Distinct parafacial regions in control of breathing in adult rats

Robert T. R. Huckstepp, Kathryn P. Cardoza, Lauren E. Henderson, Jack L. Feldman

Department of Neurobiology, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, California, United States of America

Current address: School of Life Sciences, University of Warwick, Coventry, United Kingdom.

* feldman@g.ucla.edu

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 pFv and pFl. Transverse view of medulla at Bregma -11.25 mm. Red circles show locations of injection sites for pFv and pFl. Green dashed box is magnified in C. B) Ventral view of medullary surface with location of pFv and pFl 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 into the pFV and pFL. Py–Pyramidal tract, SP-5 –Spinal trigeminal tract, 7n –Facial nucleus. https://doi.org/10.1371/journal.pone.0201485.g001
breathing is driven by a dual oscillator system [10]. We identified two neighboring parafacial regions, lateral (pFL) and ventral (pFV) that appear to be functionally distinct components of the bCPG [11]. We hypothesized that the pFL is a conditional expiratory oscillator that is inhibited at rest [8, 11, 12], whereas the pFV 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 pFL and pFV, we selectively modulated their excitability and measured the effects on ventilation in spontaneously breathing vagotomized urethane anesthetized adult rats.

We conclude that: i) pFL 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) pFV 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 VT. Interestingly no subpopulation of pFV neurons appears capable of directly inducing active expiration; instead the pFV modulates active expiration generated elsewhere, presumably by effects in the pFL 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 isoflurane 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 basiococcipital craniotomy exposed the ventral medullary surface, and the dura was resected. After bilateral

| PARAMETER | DATA STRUCTURE | TYPE OF TEST | EFFECT SIZE | POWER | REQUIRED SAMPLE SIZE |
|-----------|----------------|--------------|-------------|-------|-----------------------|
| f         | Non-parametric | 2-sided Wilcoxon signed-rank test | 1.5         | 0.94  | n = 8                 |
| T₁        | Non-parametric | 2-sided Wilcoxon signed-rank test | 1.7         | 0.95  | n = 7                 |
| T₂        | Non-parametric | 2-sided Wilcoxon signed-rank test | 1.8         | 0.92  | n = 6                 |
| Vₜ        | Non-parametric | 2-sided Wilcoxon signed-rank test | 1.4         | 0.94  | n = 8                 |
| DiaEMG    | Non-parametric | 2-sided Wilcoxon signed-rank test | 1.4         | 0.94  | n = 8                 |
| GGEMG     | Non-parametric | 2-sided Wilcoxon signed-rank test | 1.7         | 0.95  | n = 7                 |
| AbdEMG    | Non-parametric | 2-sided Wilcoxon signed-rank test | 18.5        | 1.0   | n = 3                 |

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A

\[ V_T \]

\[ \dot{\phi}_{GG_{EMG}} \]

\[ \dot{\phi}_{Dia_{EMG}} \]

\[ \dot{\phi}_{Abd_{EMG}} \]

B

\[ f \]

\[ B_i \]

\[ B_{ii} \]

Bi

\[ V_T \]

\[ \dot{\phi}_{GG_{EMG}} \]

\[ \dot{\phi}_{Dia_{EMG}} \]

\[ \dot{\phi}_{Abd_{EMG}} \]

Bii

\[ 8 \text{ mL} \]

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

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

\[ 40 \text{ BPM} \]

\[ 10 \]

\[ \text{5 mins} \]

\[ \text{1 sec} \]

C

Normalized data

\[ \text{Rst f} \]

\[ \text{Rst Ti} \]

\[ \text{Rst Te} \]

\[ \text{Rst VT} \]

\[ Rst \dot{\phi}_{Dia_{EMG}} \]

\[ Rst \dot{\phi}_{GG_{EMG}} \]

\[ Rst \dot{\phi}_{Abd_{EMG}} \]

\[ Rst V_e \]
Fig 2. B+SpFL induces active expiration. 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 B+SpFL. Bi) Rest. Bii) Following B+SpFL. Grey vertical boxes demark period of each breath taken up by inspiration (I; light gray), post-inspiration (Post; medium grey), and pre-inspiration (Pre; Dark gray). C) Comparison between ventilation at rest (Rst) and after B+SpFL 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., TpFL, Tp, VpFL, GGEMG, DiaEMG or AbdEMG regardless of whether it belonged to control or B+SpFL group. *p < 0.05. frequency–f, Tp–inspiratory period Tp, –expiratory period, tidal volume–VpFL, GGEMG–genioglossus electromyogram, DiaEMG–diaphragm electromyogram, AbdEMG–abdominal electromyogram. BPM–breaths per minute, a.u.—arbitrary units.

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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 or pFV (Fig 1). To reduce disruption of the tissue, solutions were injected at ~50 nL/min. To ensure parity of injections of different drugs, i.e., AMPA, B+S, A+N, and consistency between both sites, i.e., pFL and pFV, the bilateral injections of a drug were performed ~2 mins apart. The timing between the 2 injections of AMPA (119 ± 16 sec), the 2 injections of B+S (121 ± 10 secs), and the 2 injections of A+N (121 ± 15 secs) were not statistically different (F12, 47 = 0.01; p = 0.98; 2-way ANOVA), and no differences were found between the timings of the 2 injections in the pFV (121 ± 8 secs) and the 2 injections in the pFL (120 ± 13 secs; F12, 47 = 0.0004; p = 0.98; 2-way ANOVA). The timing between the 2 injections of Glu before (122 ± 13 secs) and after (120 ± 13 secs) vagotomy, were also not statistically different (p = 0.8; paired T-test). After each injection rats were allowed 30–45 minutes for drugs to take effect and washout, and for baseline recordings to stabilize before the next injection.

The pFL is defined as the area ventral to the lateral edge of the facial nucleus, juxtaposed to the spinal trigeminal tract [11]. The pFV is 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.

Injectons contained: i) bicuculline methylbromide (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+SpFL) or pFV (B+SpFL); ii) AMPA (20 μM; Sigma) to activate glutamatergic AMPA receptors. Injections of AMPA lead to excitation of the pFL (AMPApFL) or pFV (AMPApFV) or; iii) 2-amino-5-phosphopentanoic 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+NpFL) or pFV (A+NpFV). 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 (GluFV), 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 GluFV.

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\textsubscript{PFV} increases $f$, decreases $V_T$, 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.

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injections for B+S_{PV}. Examples of sighs are marked with arrowheads labelled with §. Post-inspiratory burst Abd_{EMG}§ are marked with arrowheads labelled with †. Bi) Rest. Bii) Following B+S_{PV}. Grey vertical boxes demarcate phases of each breath: inspiration (I; light gray), post-inspiration (Post; medium grey), and pre-inspiration (Pre; dark gray). Sigh marked by §. Post-Inspiratory Abd_{EMG}§ marked by †. C) Comparison between ventilation at rest (Rest) and after B+S_{PV} 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, T_i, T_e, V_T, GG_{EMG}, Diab_{EMG}, or Abd_{EMG}; regardless of whether it belonged to control or B+S_{PV} group. *: p < 0.05. Abbreviations defined in Fig 2.

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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, Na_2HPO_4 10, KH_2PO_4 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; ∫Diab_{EMG}, ∫GG_{EMG}, and ∫Abd_{EMG} 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
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A

$V_T$

$\int_{GG_{EMG}}$

$\int_{Dia_{EMG}}$

$\int_{Abd_{EMG}}$

$f$

Bi

Post

Pre

Bi

Post

Pre

C

Normalized data

Rst NaCl

Rst T

Rst NaCl T

Rst T

Rst NaCl

Rst NaCl

Rst NaCl

Rst NaCl

Rst NaCl

Rst NaCl

Rst NaCl

Rst NaCl

Rst NaCl

Rst NaCl

Rst NaCl

5 mL

a.u.

a.u.

a.u.

50 BPM

30

5 mL

a.u.

a.u.

a.u.

50 BPM

30

1 sec
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 VT in the following inspiration. We define sighs by their characteristic augmented VT 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 diaEMG caused by a second inspiratory effort that occurs before the initial eupneic inspiration is complete.

Data were not normally distributed. Data were therefore analyzed using the non-parametric 2-sided Wilcoxon signed-rank test with a significance level of P ≤ 0.05 and reported as median and interquartile range (IQR). Data are displayed as box and whisker plots for comparison of groups, and as line graphs for individual experiments. There were 8 biological repeats and no technical repeats in all data sets, with 2 exceptions: Statistical outliers were excluded from the data if they failed both Pierce’s criterion and Grubb’s test; this led to the removal of 1 outlier from the AbdEMG data from the AMPA_pFL and AMPA_pFL data sets.

Power analysis was calculated in G*Power3 (http://www.gpower.hhu.de/en.html [20]), using a Wilcoxon signed rank tests (matched pairs); with an α error probability of 0.05, and a power (1-β error probability) of 0.90, and effect sizes were calculated from the data (Table 1).

**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_pFL) can induce active expiration [8, 11], which we confirm here. Bilateral injection of B+S_pFL (n = 8) decreased f and T_i, increased T_E, VT, fDiaEMG, and inspiratory-related ∫GGEMG activity, and induced rhythmic expiratory bursting in ∫GGEMG and ∫AbdEMG (Fig 2), the latter a signature of active expiration, q.v., [8, 11]. Bilateral B+S_pFL had no effect on minute ventilation (V_e) due to a compensatory increase in VT 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 VT 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_pPFV) would increase V_E, as well as alter abdominal and genioglossus activity, but would not induce active expiration. Bilateral injections of B+S_pPFV (n = 8) increased f, decreased T_i, did not alter T_E, and decreased VT and ∫DiaEMG. Bilateral B+S_pPFV in anesthetized rats did not alter V_E due to a compensatory decrease in VT 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 ∫GGEMG and
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A

0.9% NaCl

\(p_{F_v}\)

\(V_T\)

\[\int_{G_{EMG}}\]

\[\int_{Dia_{EMG}}\]

\[\int_{Abd_{EMG}}\]

\(f\)

Bi

Post

Pre

8 mL

a.u.

a.u.

a.u.

Abd

50 BPM

35

Bii

Post

Pre

8 mL

a.u.

a.u.

a.u.

Abd

50 BPM

35

C

Normalized data

\[R_{st}\]

\(NaCl\)

\(T_1\)

\(TE\)

\(V_T\)

\(Dia_{EMG}\)

\(G_{EMG}\)

\(Abd_{EMG}\)

\(V_e\)
inducing both pre-inspiratory and post-inspiratory GG EMG activity (Fig 3). In 6 out of 8 experiments, B+S pFV also induced high amplitude post-inspiratory Abd EMG activity (Fig 3A† and 3Bii†), which while rhythmic was slow, occurring every 10 ± 1 breaths. This pattern of high amplitude post-inspiratory Abd EMG activity was distinct from active expiration that

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

|        | f (BPM) | T1 (secs) | TE (secs) | VT (mL) | DiaEMG (a.u.) | GGEMG (a.u.) | AbdEMG (a.u.) | VE (mL·min⁻¹) |
|--------|---------|-----------|-----------|---------|---------------|--------------|---------------|---------------|
| A) pFL |         |           |           |         |               |              |               |               |
| Rest   | 44,9    | 0.5,0.1   | 1.0,0.2   | 5.3,1.1 | 26,30         | 9,9          | 0             | 218,30        |
| B+S    | 28,3    | 0.3,0.0   | 1.8,0.1   | 7.5,1.9 | 36,26         | 17,20        | 16,27         | 219,47        |
| P      | 0.008   | 0.008     | 0.008     | 0.008   | 0.008         | 0.008        | 0.008         | 0.9           |
| Rest   | 45,8    | 0.4,0.1   | 0.9,0.3   | 5.2,1.1 | 25,22         | 6,8          | 0             | 212,52        |
| AMPA   | 32,14   | 0.3,0.1   | 1.5,0.8   | 6.1,1.0 | 29,23         | 13,10        | 2,3           | 177,7         |
| P      | 0.008   | 0.02      | 0.008     | 0.008   | 0.02          | 0.008        | 0.02          | 0.1           |
| Rest   | 54,11   | 0.3,0.1   | 0.7,0.2   | 4.2,0.7 | 28,24         | 6,8          | 0             | 215,43        |
| A+N    | 59,10   | 0.4,0.1   | 0.6,0.2   | 3.9,1.1 | 27,25         | 6,7          | 0             | 213,28        |
| P      | 0.4     | 0.4       | 0.3       | 0.8     | 0.5           | 0.2          | 0.4           | 0.9           |
| B) pFV |         |           |           |         |               |              |               |               |
| Rest   | 37,9    | 0.5,0.2   | 1.1,0.9   | 5.4,1.2 | 28,26         | 11,11        | 0             | 207,22        |
| B+S    | 50,8    | 0.3,0.1   | 0.9,0.2   | 5.0,1.9 | 24,28         | 14,9         | 7,18          | 229,80        |
| P      | 0.008   | 0.008     | 0.054     | 0.02    | 0.008         | 0.04         | 0.008         | 0.5           |
| Rest   | 42,6    | 0.5,0.1   | 1.0,0.2   | 5.2,0.7 | 25,29         | 7,77         | 0             | 210,39        |
| AMPA   | 47,4    | 0.3,0.1   | 0.9,0.2   | 5.5,1.2 | 30,28         | 13,11        | 0             | 262,40        |
| P      | 0.02    | 0.008     | 0.2       | 0.04    | 0.02          | 0.008        | 0.7           | 0.02          |
| Rest   | 48,8    | 0.43,0.1  | 0.8,0.2   | 4.7,0.9 | 28,24         | 7,8          | 0             | 217,16        |
| A+N    | 61,22   | 0.36,0.1  | 0.6,0.2   | 3.7,0.8 | 26,23         | 3,5          | 0             | 205,33        |
| P      | 0.02    | 0.02      | 0.04      | 0.008   | 0.02          | 0.008        | 0.4           | 0.3           |
| C) Glutamate-pFV |       |           |           |         |               |              |               |               |
| VI Rest| 104,53  | 0.21,0.08 | 0.3,0.1   | 2.1,0.7 | 15,13         | 3,4          | 0             | 254,34        |
| VI Glu | 87,39   | 0.23,0.09 | 0.4,0.2   | 2.4,0.9 | 16,15         | 5,5          | 0             | 232,51        |
| P      | 0.03    | 0.02      | 0.02      | 0.046   | 0.02          | 0.02         | 0.9           | 0.4           |
| Vx Rest| 46,20   | 0.34,0.26 | 0.8,0.4   | 2.8,1.9 | 20,16         | 10,16        | 0             | 163,31        |
| Vx Glu | 49,19   | 0.26,0.26 | 0.8,0.4   | 3.0,2.1 | 22,23         | 13,19        | 0             | 196,45        |
| P      | 0.03    | 0.03      | 0.4       | 0.03    | 0.03          | 0.03         | 0.2           | 0.03          |
| D) Saline |      |           |           |         |               |              |               |               |
| Rest   | 44,9    | 0.3,0.0   | 1.0,0.2   | 3.8,0.9 | 35,10         | 13,12        | 0             | 202,23        |
| pFV    | 48,9    | 0.3,0.0   | 0.9,0.2   | 4.0,0.5 | 36,12         | 14,15        | 0             | 212,28        |
| P      | 0.3     | 0.3       | 0.5       | 0.8     | 0.1           | 0.4          | 0.5           | 0.08          |
| Rest   | 48,6    | 0.3,0.0   | 0.9,0.2   | 3.9,0.7 | 35,11         | 14,13        | 0             | 48,6          |
| pF1    | 48,9    | 0.3,0.0   | 1.0,0.3   | 4.1,0.6 | 35,13         | 15,13        | 0             | 48,9          |
| P      | 0.9     | 0.9       | 1.0       | 0.5     | 0.7           | 0.5          | 0.7           | 0.6           |

A-B) Agonists and antagonists injected into the pF1 (A) and pFV (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 Abd$_{EMG}$ bursts, there was inhibition of GG$_{EMG}$ activity, showing co-ordination between GG$_{EMG}$ and Abd$_{EMG}$ during expiration (Fig 3B). In all experiments, B+S$_{pFL}$ also induced sighs i.e., augmented breaths with high amplitude inspiratory Dia$_{EMG}$ followed by prolonged T$_E$ (Fig 3A# and 3Bii#); sighs were rhythmic but slow, occurring every 12 ± 1 breaths. The high amplitude post-inspiratory Abd$_{EMG}$ activity was not coordinated with sighing.

To test for any nonspecific effects of pF$_V$ or pF$_L$ injections on breathing, we injected saline into both regions. In anesthetized rats, saline injections in the pF$_L$ (n = 8) did not alter f, T$_I$, T$_E$, T$_L$, f/Dia$_{EMG}$, GG$_{EMG}$, Abd$_{EMG}$ or V$_E$ (Fig 4). In anesthetized rats, saline injections in the pF$_V$ (n = 8) did not alter f, T$_I$, T$_E$, V$_E$, f/Dia$_{EMG}$, f/GG$_{EMG}$, f/Abd$_{EMG}$, or V$_E$ (Fig 5).

### Excitation of either pF$_L$ or pF$_V$ affects breathing pattern (Figs 6–8, Table 2)

Photostimulation of pF$_L$ neurons elicits active expiration [8]. We predicted that excitation of the pF$_L$ 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$_E$, f/Dia$_{EMG}$, inspiratory-related f/GG$_{EMG}$ activity and Abd$_{EMG}$ (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 pF$_V$ neurons by injection of glutamate increases phrenic nerve discharge amplitude and induces sighing in urethane anesthetized, paralyzed, artificially ventilated, vagotomized cats [22]; photostimulation of pF$_V$ neurons leads to increased sighing and respiratory frequency in conscious rats [23]. We predicted that excitation of the pF$_V$ with AMPA (AMPA$_{pFV}$) would induce ventilation and sighing. Bilateral injection of AMPA$_{pFV}$ (n = 8) increased f, decreased T$_I$, did not alter T$_E$, and increased V$_E$, f/Dia$_{EMG}$, and inspiratory-related f/GG$_{EMG}$, but neither induced expiratory-modulated GG$_{EMG}$ nor Abd$_{EMG}$ (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 GG$_{EMG}$ or Abd$_{EMG}$ 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 pF$_V$ (Glu$_{pFV}$) of anesthetized rats before and after vagotomy. Before vagotomy (n = 8), bilateral Glu$_{pFV}$ decreased f, increased T$_I$, T$_E$, V$_E$, f/Dia$_{EMG}$, inspiratory-related f/GG$_{EMG}$ (Fig 8), and sigh rate, but neither induced expiratory-modulated GG$_{EMG}$ nor Abd$_{EMG}$ (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$_E$, f/Dia$_{EMG}$, and inspiratory-related f/GG$_{EMG}$, but neither induced expiratory-modulated GG$_{EMG}$ nor Abd$_{EMG}$ (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
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A

\[ V_T \]

[20\mu M AMPA_{pF, v}]

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

\[ \int D\text{ia}_{EMG} \]

\[ \int A\text{bd}_{EMG} \]

\[ f \]

\[ \text{Bi} \]

\[ \text{Bii} \]

\[ \text{2 mins} \]

\[ \text{8 mL} \]

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

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

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

\[ \text{45 BPM} \]

\[ \text{30} \]

Bi

\[ \text{Post} \]

\[ \text{Pre} \]

\[ \text{Bii} \]

\[ \text{Post} \]

\[ \text{Pre} \]

\[ \text{C} \]

\[ \text{Normalized data} \]

\[ \text{Rst f} \]

\[ \text{Rst T}_I \]

\[ \text{Rst T}_E \]

\[ \text{Rst V}_T \]

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

\[ \text{Rst Gg}_{EMG} \]

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

\[ \text{Rst Ve} \]
Distinct parafacial regions in respiratory control

Fig 7. AMPA_{pFV} increases $f$ and $V_T$, but does not induce post-inspiratory activity in either abdominal muscles or in pre- and post-inspiratory activity gengiglossus 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 AMPA_{pFV}; Bi) Rest. Bii) Following AMPA_{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 AMPA_{pFV} 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., $f$, $T_I$, $T_E$, $V_T$, GG_{EMG}, Dia_{EMG}, or Abd_{EMG} regardless of whether it belonged to control or AMPA_{pFV} group. *p < 0.05. Abbreviations defined in Fig 2.

Reduced excitation of pFV and pFL have different effects on breathing (Figs 9 and 10, Table 2)

Many, if not most or all, pF_L neurons are silent at rest [8, 24]; not surprisingly, hyperpolarizing pF_L neurons at rest does not affect ventilation [11]. We predicted that reduction of pF_L excitability with local injection of a cocktail of the glutamatergic antagonists AP-5 and NBQX (A+N_{pFL}) would not affect breathing. Bilateral injections of A+N_{pFL} (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_{pFL} (Fig 9). Bilateral injections of A+N_{pFL} 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_L, likewise since A+N_{pFL} 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 inspiratory 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 chemosensory drive increases [11, 15], and that the pF_L 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_{pFL} 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)

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).
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Ai

10mM Glu$_{PFV}$

$V_T$

$\Delta G_{EMG}$

$\Delta Dia_{EMG}$

$\Delta Abd_{EMG}$

6 mL

a.u.

a.u.

150 BPM

0

5 mins

Aii

10mM Glu$_{PFV}$

$V_T$

$\Delta G_{EMG}$

$\Delta Dia_{EMG}$

$\Delta Abd_{EMG}$

6 mL

a.u.

a.u.

a.u.

55 BPM

40

2 mins

Bi

1.0

Normalized data

Rst Glu $_f$ Rst Glu $_I$ Rst Glu $_E$ Rst $V_T$

Rst $Dia_{EMG}$ Rst $G_{EMG}$ Rst $Abd_{EMG}$ Rst $V_e$

Bii

1.0

Normalized data

Rst Glu $_f$ Rst Glu $_I$ Rst Glu $_E$ Rst $V_T$

Rst $Dia_{EMG}$ Rst $G_{EMG}$ Rst $Abd_{EMG}$ Rst $V_e$
Fig 8. Glu_pFV alters, but does not induce, post-inspiratory activity in either abdominal muscles or in pre- and post-inspiratory activity in genioglossus muscles. A) Integrated traces from a single experiment, gray arrows indicate unilateral injections for Glu_pFV. Ai) Vagus intact. Aii) Vagotomized. B) Comparison between ventilation at rest (Rst) and after Glu_pFV regardless of whether it belonged to control or Glu_pFV group. *p < 0.05. Abbreviations defined in Fig 2.

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... decreased f with a compensatory increase in VT and inspiratory DiaEMG and GGEMG, and onset of expiratory bursting on GGEMG and AbdEMG, i.e., active expiration [8, 11]. Thus, these excitatory neurons have presumptive projections to neurons in the preBötC or Bötzinger Complex (pBoT C) 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 VT following the induced decrease in f, a direct excitatory projection from the pFV to the preBötC appears unlikely, but rather suggests an indirect pathway related to controlling pCO2, perhaps via the pFV. These observations are consistent with our hypothesis that the pFV 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 pFV

A+N_pFV injected into the pFV to lower its excitability, decreased VT and inspiratory-related muscle activity, likely via projections to the pBoT C 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 pCO2. As no change in phase durations or f were seen, it appears unlikely that this excitatory drive to inspiration was mediated by rhythmic preBötC neurons [38]. Rather, this observation is consistent with our hypothesis of a subpopulation of tonically active pFV neurons that provides facilitative drive to phrenic and/or other inspiratory pump motoneurons to affect VT, but do not contribute directly to regulating f or inspiratory drive to genioglossal motoneurons [11]. Instead it is more likely that the pFV affects VT through its projections to the rVRG [39], the premotor bulbo spinal relay to the phrenic nucleus for inspiratory drive [40], as this will alter VT without directly altering other inspiratory parameters, i.e., f and GGEMG.

B+S_pFV to increase pFV excitability, increased f, most likely through projections to the preBötC [38, 41], presumably to the same neurons that lead to an increase in f following optogenetic photostimulation of the pFV [42, 43]. B+S_pFV also increased inspiratory-related GGEMG, likely through pFV 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_pFV attenuated DiaEMG and VT, this appeared secondary to the reduction in f and thus was most likely due to chemosensory feedback to control pCO2. This further supports our hypothesis of a subpopulation of tonically suppressed pFV neurons that provide facilitative drive to modulate f, but does not contribute directly to VT.

Unlike B+S_pFV, AMPA_pFV potentiated VT and DiaEMG activity, most likely through excitation of the neurons that were attenuated by A+N_pFV and project to the rVRG. AMPA_pFV also increased f and inspiratory-related GGEMG most likely through excitation of neurons that project to the preBötC and parahypoglossal region that were activated following B+S_pFV. As B +S_pFV and AMPA_pFV each led to different patterns of breathing with neither similar to the effects of activating the pFV, we suggest that there are at least two relevant pFV subpopulations, one expressing inhibitory receptors and one that does not, and that both of these subgroups are distinct from the pFV.
Similar to stimulation of pF\textsubscript{V} neurons in awake behaving vagus intact rats [23] and in vagotomized urethane anesthetized cats [22], disinhibition with B+S\textsubscript{pFV} elicited sighs in vagotomized rats (Fig 3Bi\#), as did excitation with Glu\textsubscript{pFV} 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 \( V_T \) was low, i.e., in vagus-intact rats or following a reduction in amplitude caused by B+S\textsubscript{pFV} in vagotomized rats, sustained increases in sigh activity could be seen. This confirms our recent study showing a cluster of neurons in the pF\textsubscript{V} that release bombesin-like neuropeptides that affect sighing through the activation of cognate receptors in the preBot\textsubscript{C} [16].

Hyperpolarizing pF\textsubscript{V} neurons during hypercapnia and hypoxia affects the amplitude of Abd\textsubscript{EMG} and GG\textsubscript{EMG}, but not \( V_T \) or \( f \) [11], likely through direct projections to the cVRG [15] and parahypoglossal region [39]. Interestingly, B+S\textsubscript{pFV} induced high amplitude post-inspiratory activity on both GG\textsubscript{EMG} and Abd\textsubscript{EMG}, likely through the same projections, supporting our previous finding that the pF\textsubscript{V} provides excitatory drive to expiratory premotor nuclei independent of its projections to the preBot\textsubscript{C} [11]. Interestingly, no perturbation of pF\textsubscript{V} excitability induced active expiration, while hyperpolarization of the pF\textsubscript{V} reduces active expiration during chemosensory stimulation [11, 13]. We conclude that the pF\textsubscript{V} provides can modulate expiratory activity generated elsewhere, but cannot itself induce active expiration.

Interestingly, most manipulations which changed either \( f \) or \( V_T \) led to compensatory changes, presumably to regulate \( V_T \) to control pCO\textsubscript{2} to within the normal range. For example, reducing excitation in the pF\textsubscript{V} reduced activity of neurons that influence diaphragmatic (pre)motoneurons, which are constitutively active at rest. Thus, this manipulation reduced \( V_T \), but had no effect on \( f \) as the pF\textsubscript{V} neurons that influence \( f \) were supressed at rest and therefore their activity could not be affected by A+N; this allows for other brain regions to affect preBot\textsubscript{C} rhythmogenic neurons to increase \( f \) to compensate for the reduction in \( V_T \). Only one manipulation, glutamatergic activation of the pF\textsubscript{V} (with either AMPA or Glu) changed \( V_F \). We believe that this is because glutamatergic activation of the pF\textsubscript{V} RTN leads to activation of the tonically suppressed neurons that activate preBot\textsubscript{C} rhythmogenic neurons; furthermore this manipulation also excites the neurons that are active at rest that influence diaphragmatic (pre)motoneurons, consequently altering both \( f \) and \( V_T \) simultaneously.

Summary

We propose that there are at least 6 subpopulations of parafacial neurons (Fig 11). The pF\textsubscript{L} is a conditional expiratory oscillator, with a functionally homogeneous population of neurons that drive active expiration (Fig 11). By contrast, the pF\textsubscript{V} 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 \( V_T \) 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 6\textsuperscript{th} subpopulation bombesin-peptide, i.e., GRP, neurons in the dorsocaudal parafacial (pF\textsubscript{DC}) 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+N
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, T\text{I}, T\text{E}, V\text{T}, GG\text{EMG}, Di\text{EMG}, or Abd\text{EMG}; regardless of whether it belonged to control or A+N
\text{pFV} group. *: p < 0.05. Abbreviations defined in Fig 2.

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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 pF\text{L}; 2) parafacial Dorsocaudal (pF\text{DC}) contains GRP positive neurons contributing to basal sigh rhythm. 3) pF\text{L} 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) pF\text{V} contains neurons and glia that contribute to CO\text{2}/pH regulation and integrates sensory afferents affecting breathing, including basal sigh rate, via excitatory connections to preBöC and breathing premotor and motor neurons. pF\text{V} contains 4 subpopulations: i) tonically active neurons that modulate V\text{T} 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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Author Contributions

Conceptualization: Robert T. R. Huckstepp, Jack L. Feldman.

Data curation: Robert T. R. Huckstepp, Kathryn P. Cardoza, Lauren E. Henderson.

Formal analysis: Robert T. R. Huckstepp.

Funding acquisition: Jack L. Feldman.

Methodology: Robert T. R. Huckstepp, Jack L. Feldman.

Writing – original draft: Robert T. R. Huckstepp, Jack L. Feldman.

Writing – review & editing: Robert T. R. Huckstepp, Kathryn P. Cardoza, Lauren E. Henderson, Jack L. Feldman.

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