the facial nucleus, at a central location between the pyramidal tract and the spinal trigeminal tract [11]. Coordinates: lateral from the basilar artery, rostral from the rostral hypoglossal nerve rootlet, and dorsal from the ventral surface (in mm); pFL: 1.8, 0.6, 0.1, and pFV: 2.5, 0.9, 0.2.

Injections contained: i) baclofen (250 μM; Tocris) and strychnine hydrochloride (250 μM; Sigma) (B+S) to antagonize GABA_A and glycine receptors, respectively. Injections of B+S led to disinhibition of the pFL (B+S_pFL) or pFV (B+S_pFV); ii) AMPA (20 μM; Sigma) to activate glutamatergic AMPA receptors. Injections of AMPA lead to excitation of the pFL (AMPA_pFL) or pFV (AMPA_pFV) or; iii) 2-amino-5-phosphopentoic acid (AP-5; 1mM; Sigma) and 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX; 1mM; Sigma) (A+N) to antagonize glutamatergic NMDA and AMPA receptors, respectively. Injections of A+N reduced excitation in the pFL (A+N_pFL) or pFV (A+N_pFV). All drugs were diluted in sterile saline balanced with NaOH to pH 7.35.

In one set of experiments, a ventral approach to the medulla was performed in vagus-intact rats. After a resting period to allow breathing to stabilize, rats received 100–200 nL bilateral injections of glutamate (10 mM; Sigma) administered at ~50 nL/min into the pFV (Glu_pFV), following which breathing was allowed to recover. After breathing returned to baseline levels, rats were bilaterally vagotomized at the mid-cervical level. Breathing was allowed to stabilize (~30–60 mins), following which rats received a second bilateral injection of Glu_pFV.

Care was taken to reduce any transient effects of mechanical stimulation when placing the pipette into the tissue. As experimental controls to determine whether insertion of the pipette and injection of solution per se had effects, we tested the effects of saline injections. All injections contained fluorescent beads (red fluoSpheres; Invitrogen) to allow for post-hoc identification of injection sites.
Fig 3. **B + S**<sub>PFV</sub> increases *f*, decreases *V<sub>T</sub>* and induces post-inspiratory activity in abdominal muscles and pre- and post-inspiratory activity in gengioglossus muscles. A) Integrated traces from a single experiment. Black arrows at bottom indicate epochs in expanded traces (Bi and Bii), gray arrows at top indicate unilateral.
Localization of injection sites (Fig 1)

Rats were sacrificed by overdose of urethane and transcardially perfused with saline followed by cold (4˚C) paraformaldehyde (PFA; 4%). The medulla was harvested and postfixed in 4% PFA overnight at 4˚C, then cryoprotected in sucrose (30%) in standard PBS (1–3 days at 4˚C). PBS contained (mM): NaCl 137, KCl 2.7, Na2HPO4 10, KH2PO4 1.8, pH 7.4. Brainstems were transversely sectioned at 40 μm. Free-floating sections were incubated overnight in PBS containing 0.1% Triton X-100 (PBT) and mouse anti-NeuN primary antibody (1:500; EMD Millipore) or goat anti-cholineacetyl transferase (ChAT; 1:100; EMD Millipore). The tissue was washed in PBS, 6 times for 5 minutes per wash, and then incubated separately for 2–4 hours in a solution of PBT containing either donkey anti-mouse Alexa Fluor 647 secondary antibody (1:250; Jackson ImmunoResearch Laboratories, Inc.) or donkey anti-goat Alexa Fluor 488 (1:250; Invitrogen), for NeuN and ChAT, respectively. The tissue was washed in PBS, 6 times for 5 minutes. Slices were mounted onto polylysine-coated slides, dehydrated overnight at 22˚C, and coverslipped using Cytoseal 60 (Electron Microscopy Sciences). Slides were analyzed using a fluorescent microscope with AxioVision acquisition software (AxioCam2, Zeiss).

Data analysis and statistics

EMG signals and airflow measurements were collected using preamplifiers (P5; Grass Instruments) connected to a Powerlab AD board (ADInstruments) in a computer running LabChart software (ADInstruments), and were sampled at 400 Hz/channel. High pass filtered (>0.1 Hz) flow head measurements were used to calculate: tidal volume (V_T, peak amplitude of the integrated airflow signal during inspiration; pressure sensors were calibrated with a 3 mL syringe); V_T is expressed as mL. Inspiratory duration (T_I, beginning of inspiration until peak V_T), expiratory duration (T_E, peak V_T to the beginning of the next inspiration), and f (1/[T_I+T_E]); T_I and T_E, are expressed in secs (s), and f is expressed as breaths per minute (BPM). Minute ventilation (V_e) was calculated as f x V_T, and is expressed as mL/min. EMG data were integrated (τ = 0.05 s; ∫DiaEMG, ∫GGEMG, and ∫AbdEMG in arbitrary units (a.u.)) and the peak amplitude of each signal computed for each cycle.

To obtain control values, all parameters were averaged for 20 respiratory cycles preceding each injection. To measure drug effect, 20 cycles were averaged during a period where the injection had its greatest effect on the airflow channel. Measurements were only made of the initial response to the drug, usually within the first 5 minutes following the 2nd injection, at a similar time as the expanded traces in the figures (marked in each figure by a black arrow with a black dotted line). Care was taken to avoid measurements where reflexive changes had taken place, for example, where the drug caused an initial decrease in breathing followed by a compensatory increase in breathing as the compound wore off. In these cases, measurements were taken at the peak effect during initial decrease and not during the reflexive increase that followed. Data was analyzed offline and exported to Excel™ (Microsoft) for further analysis. All statistical tests were performed using Igor Pro™ (WaveMetrics), except 2-way ANOVAs which were performed in OriginPro™ (OriginLab).

As described above, for each rat we calculated the average of 20 cycles preceding the stimulus (control), and the average of 20 cycles during the stimulus (stimulus). Both groups, the control...
values and their associated stimulus value for every rat, were combined into a single data set. To facilitate graphical comparisons data was normalized to the highest value in the data set regardless of whether it belonged to control or stimulus group. Therefore, the highest value in the data set, whether it be control or stimulus, was 1.0.

We define active expiration as the epoch of appearance of burst activity in expiratory muscles, i.e., abdominals, that leads to forced air outflow, typically during late expiration, and consequently, increased \( V_T \) in the following inspiration. We define sighs by their characteristic augmented \( V_T \) caused by a second inspiratory effort that occurs before the initial eupneic inspiration is complete. These augmented breaths result from largely from high amplitude inspiratory \( \Delta V \) caused by a second inspiratory effort that occurs before the initial eupneic inspiration is complete.

These augmented breaths result from largely from high amplitude inspiratory \( \Delta V \) caused by a second inspiratory effort that occurs before the initial eupneic inspiration is complete.

Results

Disinhibition of \( pF_L \) or \( pF_V \) affect breathing pattern (Figs 2–5, Table 2)

Disinhibition of \( pF_L \) neurons by the GABAergic antagonist bicuculline and the glycine antagonist strychnine (B+S\( \text{pFL} \)) can induce active expiration [8, 11], which we confirm here. Bilateral injection of B+S\( \text{pFL} \) (\( n = 8 \)) decreased \( f \) and \( T_i \), increased \( T_e \), \( V_T \), \( \int \Delta V_{\text{EMG}} \), and inspiratory-related \( \int G_{\text{EMG}} \) activity, and induced rhythmic expiratory bursting in \( \int G_{\text{EMG}} \) and \( \int \Delta V_{\text{EMG}} \) (Fig 2), the latter a signature of active expiration, q.v., [8, 11]. Bilateral B+S\( \text{pFL} \) had no effect on minute ventilation \( (V_e) \) due to a compensatory increase in \( V_T \) in response to the changes in \( f \) elicited by the antagonism of the inhibitory receptors.

Disinhibition of \( pF_V \) neurons by unilateral injection of bicuculline increases \( V_T \) with a reciprocal decrease in \( f \) in awake rats [21]. Furthermore, \( pF_V \) appears to facilitate active expiration through projections to abdominal and genioglossus motoneurons, but does not itself induce active expiration [11]. We therefore expected that \( pF_V \) disinhibition with a cocktail of bicuculline and strychnine (B+S\( \text{pPV} \)) would increase \( V_e \), as well as alter abdominal and genioglossus activity, but would not induce active expiration. Bilateral injections of B+S\( \text{pPV} \) (\( n = 8 \)) increased \( f \), decreased \( T_i \), did not alter \( T_e \), and decreased \( V_T \) and \( \int \Delta V_{\text{EMG}} \). Bilateral B+S\( \text{pPV} \) in anesthetized rats did not alter \( V_e \) due to a compensatory decrease in \( V_T \) in response to an increase in \( f \) elicited by the antagonism of inhibitory receptors, which is the opposite response to unilateral injection of bicuculline in the same region in awake rats [21]. \( pF_V \) disinhibition had multiple effects on genioglossus activity, increasing inspiratory-related \( \int G_{\text{EMG}} \) and
inducing both pre-inspiratory and post-inspiratory \( f \) GGEMG activity (Fig 3). In 6 out of 8 experiments, B+SpFV also induced high amplitude post-inspiratory AbdEMG activity (Fig 3A† and 3Bii†), which while rhythmic was slow, occurring every 10 ± 1 breaths. This pattern of high amplitude post-inspiratory AbdEMG activity was distinct from active expiration that

Table 2. Median and interquartile range for all recorded variables.

<table>
<thead>
<tr>
<th></th>
<th>( f ) (BPM)</th>
<th>( T_I ) (secs)</th>
<th>( T_E ) (secs)</th>
<th>VT (mL)</th>
<th>DiaEMG (a.u.)</th>
<th>GGEMG (a.u.)</th>
<th>AbdEMG (a.u.)</th>
<th>VE (mL/min)</th>
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<td>17, 20</td>
<td>16, 27</td>
<td>219, 47</td>
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<td>0.008</td>
<td>0.008</td>
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<td>0.008</td>
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<td>6, 8</td>
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<td>2,3</td>
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<td>0.3</td>
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<td>0.7</td>
<td>0.5</td>
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<td>0.6</td>
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A-B) Agonists and antagonists injected into the pF_L (A) and pF_V (B). C) Glutamate injected into the pFv of vagus-intact (VI) and vagotomized (Vx) rats. D) Saline injected into the pFv or pFL. All tables display data as: median, IQR.

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occurs between every inspiration at a lower amplitude (see Fig 2 and [8, 11]. Interestingly, coincident with high amplitude post-inspiratory AbdEMG bursts, there was inhibition of GGEMG activity, showing co-ordination between GGEMG and AbdEMG during expiration (Fig 3B). In all experiments, B+S_pFL also induced sighs i.e., augmented breaths with high amplitude inspiratory DiaEMG followed by prolonged TE (Fig 3A# and 3Bii#); sighs were rhythmic but slow, occurring every 12 ± 1 breaths. The high amplitude post-inspiratory AbdEMG activity was not coordinated with sighing.

To test for any nonspecific effects of pFV or pFL injections on breathing, we injected saline into both regions. In anesthetized rats, saline injections in the pFL (n = 8) did not alter f, T_i, T_E, V_T, DiaEMG, GGEMG, AbdEMG, or V_E (Fig 4). In anesthetized rats, saline injections in the pFV (n = 8) did not alter f, T_i, T_E, V_T, DiaEMG, GGEMG, AbdEMG, or V_E (Fig 5).

Excitation of either pFL or pFV affects breathing pattern (Figs 6–8, Table 2)

Photostimulation of pFL neurons elicits active expiration [8]. We predicted that excitation of the pFL with the glutamatergic agonist AMPA (AMPA_pFL) would also elicit active expiration. Bilateral injections of AMPA_pFL (n = 8) decreased f and T_p, and increased T_E, V_T, DiaEMG, GGEMG activity and AbdEMG (Fig 6), the latter a signature of active expiration, q.v., [8, 11]. Like B+S_pFL, bilateral injections of AMPA_pFL did not affect V_E, presumably due to a compensatory increase in V_T in response to the decrease in f.

Excitation of pFV neurons by injection of glutamate increases phrenic nerve discharge amplitude and induces sighing in urethane anesthetized, paralyzed, artificially ventilated, vagotomized cats [22]; photostimulation of pFV neurons leads to increased sighing and respiratory frequency in conscious rats [23]. We predicted that excitation of the pFV with AMPA (AMPA_pFV) would increase ventilation and sighing. Bilateral injection of AMPA_pFV (n = 8) increased f, decreased T_i, did not alter T_E, and increased V_T, DiaEMG, and inspiratory-related GGEMG but neither induced expiratory-modulated GGEMG nor AbdEMG (Fig 7). Unlike B +S_pFV, bilateral injections of AMPA_pFV increased V_E due to increases in both V_T and f. In 5 out of 8 rats, before AMPA_pFV caused V_T to reach maximal amplitude it induced 1–2 sigh like events, but with no associated GGEMG or AbdEMG activity (data not shown).

The lack of induction of sighing could have been due to either the increased V_T in vagotomized rats, or due to the lack of activation of other glutamatergic receptors, e.g., NMDA, mGluR, etc, in addition to AMPA receptors. To explore these possibilities, in separate experiments, we injected glutamate into the pFV (Glu_pFV) of anesthetized rats before and after vagotomy. Before vagotomy (n = 8), bilateral Glu_pFV decreased f, increased T_i, T_E, V_T, DiaEMG, inspiratory-related GGEMG, and sigh rate, but neither induced expiratory-modulated GGEMG nor AbdEMG (Fig 8). Bilateral injections of Glu_pFV did not affect V_E due to a compensatory decrease in f in response to an increase in V_T, elicited by the activation of glutamate receptors. Following vagotomy, bilateral Glu_pFV increased f, decreased T_i, did not alter T_E, and increased V_T, DiaEMG, and inspiratory-related GGEMG, but neither induced expiratory-modulated GGEMG nor AbdEMG (Fig 8), similar to AMPA_pFV (Fig 7). Like AMPA_pFV, bilateral injections of Glu_pFV increased V_E due to increases in both V_T and f. In 3 out of 6 vagotomized
Distinct parafacial regions in respiratory control

A

\[ 20 \mu M \text{AMPA}_{PF_v} \]

\[ V_T \quad \text{8 mL} \]

\[ \int \text{G}_{EMG} \quad \text{a.u.} \]

\[ \int \text{Dia}_{EMG} \quad \text{a.u.} \]

\[ \int \text{Abd}_{EMG} \quad \text{a.u.} \]

\[ f \quad 45 \text{ BPM} \]

\[ \text{2 mins} \]

Bi

Post

Pre

\[ V_T \]

\[ \int \text{G}_{EMG} \]

\[ \int \text{Dia}_{EMG} \]

\[ \int \text{Abd}_{EMG} \]

\[ f \quad 8 \text{ mL} \]

\[ \text{a.u.} \]

\[ \text{a.u.} \]

\[ \text{a.u.} \]

\[ 45 \text{ BPM} \]

\[ 30 \]

\[ 1 \text{ secs} \]

Bii

Post

Pre

\[ V_T \]

\[ \int \text{G}_{EMG} \]

\[ \int \text{Dia}_{EMG} \]

\[ \int \text{Abd}_{EMG} \]

\[ f \quad 8 \text{ mL} \]

\[ \text{a.u.} \]

\[ \text{a.u.} \]

\[ \text{a.u.} \]

\[ 45 \text{ BPM} \]

\[ 30 \]

C

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rats, before Glu_pFV caused V_T to reach maximal amplitude it induced 3–6 sigh-like events but with no associated GG_EMG or Abd_EMG (data not shown).

**Reduced excitation of pF_V and pF_L have different effects on breathing (Figs 9 and 10, Table 2)**

Many, if not most or all, pF_I neurons are silent at rest [8, 24]; not surprisingly, hyperpolarizing pF_I neurons at rest does not affect ventilation [11]. We predicted that reduction of pF_I excitability with local injection of a cocktail of the glutamatergic antagonists AP-5 and NBQX (A+N_pF) would not affect breathing. Bilateral injections of A+N_pF (n = 8) had no effect on f, T_i, T_e, V_T, f/Dia_EMG or f/GG_EMG; Abd_EMG silent at rest, remained so after A+N_pF (Fig 9). Bilateral injections of A+N_pF did not affect V_E as it neither affected V_T nor f.

By contrast, pF_V neurons are active at rest, providing excitatory drive for quiet breathing [25–29]; hyperpolarizing pF_V neurons reduces ventilation [5, 11, 13]. We predicted that reduction of pF_V excitability with local injection of AP-5 and NBQX (A+N_pFV), would reduce ventilation. Bilateral A+N_pFV (n = 8) increased f, decreased T_i and T_e, V_T, f/Dia_EMG, and f/GG_EMG; Abd_EMG silent at rest, remained so after A+N_pFV (Fig 10). Bilateral injections of A+N_pFV did not affect V_E due to a compensatory increase in f in response to a decrease in V_T, elicited by the activation of glutamate receptors. That no injection into the pF_V induced active expiration is indicative that the injectate did not spread to the adjacent pF_I, likewise since A+N_pF did not induce any changes in breathing, this indicates the injectate did not spread to the adjacent pF_V.

**Discussion**

Since the putative identification of a conditional expiratory oscillator in the rostral medulla [10, 12, 30], attention has focused on regions surrounding the facial nucleus as its location [8, 11, 15, 24, 31]. We identified two functionally separate parafacial regions: the pF_V and pF_L [11]. We propose that the pF_V provides a critical generic drive to breathe, driving inspiration at rest and facilitating both inspiration and expiration when chemoosensory drive increases [11, 15], and that the pF_I is silent at rest, but once activated, drives active expiration [8, 11]. Additionally, there appears to be a third parafacial region, more dorsocaudal, containing neurons expressing gastrin releasing peptide that modulates baseline sigh rate [16]. Thus, there appear to be several distinct parafacial regions contributing to the bCPG. To further investigate the role of parafacial neurons, and the neuronal composition of parafacial regions at the ventral medullary surface, we pharmacologically altered the excitability of pF_V and pF_L neurons and measured the effects on breathing.

**Further support of the hypothesis of the pF_L as the source for active expiration**

Antagonizing ionotropic glutamate receptors with A+N_pF did not alter any respiratory parameter, i.e., no change in f, T_i, T_e, V_T, Dia_EMG, GG_EMG, or Abd_EMG; supporting our previous observation that these neurons are silent at rest, q.v., [8, 11]. In the pF_L, excitation (with AMPA) or disinhibition (by antagonizing ionotropic GABA and glycine receptors with B+S)
Fig 8. Glu\textsubscript{genioglossus} muscles. A) Integrated traces from a single experiment, gray arrows indicate unilateral injections for Glu\textsubscript{genioglossus}. Ai) Vagus intact. Aii) Vagotomized. B) Comparison between ventilation at rest (Rst) and after Glu\textsubscript{genioglossus} regardless of whether it belonged to control or Glu\textsubscript{genioglossus} group. \( ^* \): p < 0.05. Abbreviations defined in Fig 2.

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decreased \( f \) with a compensatory increase in \( V_T \) and inspiratory \( DiaEMG \) and \( GGEMG \), and onset of expiratory bursting on GG\textsubscript{EMG} and Abd\textsubscript{EMG}, i.e., active expiration [8, 11]. Thus, these excitatory neurons have presumptive projections to neurons in the preB\text{"o}tC or B\text{"o}tC [32, 33] that inhibit inspiration during expiration, i.e., reciprocal inhibition, and to excitatory premotoneurons in the caudal ventral respiratory group (cVRG) that project to abdominal muscle motoneurons [34–36].

Given the delayed increase in \( V_T \) following the induced decrease in \( f \), a direct excitatory projection from the pF\textsubscript{L} to the preB\text{"o}tC appears unlikely, but rather suggests an indirect pathway related to controlling pCO\textsubscript{2}, perhaps via the pF\textsubscript{V}. These observations are consistent with our hypothesis that the pF\textsubscript{L} is a conditional expiratory oscillator with neurons that are tonically inhibited at rest that can be turned on either by disinhibition and/or excitation.

**Multifunctional role of the pF\textsubscript{V}**

A+N\textsubscript{pFV} injected into the pF\textsubscript{V} to lower its excitability, decreased \( V_T \) and inspiratory-related muscle activity, likely via projections to the preB\text{"o}tC and/or the rostral ventral respiratory group (rVRG) [37]. The associated delayed increase in \( f \) could again be explained as intrinsic to the slower time course of chemosensory feedback to maintain pCO\textsubscript{2}. As no change in phase durations or \( f \) were seen, it appears unlikely that this excitatory drive to inspiration was mediated by rhythmic preB\text{"o}tC neurons [38]. Rather, this observation is consistent with our hypothesis of a subpopulation of tonically active pF\textsubscript{V} neurons that provides facilitative drive to phrenic and/or other inspiratory pump motoneurons to affect \( V_T \), but do not contribute directly to regulating \( f \) or inspiratory drive to genioglossal motoneurons [11]. Instead it is more likely that the pF\textsubscript{V} affects \( V_T \) through its projections to the rVRG [39], the premotor bulbospinal relay to the phrenic nucleus for inspiratory drive [40], as this will alter \( V_T \) without directly altering other inspiratory parameters, i.e., \( f \) and GG\textsubscript{EMG}.

B+S\textsubscript{pFV} to increase pF\textsubscript{V} excitability, increased \( f \), most likely through projections to the preB\text{"o}tC [38, 41], presumably to the same neurons that lead to an increase in \( f \) following optogenetic photostimulation of the pF\textsubscript{V} [42, 43]. B+S\textsubscript{pFV} also increased inspiratory-related GG\textsubscript{EMG}, likely through pF\textsubscript{V} projections to the parahypoglossal region (pXII) [39], which appears to be the premotor relay for inspiratory drive to the XII nucleus [44]. Though B+S\textsubscript{pFV} attenuated Dia\textsubscript{EMG} and \( V_T \), this appeared secondary to the reduction in \( f \) and thus was most likely due to chemosensory feedback to control pCO\textsubscript{2}. This further supports our hypothesis of a subpopulation of tonically suppressed pF\textsubscript{V} neurons that provide facilitative drive to modulate \( f \), but does not contribute directly to \( V_T \).

Unlike B+S\textsubscript{pFV}, AMPA\textsubscript{pFV} potentiated \( V_T \) and Dia\textsubscript{EMG} activity, most likely through excitation of the neurons that were attenuated by A+N\textsubscript{pFV} and project to the rVRG. AMPA\textsubscript{pFV} also increased \( f \) and inspiratory-related GG\textsubscript{EMG} most likely through excitation of neurons that project to the preB\text{"o}tC and parahypoglossal region that were activated following B+S\textsubscript{pFV}. As B+S\textsubscript{pFV} and AMPA\textsubscript{pFV} each led to different patterns of breathing with neither similar to the effects of activating the pF\textsubscript{L}, we suggest that there are at least two relevant pF\textsubscript{V} subpopulations, one expressing inhibitory receptors and one that does not, and that both of these subgroups are distinct from the pF\textsubscript{L}.

Distinct parafacial regions in respiratory control
Fig 9. A+NpFl does not affect breathing. A) Integrated traces from a single experiment. Black arrows at bottom indicate epochs in expanded traces (Bi and Biil), grey arrows at top indicate unilateral injections for A+NpFl. Bi) Rest. Biil) Following A+NpFl. Grey vertical boxes demarcate phases of each breath: inspiration (I; light gray), post-inspiration (Post; medium grey), and pre-inspiration (Pre; Dark gray). C) Comparison between ventilation at rest (Rst) and after A+NpFl injection. Lines connect data from individual experiments, box and whisker plots show combined data. Data are normalized to highest value for each parameter, i.e., f, Ti, Te, VT, GGEMG, DiaEMG, AbdEMG, or AbdEMG, regardless of whether it belonged to control or A+NpFl group. Abbreviations defined in Fig 2.

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Similar to stimulation of pFV neurons in awake behaving vagus intact rats [23] and in vagotomized urethane anesthetized cats [22], disinhibition with B+S_pFl elicited sighs in vagotomized rats (Fig 3Biί#), as did excitation with Glu_pFl in vagus-intact rats (Fig 8A). In vagotomized rats the amplitude of normal breaths is considerably larger than vagus-intact rats, with the consequence that sighs are masked. Accordingly, when VT was low, i.e., in vagus-intact rats or following a reduction in amplitude caused by B+S_pFl in vagotomized rats, sustained increases in sigh activity could be seen. This confirms our recent study showing a cluster of neurons in the pFV that release bombesin-like neuropeptides that affect sighing through the activation of cognate receptors in the preBotC [16].

Hyperpolarizing pFV neurons during hypercapnia and hypoxia affects the amplitude of AbdEMG and GGEMG, but not VT or f [11], likely through direct projections to the cVRG [15] and parahypoglossal region [39]. Interestingly, B+S_pFl induced high amplitude post-inspiratory activity on both GGEMG and AbdEMG, likely through the same projections, supporting our previous finding that the pFV provides excitatory drive to expiratory premotor nuclei independent of its projections to the preBotC [11]. Interestingly, no perturbation of pFV excitability induced active expiration, while hyperpolarization of the pFV reduces active expiration during chemosensory stimulation [11, 13]. We conclude that the pFV provides can modulate expiratory activity generated elsewhere, but cannot itself induce active expiration.

Interestingly, most manipulations which changed either f or VT led to compensatory changes, presumably to regulate VT to control pCO2 to within the normal range. For example, reducing excitation in the pFV reduced activity of neurons that influence diaphragmatic (pre) motoneurons, which are constitutively active at rest. Thus, this manipulation reduced VT, but had no effect on f as the pFV neurons that influence f were suppressed at rest and therefore their activity could not be affected by A+N; this allows for other brain regions to affect preBotC rhythmogenic neurons to increase f to compensate for the reduction in VT. Only one manipulation, glutamatergic activation of the pFV (with either AMPA or Glu) changed VT. We believe that this is because glutamatergic activation of the pFV RTN leads to activation of the tonically suppressed neurons that activate preBotC rhythmogenic neurons; furthermore this manipulation also excites the neurons that are active at rest that influence diaphragmatic (pre)motoneurons, consequently altering both f and VT simultaneously.

Summary

We propose that there are at least 6 subpopulations of parafacial neurons (Fig 11). The pFV is a conditional expiratory oscillator, with a functionally homogeneous population of neurons that drive active expiration (Fig 11). By contrast, the pFV provides a critical generic facilitatory drive to breathe, and consists of at least 4 functionally distinct subpopulations of neurons: i) a tonically active subpopulation that drives VT via the diaphragm; ii) one subpopulation of tonically suppressed neurons that modulate f; and; iii) a second subpopulation of tonically suppressed neurons that provide drive to abdominal and genioglossus expiratory motor pools, iv) a subpopulation of bombesin-peptide, i.e., NMB, neurons of the hypothesized peptidergic sigh circuit [16]. In addition, there is a 6th subpopulation bombesin-peptide, i.e., GRP, neurons in the dorsocaudal parafacial (pFDC) that also can modulate basal sigh rate [16].
Fig 10. A+N_{pFv} decreases V_T, and reduces output of inspiratory muscles. A) Integrated traces from a single experiment. Black arrows at bottom indicate epochs in expanded traces (Bi and Bii), gray arrows at top indicate unilateral injections for A+N_{pFv}. Bi) Rest. Bii) Following A+N_{pFv}. Grey vertical boxes demark phases of each
breath: inspiration (I; light gray), post-inspiration (Post: medium grey), and pre-inspiration (Pre: Dark gray). C) Comparison between ventilation at rest (Rst) and after A+NpFV injection. Lines connect data from individual experiments, box and whisker plots show combined data. Data are normalized to highest value for each parameter, i.e., f, Ti, Te, VT, GGBMG, DIaEMG, or AbdEMG regardless of whether it belonged to control or A+NpFV group. : p < 0.05. Abbreviations defined in Fig 2.

Fig 11. Schematic of minimal bCPG, which consists of 4 essential components. 1) preBötzinger Complex (preBöC) drives inspiration by exciting inspiratory premotor neuronal populations projecting to inspiratory muscles, e.g., diaphragm and tongue, and inhibits pFl; 2) parafacial Dorsocaudal (pFDc) contains GRP positive neurons contributing to basal sigh rhythm. 3) pFL drives active expiration by exciting expiratory premotor neuronal populations projecting to expiratory muscles, e.g., abdominals and tongue, and excites neurons that inhibit preBöC, either in preBöC or in BöC (not shown); 4) pFV contains neurons and glia that contribute to CO2/pH regulation and integrates sensory afferents affecting breathing, including basal sigh rate, via excitatory connections to preBöC and breathing premotor and motor neurons. pFV contains 4 subpopulations: i) tonically active neurons that modulate VT and diaphragm bursting at rest; ii) tonically suppressed neurons that modulate f; iii) NMB positive neurons that affect basal sigh rate, and; iv) tonically suppressed neurons that provide rhythmic drive to abdominal and genioglossus expiratory motor pools producing active expiration.

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