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Systems/Circuits

Role of Parafacial Nuclei in Control of Breathing in Adult Rats

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Contiguous brain regions associated with a given behavior are increasingly being divided into subregions associated with distinct aspects of that behavior. Using recently developed neuronal hyperpolarizing technologies, we functionally dissect the parafacial region in the medulla, which contains key elements of the central pattern generator for breathing that are important in central CO2-chemoreception and for gating active expiration. By transfecting different populations of neighboring neurons with allatostatin or HMG-D G45-coupled receptors, we analyzed the effect of their hyperpolarization on respiration in spontaneously breathing vagotomized urethane-anesthetized rats. We identify two functionally separate parafacial nuclei: ventral (pFV) and lateral (pFL). Disinhibition of the pFV with bicuculline and strychnine led to active expiration. Hyperpolarizing pFV neurons had no effect on breathing at rest, or changes in inspiratory activity induced by hypoxia and hypercapnia; however, hyperpolarizing pFL neurons attenuated active expiration when it was induced by hypercapnia, hypoxia, or disinhibition of the pFV. In contrast, hyperpolarizing pFV neurons affected breathing at rest by decreasing inspiratory-related activity, attenuating the hypoxia- and hypercapnia-induced increase in inspiratory activity, and when present, reducing expiratory-related abdominal activity. Together with previous observations, we conclude that the pFV provides a generic excitatory drive to breathe, even at rest, whereas the pFL is a conditional oscillator quiet at rest that, when activated, e.g., during exercise, drives active expiration.

Key words: active expiration; control of breathing; expiratory oscillator; parafacial respiratory group; respiration; retrotroapexoid nucleus

Introduction
Understanding how neurons are organized in microcircuits and networks is essential for comprehending behavior (Luo et al., 2008; Yuste, 2008). Here we exploit two related techniques for hyperpolarizing neurons to independently depress activity in neighboring populations of neurons and determined their role within the central pattern generator for breathing.

We hypothesize that breathing in mammals results from interactions between two oscillators (Mellen et al., 2003; Janczewski and Feldman, 2006; Feldman et al., 2013; the preBötzinger Complex (preBötC) is the kernel for inspiration (Smith et al., 1991; Tan et al., 2008), while the retrotroapexoid nucleus/parafacial respiratory group (RTN/pFRG), in whole or part, is vital for active expiration (Pagliardini et al., 2011) and CO2-chemoreception (Smith et al., 1989; Li et al., 1999; Mulkey et al., 2004).

The RTN and pFRG are parafacial regions proximal to the ventral medullary surface that project to the ventral respiratory column (Smith et al., 1989; Ellenberger and Feldman, 1990; Pagliardini et al., 2011). One notable difference between them is that in newborn rodents pFRG but not RTN neurons are respiratory rhythmic (Onimaru and Homma, 2003); this activity begins to wane during the early neonatal period (Oku et al., 2007) and late expiratory activity is completely absent in both regions in adult rodents at rest (Pagliardini et al., 2011).

The discovery of a putative conditional RTN/pFRG oscillator driving active expiration (Mellen et al., 2003; Janczewski and Feldman, 2006) raised the question whether parafacial neurons responsible for chemoreception and active expiration are identical, overlap, or separate. That disinhibition or activation of mostly lateral parafacial neurons elicits active expiration (Pagliardini et al., 2011) led us to hypothesize that the pFRG and RTN are separable in adult rats.

Given that the respective anatomical parafacial boundaries and distinctive functional contributions of the RTN and pFRG are poorly defined, we use unbiased descriptors based on position relative to the facial motor nucleus. Thus we refer to lateral and ventral parafacial regions, i.e., pFV and pFL. We virally transfected pFV and/or pFL neurons to express distinctly different exogenous receptors, either the allatostatin receptor (AlstR; Birgül et al., 1999; Callaway, 2005) or the HMG-D receptor (HMGR; Nawaratne et al., 2008; Pei et al., 2008). Once these receptors are activated, transfected neurons are sufficiently hyperpolarized to become quiescent (Callaway, 2005; Tan et al., 2006, 2008; Nawaratne et al., 2008; Ray et al., 2011).

At rest when expiration is passive (Sherrey et al., 1988; Iizuka and Fregosi, 2007; Marina et al., 2010; Pagliardini et al., 2011), hyperpolarizing pFV neurons had no effect on breathing patterns; during hypoxia or hypercapnia, which induce active expiration,
hyperpolarizing pFV neurons only attenuated expiratory activity, and disinhibition of the pFV induced active expiration, and decreased frequency (f), which led to a concomitant increase in tidal volume (VT), similar to photoactivation of pFV neurons (Pagliardini et al., 2011). These effects are consistent with the pFV acting as a conditional oscillator, quiescent at rest, that when activated drives active expiration.

Materials and Methods

Viral design and handling. Two different viruses were used: AAV-2/5 hSyn-HA-hM4D(Gi)-IRES-mCitrine (HM4DR; University of North Carolina Gene Therapy Vector Core, Chapel Hill, NC) at a titer of 8 × 10^{12} vp/ml, and; AAV-DJ synapsin-allatostatin receptor-GFP (AlstR). We designed the latter virus using Nucleotide/Blat (NCBI, Bethesda, MD), University of Santa Cruz Genome Browser (UCSC, Santa Cruz, CA), Cister (Boston University, Boston, MA), and BioEdit (Ibis Biosciences). The insert (synapsin-SV40 SD/SA-AlstR) was synthesized (GenScript), and placed into the VPK 418 pAAV-IRES-GFP plasmid vector (Cell Biolabs), modified so that it lacked the CMV promoter and β-globin intron. The plasmid was then used to create a custom AAV-DJ virus (Salk Institute, GT3 Core) at a titer of 5.8 × 10^{11} vp/ml. The viruses were aliquoted and stored at −80°C. On the day of injection, aliquots containing viruses were removed and held at 4°C. Viruses were loaded by capillary action into glass pipettes, and pipettes containing viruses were placed into an electrode holder for pressure injection.

Viral transfection of pFV, and pFL. All protocols were approved by the University of California Los Angeles Chancellor’s Animal Research Committee. Male Sprague Dawley rats (350–450 g) were anesthetized by intraperitoneal injection of ketamine (100 mg/kg; Clipper Distribution), xylazine (10 mg/kg; Lloyd), and atropine (1 mg/kg; Westward Pharmaceutical); anesthesia was maintained with isoflurane (0.5–2%; Piramal Healthcare); body temperature was maintained at a minimum of 36.5°C throughout the procedure as required. Rats were placed in a prone position in a stereotaxic frame and were anesthetized with isoflurane (0.5–2%; Piramal Healthcare); anesthesia was maintained with isoflurane (0.5–2%; Piramal Healthcare) throughout the procedure as required. Rats were placed in a prone position in a stereotaxic apparatus (Kopf Instruments) on a heating pad (TCAT 2-LV; Physitemp) and body temperature was maintained at a minimum of 36.5°C via a thermocouple. The head was leveled and glass pipettes were placed stereotactically into the pFV or pFL (Figs. 1, 2). The pFV was defined as the area ventral to the central half of the facial nucleus, between the pyramidal tract and the spinal trigeminal tract (coordinates: 1.8 mm lateral and 11.4 mm caudal from bregma, and 9.4 mm ventral from the surface of the cerebellum; Fig. 2Aii). The pFV was defined as the area ventral to the central half of the facial nucleus, between the pyramidal tract and the spinal trigeminal tract (coordinates: 1.8 mm lateral and 11.4 mm caudal from bregma, and 9.4 mm ventral from the surface of the cerebellum; Fig. 2Aii). The virus solutions were pressure injected (100–150 nl) bilaterally using a Picospritzer II (General Valve) controlled by a Master 8 pulse generator (A.M.P.I.). Pipettes were left in the pipette track.

There were four virus injection protocols (Fig. 1): (1) HM4DR in pFV neurons (pFV;HM4DR), (2) AlstR in pFV neurons (pFV;AlstR), (3) Both
pF₂;HM₄DR and pF₂;AlstR (pF₂;HM₄DR + pF₂;AlstR), and (4) HM₄DR in pF₂ neurons (pF₂;HM₄DR).

Postoperatively, rats received buprenorphine (0.1 mg/kg; Reckitt Benckiser) intraperitoneally and meloxicam (2 mg/kg; Norbrook) subcutaneously, followed by 10 d of oral antibiotics (TMS; Hi-Tech Pharmacal) and 4 d of oral meloxicam (0.05 mg/ml) in their drinking water. Rats were allowed 3–6 weeks for recovery and viral expression, with food and water ad libitum.

**Ventral approach.** Anesthesia was induced with isoflurane and maintained throughout surgery with urethane (Fig. 2Aii; 1.2–1.7 g/kg; Sigma) diluted in standard sterile saline (0.9% NaCl; Hospira) via a femoral catheter: urethane anesthesia was vital to seeing expiratory-related abdominal activity, as activity was not seen when isoflurane or ketamine were used (data not shown). Rats were placed supine onto a stereotaxic apparatus on a heating pad and core body temperature was maintained at a minimum of 36.5°C via a thermocouple. The trachea was cannulated. Respiratory flow was monitored via a flow head connected to a transducer (GM Instruments) and CO₂ via a capnograph (Type 340; Harvard Apparatus) connected to the tracheal tube. Paired EMG wire electrodes (Cooner Wire) were inserted into the genioglossus, diaphragm, and oblique abdominal muscles to record respiratory-related activity. After the anterior neck muscles were removed, a baso-occipital craniotomy exposed the ventral medullary surface, and the dura was resected. After bilateral vagotomy, the exposed tissue around the neck and the mylohyoid muscle was covered with dental putty (Reprosil; Dentsply Caulk) to prevent drying. The rat was left for 30 min to allow baseline recordings to stabilize. At resting levels, ventilation was continuous, consisting of alternation of active contraction of inspiratory muscles, e.g., diaphragm, and passive expiration. Active expiration was initiated by substituting various gas mixtures for room air, i.e., hypoxia (8% O₂ and 92% N₂) or hypercapnia (9% CO₂, 21% O₂, and 70% N₂), or by disinhibition within the pF₂ with the GABA antagonist, bicuculline methobromide or by disinhibition within the pFL with the GABA antagonist strychnine hydrochloride (250 μM; Tocris Bioscience) combined with the glycine antagonist strychnine hydrochloride (250 μM; Sigma-Aldrich) diluted in standard sterile saline.

All experiments began by testing responses to hypoxia, hypercapnia, and pF₂ disinhibition [via injection of bicuculline/strychnine (B + S) in the pF₂ (B + S)] under resting conditions (Fig. 1–iv). Rats were exposed to 1 min of hypoxia followed by a 15 min recovery period breathing room air, then changed to 2 min of hypercapnia after which rats were allowed 20 min breathing room air for baseline recordings to stabilize. Finally, rats received bilateral B + S₁ᵣ followed by a 30 min recovery period while breathing room air. pF₂ coordinates via a ventral approach were as follows: 2.5 mm lateral from the basilar artery, 0.9 mm rostral from the most rostral hypoglossal nerve rootlet, and 0.2 mm dorsal from the ventral surface. The B + S solution was pressure injected (50–100 nl) bilaterally using a Picospritzer II controlled by a Master 8 pulse generator. To avoid disruption of the tissue the B + S solution was injected at ~60 nl/min.

After experiments were performed under resting conditions, rats transfected with pF₂;AlstR or pF₂;HM₄DR + pF₂;AlstR were given a
bilateral injection of Alst (10 μM; Antagene) diluted in standard sterile saline into the pFv, to test the effects of inactivation of the pFv, i.e., pFv:AlstR rats, were given Alst to test for nonspecific effects of Alst that do not target the AlstR (Fig. 1iv), pFv, coordinates via a ventral approach were as follows: 1.8 mm lateral from the basilar artery, 0.6 mm rostral from the most rostral hypoglossal nerve rootlet, and 0.2 mm dorsal from the ventral surface. At 10 min post Alst, the response to hypoxia was re-examined. Additional Alst, with a 5 min stabilization period, preceded subsequent retrosternal of, first, the response to hypercapnia, and second, B + S/sp, then, after sufficient time for breathing to return to baseline levels (~30 min), CNO was applied to the ventral medullary surface to test the effects of pFv, inactivation (Fig. 1v)); to allow breathing to stabilize, measurements were taken after 10 min. Then, the rats were again tested for response to hypoxia, hypercapnia, and B + S/sp, with CNO reapplied 5 min before each test. As there was no difference in the responses to pFv inactivation in rats transfected with pFv:HM4DR (Fig. 1vi) or pFv:AlstR (Fig. 1v), the data from both groups were combined for analysis. As there was also no difference in the responses to Alst in rats transfected with pFv:AlstR (Fig. 1vii) or pFv:HM4DR + pFv:AlstR (Fig. 1vi), the data from both groups were combined for analysis. Thus experiments represented by Figure 1, i and ii, were compared with ixi and xii, were compared with vii + viii, and i and vi were compared with v.

Rats transfected with HM4DR in the pFv, i.e., pFv:HM4DR rats, were only tested for their response to CNO. As for other experiments, we measured the responses under resting conditions to hypoxia and hypercapnia. Then, after sufficient time for breathing to return to baseline levels (~30 min), CNO was applied to the ventral medullary surface to test the effects of pFv, inactivation (Fig. 1viii); to allow breathing to stabilize, measurements were taken after 10 min. Then, the rats were again tested for responses to hypoxia and hypercapnia, with CNO reapplied 5 min before each test. Finally, after washing off the CNO, the protocol was repeated a third time, again under resting conditions. The pre-experimental and post-experimental conditions were averaged for control data used for analysis (Fig. 1vii, viii).

Localization of transfected neurons. Rats were killed by an overdose of urethane and transcardially perfused using a peristaltic pump (Cole Palmer) with saline followed by cold (4°C) paraformaldehyde (PFA; 4%; Thermo Fischer Scientific). The medulla was harvested and postfixed in 4% PFA overnight at 4°C, then cryoprotected in sucrose (30%; Thermo Fischer Scientific) in standard PBS (1–3 at 4°C). PBS contained the following (in mM): 137 NaCl, 2.7 KCl, 10 Na2HPO4, and 1.8 KH2PO4, adjusted to pH 7.4 with HCl (all reagents from Thermo Fischer Scientific). Brainstems were transversely sectioned at 40 μm with a cryostat (Leica Biosystems). Free-floating sections were incubated overnight in PBS containing 0.1% Triton X-100 (PBT) and the following primary antibodies (1:500): mouse anti-NeuN (EMD Millipore), rabbit anti-neurokinin 1 receptor (NK1R; EMD Millipore), and chicken anti-GFP (Aves Labs). The tissue was washed in PBS six times for 5 min per wash and then incubated separately for 2–4 h in solution of PBT containing the following secondary antibodies (1:250): donkey anti-mouse Alexa Fluor 647, donkey anti-rabbit rhodamine red, and donkey anti-chicken Alexa Fluor 488 (Jackson ImmunoResearch). The tissue was then washed in PBS six times for 5 min per wash. Slides were mounted onto polylysine-coated slides, dehydrated overnight at room temperature, and coverslipped using Cytoseal 60 (Electron Microscopy Sciences) mounting medium. Slides were observed under an AxioCam 2 Zeiss fluorescent microscope with AxioVision acquisition software (Zeiss). Images were acquired and exported as TIFF files.

To calculate the transfection efficiencies of the two viruses, we counted transfected neurons from a single representative 40 μm section at the core of the pFv:HM4DR (n = 3) and pFv:AlstR (n = 3) injection sites from different rats. Images of these sections were opened in CorelDRAW.
GGEMG activity is not present at rest, but could be initiated during active expiration. As expiration
GGEMG activity was not consistently induced by any of the above manipulations, either in the same preparation or between different preparations, these data were not included for further analysis. To obtain control values, the 20 cycles preceding each experimental manipulation for all parameters were averaged. Under hypoxic or hypercapnic conditions, measurements from the 20 cycles preceding stimulus cessation were averaged. As the time of the peak response to B + S_pFL was different on each channel (airflow, DiaEMG, GGEMG, and AbdEMG), for values of the effect of B + S_pFL, 20 values were averaged at the peak of change on each channel separately; all measurements from the integrated airflow channel (f, V_T, T_In, and T_Ex) were made from the same 20 cycles. Data were analyzed off-line, and then exported to Excel for further analysis. All statistical tests were performed using Igor Pro. In one pFL:HM4DR experiment, data were excluded from analysis from the GGEMG channel, as the baseline was unstable in the period preceding the hypcapnic challenge.

For each variable, we calculated the ratio change induced by each stimulus under each condition. We divided the average of 20 cycles at the peak of the stimulus by the average of 20 cycles preceding the stimulus. These ratios were then used for statistical comparison. For example, to compute the ratio change for the effects of hyperpolarizing pFL:HM4DR neurons with CNO on respiratory changes induced by B + S_pFL (1) we computed the ratio of the change of each variable induced by B + S_pFL compared with its control, which we designate as [B + S_pFL]/Ctl; (2) we computed the ratio of the change of each variable induced by B + S_pFL in the presence of CNO compared with its control in the presence of CNO, which we designate as (B + S_pFL + CNO)/(Ctl + CNO); and (3) we compared the ratio changes of both groups, i.e., [B + S_pFL]/Ctl vs [(B + S_pFL + CNO)/(Ctl + CNO)]. If the ratio change in the presence of CNO was significantly closer to 1, then we conclude hyperpolarizing pFL:HM4DR neurons reduced the effect of changes induced by B + S_pFL. If the ratio change in the presence of CNO was significantly further away from 1, then we conclude hyperpolarizing pFL:HM4DR neurons increased the effect of changes induced by B + S_pFL. Ratio comparisons were then performed using one-way ANOVA in Igor Pro. If p < 0.05, *p < 0.01, **p < 0.005.

As described above, for each rat we calculated the average of 20 cycles preceding the stimulus (X_control), and the average of 20 cycles during the stimulus (X_stimulus). Both groups, the X_control values and their associated
Hyperpolarizing pFL neurons at rest had no effect on breathing

Based on our hypothesis that in adult rat the pF_{V} is a conditional expiratory oscillator that is inactive at rest (Janczewski and Feldman, 2006; Pagliardini et al., 2011), we predicted little or no effect of hyperpolarizing pFL neurons on resting ventilation. In anesthetized rats at rest transfected with HM4DR in the lateral parafacial, i.e., pF_{V}; HM4DR rats (Fig. 1Ai,B), CNO increased frequency (f) and decreased expiratory period (T_{E}), with no significant effect on inspiratory period (T_{I}), tidal volume (V_{T}), DiaEMG, or GGEMG; AbdEMG silent at rest, remained so after CNO (n = 13; Fig. 4Ai,Bi,D). We assessed whether the effects of CNO on resting ventilation were due exclusively to actions via pF_{V};HM4DR-transfected neurons or confounded by nonspecific effects. In anesthetized rats at rest with no HM4DRs (i.e., pF_{V};AlstR rats; Fig. 1xi), CNO increased f and decreased T_{E} with no significant effect on T_{I}, V_{T}, DiaEMG and GGEMG; AbdEMG silent at rest, remained so after CNO (n = 7; Fig. 4Ai,Bii,D). To determine whether hyperpolarizing pF_{V};HM4DR neurons had additional effects on f and T_{E} beyond the nonspecific effects seen in pF_{V};AlstR rats, we computed for each measured variable, i.e., f, T_{I}, T_{E}, V_{T}, DiaEMG, GGEMG, AbdEMG, the ratio of its value after CNO divided by its value in the dataset, whether it be a ratio of 1.

Results

Targeting of specific parafacial regions

By carefully choosing stereotaxic coordinates we were able to target viral injections into two anatomically separate regions that we designate as the parafacial ventral (pF_{V}) and parafacial lateral (pF_{L}; Fig. 2). Using anatomical markers we confirmed the location of our injections and that they were restricted to defined areas with no overlapping transfection of neurons (Fig. 2B,C). The pF_{V} was defined as the area ventral to the lateral edge of the facial nucleus, juxtaposed to the spinal trigeminal tract (Fig. 2Ai,B). The pF_{L} was defined as the area ventral to the caudal half of the facial nucleus, between the pyramidal tract and the spinal trigeminal tract (Fig. 2Ai,C,D). In representative individual 40 µm sections from the core of the synapsin-HM4DR injection site in the pF_{V} of different rats, 135 ± 20 neurons expressed mCitrine representing 78 ± 9% of neurons (n = 3). Similarly, in representative individual 40 µm sections from the core of the synapsin-AlstR injection site in the pF_{L} of different rats, 129 ± 21 neurons expressed GFP, representing 76 ± 6% of neurons (n = 3). Therefore, the transfection efficiencies of the two viruses in each area were similar for both the number of transfected neurons (p = 0,5), and the percentage of neurons transfected (p = 0,3).

Hyperpolarizing pF_{L} neurons at rest

Hyperpolarizing pF_{L} neurons reduced effects of hypercapnia (9% CO_{2}) on AbdEMG only. A, Integrated traces from a single experiment: shaded area shows period of hypercapnia. Ai, Rest; Aii, During application of CNO to medullary surface (present for entire trace). B, Comparison of respiratory variables before and after hypercapnia in pF_{L};HM4DR rats at rest (Bii) and in the presence of CNO (Bi). Lines connect data from individual experiments, and box-and-whisker plots show combined data. Data in Bi and Bii are normalized to highest value for that parameter, i.e., f, T_{I}, T_{E}, V_{T}, DiaEMG, GGEMG, AbdEMG, regardless of whether it belonged to control or 9% CO_{2} group. C, Comparison between ratio changes induced by hypercapnia in pF_{L};HM4DR rats at rest and in the presence of CNO. Data in C are expressed as ratios of resting values, and red horizontal dashed line represents a ratio of 1. D, Table containing median, IQR, and p values, from data represented in B. *p < 0,05, **p < 0,01, ***p < 0,005.

\[ \frac{\text{Experimental value}}{\text{Control value}} \]

value for every rat, were combined into a single dataset. To facilitate graphical comparisons, excluding ratio changes (see above), data were normalized to the highest value in the dataset regardless of whether it belonged to \( \bar{X}_{\text{control}} \) or \( \bar{X}_{\text{stimulus}} \) group. Therefore the highest value in the dataset, whether it be \( \bar{X}_{\text{control}} \) or \( \bar{X}_{\text{stimulus}} \) was 1.0.

For the purpose of analysis, active expiration was defined by the presence of bursts of AbdEMG activity above tonic levels between inspiratory bursts, similar to previous studies (Pagliardini et al., 2011). As usually seen in smaller sample sizes (n < 30), recorded data were often skewed, and thus were treated as nonparametric, for which the median and interquartile range (IQR) better represent the data than mean, and SD or SE. We used nonparametric tests for our statistical analysis, as these tests make fewer assumptions about the data and would not require us to exclude statistical outliers. Statistical tests are Wilcoxon signed-rank tests unless otherwise stated, with a significance level of p ≤ 0.05. In Results, we have displayed the data as box-and-whisker plots for comparison of group data and as line graphs for individual experiments.
its value before CNO, i.e., “ratio change” (see Materials and Methods, Data analysis and statistics), in rats with versus without HM4DRs, i.e., pF:\(\text{HM4DR}\) \(\{\text{rest + CNO}\}/\text{rest}\) vs pF:\(\text{ctl}\):AltR \(\{\text{rest + CNO}\}/\text{rest}\). The ratio changes between the pF:\(\text{ctl}\):HM4DR and pF:\(\text{ctl}\):AlstR rat groups were not significantly different for any variable \((n = 20, \text{Kruskal–Wallis}; \text{Fig. } 4C,D)\). As the effects of CNO on resting ventilation were similar whether HM4DRs were present or not, the CNO-induced changes at rest were not due to activation of HM4DRs, but instead to some nonspecific effect of CNO or its vehicle. In subsequent experiments, to control for nonspecific effects of CNO, we compared the effects of CNO on the responses to different stimuli in rats without HM4DRs to rats with HM4DRs.

Disinhibition of pF\(i\) has profound effects on respiration

pF\(i\) neurons can be disinhibited by injection of a mixture of the GABA\(A\) and glycine antagonists bicuculline and strychnine \((B + S_{\text{pF}i})\), which results in a transformation in breathing from passive to active expiration (Pagliardini et al., 2011). Hyperpolarization of pF:\(i\):HM4DR neurons should reduce the depolarizing effects of B + S\(p_{\text{pF}i}\). In anesthetized rats at rest transfected with HM4DR in the lateral parafacial, i.e., pF:\(i\):HM4DR rats (Fig. 1\(i\)ii)), B + S\(p_{\text{pF}i}\) decreased \(T_E\) and \(V_T\), increased \(V_{Ei}\), DI\(a_{\text{EMG}}\) and GG\(E_{\text{EMG}}\) and induced expiratory-related Abd\(E_{\text{EMG}}\) \((n = 13\); Fig. 5\(Ai\)iii,Bii,D), the latter a signature of active expiration, q.v. (Pagliardini et al., 2011). The same trends \((n = 13; \text{Fig. } 5\text{A}i\text{ii},\text{B}ii)\) were seen in the presence of CNO (Fig. 1\(ix\)). However, in the presence of CNO following B + S\(p_{\text{pF}i}\), the ratio changes \([(B + S_{\text{pF}i} + \text{CNO})/(\text{Ctl} + \text{CNO})]\) for all measured variables were significantly closer to 1 than those in the absence of CNO \([B + S_{\text{pF}i}/\text{Ctl}]\) \((n = 13; \text{Fig. } 5\text{C}i\text{D})\). As CNO in the absence of HM4DRs did not affect the response to B + S\(p_{\text{pF}i}\) (see next paragraph), these results indicate that hyperpolarizing pF\(i\):HM4DR neurons with CNO attenuated the effects of B + S\(p_{\text{pF}i}\) on all variables.

CNO does not affect the response to B + S\(p_{\text{pF}i}\) in the absence of CNO–sensitive HM4DRs

In anesthetized rats at rest with no HM4DRs, i.e., pF:\(i\):AlstR rats (Fig. 1\(iii\)ii)), B + S\(p_{\text{pF}i}\) decreased \(T_E\) \((p = 0.02)\) and \(T_f\) \((p = 0.02)\), increased \(V_{Ei}\) \((p = 0.008)\), \(V_T\) \((p = 0.02)\), DI\(a_{\text{EMG}}\) \((p = 0.008)\), and GG\(E_{\text{EMG}}\) \((p = 0.008)\), and induced expiratory-related Abd\(E_{\text{EMG}}\) \((p = 0.02; n = 7); \text{data not shown}\). The same trends \((n = 7; f : p = 0.02; T_E : p = 0.02; T_E : p = 0.008; V_{Ei} : p = 0.02; D_{a_{\text{EMG}}} : p = 0.008); G_{E_{\text{EMG}}} : p = 0.008); A_{b_{\text{EMG}}} : p = 0.02; \text{data not shown}\) were seen in the presence of CNO (Fig. 1\(x\)). Importantly, in the presence of CNO following B + S\(p_{\text{pF}i}\), the ratio changes \([(B + S_{\text{pF}i} + \text{CNO})/(\text{Ctl} + \text{CNO})]\) for all measured variables were not significantly different to those in the absence of CNO \([B + S_{\text{pF}i}/\text{Ctl}]\) \((n = 7; f : p = 0.8; T_E : p = 0.2; T_f : p = 0.7; V_{Ei} : p = 1.0; D_{a_{\text{EMG}}} : p = 1.0; G_{E_{\text{EMG}}} : p = 0.7); A_{b_{\text{EMG}}} : p = 0.6); \text{data not shown}\). Thus any nonspecific effects of CNO on \(f\) did not affect the response to B + S\(p_{\text{pF}i}\).

Hyperpolarizing pF\(i\) neurons during hypercapnia only affect Abd\(E_{\text{EMG}}\)

Hypercapnia increases ventilation by increasing \(V_T\) without a concurrent change in \(f\) (Stunden et al., 2001; Putnam et al., 2005), and elicits robust expiratory-related abdominal activity (Iizuka and Fregosi, 2007; Marina et al., 2010), i.e., active expiration. These effects were reduced by hyperpolarizing parafacial neurons (Marina et al., 2010). In anesthetized rats at rest transfected with...
CNO does not affect the response to hypercapnia in the absence of CNO-sensitive HM4DRs

In anesthetized rats at rest with no HM4DRs, i.e., pFV:AlstR rats (Fig. 1ii), hypercapnia did not affect \( f \) (\( p = 1.0 \)); decreased \( T_i \) (\( p = 0.04 \)); did not affect \( T_e \) (\( p = 0.6 \)); increased \( V_t \) (\( p = 0.02 \)); Di\( \text{a}_{\text{EMG}} \) (\( p = 0.008 \)); and GG\( \text{EMG} \) (\( p = 0.008 \)); and induced expiratory-related Abd\( \text{EMG} \) (\( p = 0.008; n = 7; \) data not shown). The same trends (\( n = 7; f; p = 0.6; T_i; p = 0.02; T_e; p = 0.3; V_t; p = 0.02; \) Di\( \text{a}_{\text{EMG}}; p = 0.008; \) GG\( \text{EMG}; p = 0.008; \) Abd\( \text{EMG}; p = 0.008; \) data not shown) were seen in the presence of CNO (Fig. 1xi). Importantly, during hypercapnia in the presence of CNO, the ratio changes [hypercapnia/CNO]/(Ctl in CNO) for all measured variables were not significantly different to those in the absence of CNO [hypercapnia/Ctl] (\( n = 7; f; p = 1.0; T_i; p = 0.3; T_e; p = 0.7; V_t; p = 0.9; \) Di\( \text{a}_{\text{EMG}}; p = 0.4; \) GG\( \text{EMG}; p = 0.1; \) Abd\( \text{EMG}; p = 0.8; \) data not shown). Thus any nonspecific effects of CNO on \( f \) did not affect the response to hypercapnia.

Hyperpolarizing pFV neurons during hypoxia only affect Abd\( \text{EMG} \)

Hypoxia increases ventilation by increasing \( V_t \) with a concurrent change in \( f \), and elicits robust expiratory-related abdominal activity (Sherrey et al., 1988; Iizuka and Fregosi, 2007). In anesthetized rats at rest transfected with HM\( _4 \)DR in the lateral parafacial, i.e., pFV:HM\( _4 \)DR rats (Fig. 1i,ii), hypoxia increased \( f \); decreased \( T_i \) and \( T_e \); increased \( V_t \), Di\( \text{a}_{\text{EMG}} \), and GG\( \text{EMG} \); and induced expiratory-related Abd\( \text{EMG} \) (\( n = 13; \) Fig. 7Ai,Bi,D). The same trends (\( n = 13; \) Fig. 7Ai, Bi, D) were seen in the presence of CNO (Fig. 1ix). However, in the presence of CNO during hypoxia only the ratio change [hypoxia + CNO]/(Ctl + CNO) for expiratory-related Abd\( \text{EMG} \) was significantly closer to 1 than the ratio change in the absence of CNO [hypoxia/Ctl] (\( n = 13; \) Fig. 7C,D). CNO in the absence of HM\( _4 \)DRs did not affect the response to hypoxia (see next paragraph). Thus hyperpolarizing pFV:HM\( _4 \)DR neurons with CNO only attenuated the effects of hypoxia on expiratory-related Abd\( \text{EMG} \).

CNO does not affect response to hypoxia in absence of CNO-sensitive HM\( _4 \)DRs

In anesthetized rats at rest with no HM\( _4 \)DRs, i.e., pFV:AlstR rats (Fig. 1iii), hypoxia increased \( f \) (\( p = 0.02 \)); decreased \( T_i \) (\( p = 0.04 \)) and \( T_e \) (\( p = 0.04 \)); increased \( V_t \) (\( p = 0.02 \)); Di\( \text{a}_{\text{EMG}} \) (\( p = 0.008 \)); and GG\( \text{EMG} \) (\( p = 0.008 \)); and induced expiratory-related Abd\( \text{EMG} \) (\( p = 0.008; n = 7; \) data not shown). The same trends (\( n = 7; f; p = 0.6; T_i; p = 0.02; T_e; p = 0.3; V_t; p = 0.02; \) Di\( \text{a}_{\text{EMG}}; p = 0.008; \) GG\( \text{EMG}; p = 0.008; \) Abd\( \text{EMG}; p = 0.008; \) data not shown) were seen in the presence of CNO (Fig. 1xi). Importantly, during hypoxia in the presence of CNO, the ratio changes [hypoxia/CNO]/(Ctl in CNO) for all measured variables were not significantly different to those in the absence of CNO [hypoxia/Ctl] (\( n = 7; f; p = 1.0; T_i; p = 0.3; T_e; p = 0.7; V_t; p = 0.9; \) Di\( \text{a}_{\text{EMG}}; p = 0.4; \) GG\( \text{EMG}; p = 0.1; \) Abd\( \text{EMG}; p = 0.8; \) data not shown). Thus any nonspecific effects of CNO on \( f \) did not affect the response to hypoxia.

HM\( _4 \)DR in the lateral parafacial, i.e., pFV:HM\( _4 \)DR rats (Fig. 1i,ii), hypercapnia did not affect \( f \); decreased \( T_i \); did not affect \( T_e \); increased \( V_t \), Di\( \text{a}_{\text{EMG}} \), and GG\( \text{EMG} \); and induced expiratory-related Abd\( \text{EMG} \) (\( n = 13; \) Fig. 6Ai,Bi,D). The same trends (\( n = 13; \) Fig. 6Ai, Bi, D) were seen in the presence of CNO (Fig. 1xx). However, in the presence of CNO during hypercapnia only the ratio change [hypercapnia + CNO]/(Ctl + CNO) for expiratory-related Abd\( \text{EMG} \) was significantly closer to 1 than the ratio change in the absence of CNO [hypercapnia/Ctl] (\( n = 13; \) Fig. 6C,D). CNO in the absence of HM\( _4 \)DRs did not affect the response to hypercapnia (see next paragraph). Thus hyperpolarizing pFV:HM\( _4 \)DR neurons with CNO only attenuated the effects of hypercapnia on expiratory-related Abd\( \text{EMG} \).
0.02; $T_i^p = 0.04$; $T_e^p = 0.04$; $V_t^p = 0.02$; $\text{Dia}_{\text{EMG}}^p = 0.0008$; $\text{GG}_{\text{EMG}}^p = 0.0008$; $\text{Abd}_{\text{EMG}}^p = 0.0008$; data not shown) were seen in the presence of CNO ([hypoxia + CNO]/[Ctl + CNO]) for all measured variables were not significantly different to the ratio changes ([hypoxia/Ctl] for allatostatin bilaterally in the absence of CNO [hypoxia/Ctl] $(n = 7; f = 0.5$; $T_i^p = 0.1$; $T_e^p = 0.9$; $V_t^p = 0.3$; $\text{Dia}_{\text{EMG}}^p = 0.9$; $\text{GG}_{\text{EMG}}^p = 0.2$; $\text{Abd}_{\text{EMG}}^p = 0.7$; data not shown). Thus any nonspecific effect of CNO on $f$ did not affect the response to hypoxia.

### Hyperpolarizing pFV neurons at rest decrease $V_t^p$ and $\text{Dia}_{\text{EMG}}^p$

Neurons ventral to the facial nucleus are hypothesized to provide significant ventilatory drive at rest (Smith et al., 1989; Ellenberger and Feldman, 1990; Nattie, 2000; Guyenet et al., 2005), and hyperpolarizing neurons ventral to the facial nucleus decrease resting ventilation (Nattie and Li, 2002; Li et al., 2006) and reduce phrenic nerve activity (Marina et al., 2010). Thus we predicted that hyperpolarizing pFV neurons would reduce $\text{Dia}_{\text{EMG}}^p$ and consequently $V_t^p$. In anesthetized rats at rest transfected with AlstR in the ventral parafacial, i.e., pFV:AlstR rats ([Fig. 1v,iv]), allatostatin bilaterally injected into the pFV (Alst) decreased $V_t^p$ and $\text{Dia}_{\text{EMG}}^p$, with no significant effect on $f$, $T_i^p$, $T_e^p$, and $\text{GG}_{\text{EMG}}^p$; $\text{Abd}_{\text{EMG}}^p$, silent at rest, remained so after Alst $(n = 13$; [Fig. 8Ai,Bi,D]).

We assessed whether the effects of injecting Alst into the pFV on resting ventilation were due exclusively to actions via pFV:AlstR neurons or confounded by nonspecific effects of Alst or vehicle. In anesthetized rats at rest with no AlstRs, i.e., pFV:HM4DR rats ([Fig. 1v]), Alst did not affect $f$, $T_i^p$, $T_e^p$, $V_t^p$, $\text{Dia}_{\text{EMG}}^p$, and $\text{GG}_{\text{EMG}}^p$; $\text{Abd}_{\text{EMG}}^p$, silent at rest, remained so after Alst $(n = 7$; [Fig. 8Aii,Bii,D]). Therefore, following Alst under resting conditions only the ratio changes, i.e., pFV:AlstR $[(\text{rest + Alst})/\text{rest}]$ vs pFV:HM4DR $[(\text{rest + Alst})/\text{rest}]$, for $V_t^p$ and $\text{Dia}_{\text{EMG}}^p$ in pFV:AlstR rats were significantly different to those in pFV:HM4DR rats $(n = 20$; Kruskal–Wallis: [Fig. 8C, D]). Thus hyperpolarizing pFV:AlstR neurons reduced $V_t^p$ and $\text{Dia}_{\text{EMG}}^p$, consistent with the hypothesis that the pFV contributes to respiratory drive at rest (Smith et al., 1989; Ellenberger and Feldman, 1990).

### Hyperpolarizing pFV neurons during B + $S_{\text{PFL}}$ only attenuates $\text{Abd}_{\text{EMG}}$

We wanted to ascertain if, and how, the pFV and pFV interact. Thus we hyperpolarized pFV neurons followed by B + $S_{\text{PFL}}$. In anesthetized rats at rest transfected with AlstR in the ventral parafacial, i.e., pFV:AlstR rats ([Fig. 1i,iii]), B + $S_{\text{PFL}}$ decreased $f$ and $T_i^p$; increased $T_e^p$, $V_t^p$, $\text{Dia}_{\text{EMG}}^p$, and $\text{GG}_{\text{EMG}}^p$ and induced expiratory-related $\text{Abd}_{\text{EMG}}^p$ $(n = 13$; Results). The same trends $(n = 13$; [Fig. 9Aii,Bii,D]) were seen in the presence of Alst ([Fig. 1vii,vi]). However, in the presence of Alst following B + $S_{\text{PFL}}$ only the ratio change $[(B + S_{\text{PFL}} + \text{Alst})/(\text{Ctl + Alst})]$ for expiratory-related $\text{Abd}_{\text{EMG}}^p$ was significantly closer to 1 than the ratio change in the absence of Alst $[(B + S_{\text{PFL}})/(\text{Ctl})]$ $(n = 13$; [Fig. 9C,D]). Alst in the absence of AlstRs did not affect the response to B + $S_{\text{PFL}}$ (see next paragraph). Thus although B + $S_{\text{PFL}}$ had profound effects on breathing patterns, the sole effect of hyperpolarizing pFV neurons was to reduce the $B + S_{\text{PFL}}$-induced increase in expiratory-related $\text{Abd}_{\text{EMG}}^p$.

Alst does not affect the response to B + $S_{\text{PFL}}$ in the absence of AlstRs

In anesthetized rats at rest with no AlstRs, i.e., pFV:HM4DR rats ([Fig. 1i]), B + $S_{\text{PFL}}$ decreased $f$ $(p = 0.02)$ and $T_i^p$ $(p = 0.04)$;
with AlstR in the ventral parafacial, i.e., pFV:AlstR rats (Fig. 1i,iii), hypercapnia did not affect f; decreased T\(_E\); did not affect T\(_i\); increased V\(_T\), Dia\(_{EMG}\), and GG\(_{EMG}\); and induced expiratory-related Abd\(_{EMG}\) (n = 13; Fig. 10Ai,Bii,D). The same trends (n = 13; Fig. 10Aii,Bii,D) were seen in the presence of Alst (Fig. 1v,vi,vi). However, in the presence of Alst during hypercapnia, only the ratio changes [(hypercapnia + Alst)/(Ctl + Alst)] for inspiratory-related GG\(_{EMG}\) and expiratory-related Abd\(_{EMG}\) were significantly closer to 1 than the ratio changes in the absence of Alst [hypercapnia/Ctl] (n = 13; Fig. 10C,D), Alst in the absence of AlstRs did not affect the response to hypercapnia (see next paragraph). Thus hyperpolarizing pFV neurons during hypercapnia decreased both inspiratory-related GG\(_{EMG}\) and expiratory-related Abd\(_{EMG}\) activity.

Alst does not affect the response to hypercapnia in the absence of AlstRs

In anesthetized rats at rest with no AlstRs, i.e., pF\(_V\):H\(_M\)\(_D\)R rats (Fig. 1), hypercapnia did not affect f (p = 0.8); decreased T\(_i\) (p = 0.04); did not affect T\(_E\) (p = 0.3); increased V\(_T\) (p = 0.02), Dia\(_{EMG}\) (p = 0.02), and GG\(_{EMG}\) (p = 0.02); and induced expiratory-related Abd\(_{EMG}\) (p = 0.02; n = 7; data not shown). The same trends (n = 7; f; p = 0.9; T\(_i\); p = 0.02; T\(_E\); p = 0.8; V\(_T\); p = 0.02; Dia\(_{EMG}\); p = 0.02; GG\(_{EMG}\); p = 0.02; Abd\(_{EMG}\); p = 0.02; data not shown) were seen in the presence of Alst (Fig. 1v). Importantly, in the presence of Alst during hypercapnia, the ratio changes [(hypercapnia + Alst)/(Ctl + Alst)] for all measured variables was not significantly different to those in the absence of Alst [hypercapnia/Ctl] (n = 7; f; p = 0.5; T\(_i\); p = 0.2; T\(_E\); p = 0.3; V\(_T\); p = 0.08; Dia\(_{EMG}\); p = 0.4; GG\(_{EMG}\); p = 0.5; Abd\(_{EMG}\); p = 0.7; data not shown). Thus Alst in the absence of AlstR did not affect the response to hypercapnia.

Hyperpolarizing pF\(_V\) neurons during hypoxia attenuates GG\(_{EMG}\) and Abd\(_{EMG}\)

Using a similar sequence of protocols, we next tested the effect of hypoxia at rest. In anesthetized rats at rest transfected with AlstR in the ventral parafacial, i.e., pF\(_V\):AlstR rats (Fig. 1i,iii) under resting conditions, hypoxia increased f; decreased T\(_i\) and T\(_E\); increased V\(_T\), Dia\(_{EMG}\), and GG\(_{EMG}\); and induced expiratory-related Abd\(_{EMG}\) (n = 13; Fig. 11Ai,Bii,D). The same trends (n = 13; Fig. 11Aii,Bii,D) were seen in the presence of Alst (Fig. 1v,vi,vi). However, in the presence of Alst during hypoxia, only the ratio changes [(hypoxia + Alst)/(Ctl + Alst)] for inspiratory-related GG\(_{EMG}\) and expiratory-related Abd\(_{EMG}\) were significantly closer to 1 than the ratio changes in the absence of Alst [hypoxia/Ctl] (n = 13; Fig. 11C,D). Alst in the absence of AlstRs did not affect the response to hypoxia (see next paragraph). Thus hyperpolarizing pF\(_V\) neurons during hypoxia decreased both inspiratory-related GG\(_{EMG}\) and expiratory-related Abd\(_{EMG}\) activity.

Hyperpolarizing pF\(_V\) neurons during hypercapnia attenuates GG\(_{EMG}\) and Abd\(_{EMG}\)

To assess the role of the pF\(_V\) in chemoreflexes, we first tested the response to hypercapnia. In anesthetized rats at rest transfected with AlstR in the ventral parafacial, i.e., pF\(_V\):AlstR rats (Fig. 1i,iii) under resting conditions, hypoxia increased f; decreased T\(_i\) and T\(_E\); increased V\(_T\), Dia\(_{EMG}\), and GG\(_{EMG}\); and induced expiratory-related Abd\(_{EMG}\) (n = 13; Fig. 11Ai,Bii,D). The same trends (n = 13; Fig. 11Aii,Bii,D) were seen in the presence of Alst (Fig. 1v,vi,vi). However, in the presence of Alst during hypercapnia, only the ratio changes [(hypercapnia + Alst)/(Ctl + Alst)] for inspiratory-related GG\(_{EMG}\) and expiratory-related Abd\(_{EMG}\) were significantly closer to 1 than the ratio changes in the absence of Alst [hypercapnia/Ctl] (n = 13; Fig. 11C,D). Alst in the absence of AlstRs did not affect the response to hypoxia (see next paragraph). Thus hyperpolarizing pF\(_V\) neurons during hypercapnia decreased both inspiratory-related GG\(_{EMG}\) and expiratory-related Abd\(_{EMG}\) activity.
Alst does not affect the response to hypoxia in the absence of AlstRs

In anesthetized rats at rest with no AlstRs, i.e., PfV,HM4DR rats (Fig. 1i), hypoxia increased f (p = 0.02); decreased T1 (p = 0.02) and T2 (p = 0.02); increased VT (p = 0.03), DIaEMG (p = 0.03), and GGEMG (p = 0.02); and induced expiratory-related AbdEMG activity (p = 0.02; n = 7; data not shown). The same trends (n = 7; f = 0.02; T1f = p = 0.01; T2f: p = 0.02; VTf: p = 0.02; DIaEMGf: p = 0.02; GGEMGf: p = 0.008; AbdEMGf: p = 0.02; data not shown) were seen in the presence of Alst (Fig. 1v). Importantly, in the presence of Alst, during hypoxia, the ratio changes [(hypoxia + Alst)/(Ctl + Alst)] for all measured variables were not significantly different to those in the absence of Alst [hypoxia/Ctl] (n = 7; f = 1.0; T1: p = 0.3; T2: p = 1.0; VT: p = 0.8; DIaEMG: p = 0.2; GGEMG: p = 0.2; AbdEMG: p = 0.2; data not shown). Thus, Alst in the absence of AlstR did not affect the response to hypoxia.

AlstR and HM4DR are equally effective in our experimental paradigms

Differences in the effects of hyperpolarizing PfV or PfV neurons could be due to the differences in the effects of activating HM4DRs versus AlstRs, and not a functional distinction between PfV and PfV. To assess this, we transfected the PfV with HM4DRs.

We first tested the response to capnopenia. In anesthetized rats at rest transfected with HM4DR in the ventral parafacial, i.e., PfV:HM4DR rats (Fig. 1iv,xii), hypercapnia did not affect f; decreased T1f; did not affect T2f; increased VTf; DIaEMGf and GGEMGf; and induced expiratory-related AbdEMGf (n = 13; Fig. 12Ai,Bi,D). The same trends (n = 13; Fig. 12Aii,Bii,D) were seen in the presence of CNO (Fig. 1viii). However, in the presence of CNO, during hypercapnia, only the ratio changes [(hypercapnia + CNO)/(Ctl + CNO)] for inspiratory-related GGEMG and expiratory-related AbdEMG were significantly closer to 1 than the ratio changes in the absence of CNO [hypoxia/Ctl] (n = 13; Fig. 12C,D). Thus, hyperpolarizing PfV:HM4DR neurons during hypercapnia affected both inspiratory-related GGEMG and expiratory-related AbdEMG. Regardless of whether the PfV neurons were hyperpolarized via the activation of AlstRs or HM4DRs, the ratio changes, i.e., [(hypercapnia + Alst)/(Ctl + Alst)] vs [(hypercapnia + CNO)/(Ctl + CNO)], were similar for inspiratory-related GGEMG and expiratory-related AbdEMG (n = 21; PfV:AlstR: 1.2 IQR 0.7; PfV:HM4DR: 1.6 IQR 1.6; Kruskal–Wallis: p = 0.2; data not shown).
HM4DR: 9.2 IQR 8.2; Kruskal–Wallis; pFV:HM4DR rats at rest and in presence of CNO. Data in
troughs in the same rat we could discriminate these differ-
to illuminate their differences in function. By making these per-
followed by exogenous application of their ligand (Alst or CNO),
regardless of whether it belonged to control or 9% CO2 group.

Figure 12. Hyperpolarizing pFV neurons during hypercapnia (9% CO2) with HM4DR only affects GGEMG and AbdEMG. A. Integrated traces from a single experiment: shaded area shows period of hypercapnia. Ai. Rest. Ai. During application of CNO to medullary surface (present for entire trace). B. Comparison of respiratory variables before and after hypercapnia in pFV:HM4DR rats at rest (Bi) and in the presence of CNO (Bi). Lines connect data from individual experiments, and box-and-whisker plots show combined data. Data in Bi and Bi are normalized to highest value for that parameter, i.e., f, Ti, Vt, GGEMG, DIaEMG or AbdEMG
regardless of whether it belonged to control or 9% CO2 group. C. Comparison between ratio changes induced by hypercapnia in pFV:HM4DR rats at rest and in presence of CNO. Data in C are expressed as ratios of resting values, and red horizontal dashed line represents a ratio of 1. D. Table containing median, IQR, and p values, from data represented in B. *p < 0.05, **p < 0.01, ***p < 0.005. Bi = 7 for GGEMG.

HM4DR: 1.6 IQR 0.5; Kruskal–Wallis; p = 0.5; data not shown) and
expiratory-related AbdEMG (n = 21; pFV:AlstR: 4.8 IQR 5.8; pFV:HM4DR: 9.2 IQR 8.2; Kruskal–Wallis; p = 0.2; data not shown). Thus the disparities between the pFV and pFV in terms of the
response to hypercapnia and hypoxia are due to functional differences between the pFV and pFV.

Discussion
We investigated the respective contributions of two adjacent
parafacial regions in controlling breathing pattern. To do this we
combined two recently developed technologies for hyperpolariz-
ing neurons, i.e., transfection of neurons with AlstRs or HM4DRs
followed by exogenous application of their ligand (Alst or CNO),
to illuminate their differences in function. By making these perturbations in the same rat we could discriminate these differ-
ences. Although we focus on breathing, this approach can be used in any region of
the brain to parse out differences in function among neighboring subpopulations, and where distinguishing genetic markers
can be exploited, one could discriminate between overlapping populations.

Breathing in mammals is a complex behavior with critical sites in the brains-
for rhythm and pattern generation and for sensory processing. An emerging
picture is that distinct regions have specific
functional roles (Feldman et al., 2013), such as in CO2-chemoreception
(Guyenet et al., 2010; Hodges and Richer-
sion, 2010; Huckstepp and Dale, 2011; Nattie, 2011), or in generation of expira-
tory (Onimaru and Homma, 2003; Jancz-
zweski and Feldman, 2006; Pagliardini et al., 2011) or inspiratory (Smith et al., 1991; Tan et al., 2008) rhythm. Appropriate
parcellation of distinct respiratory functions to definable brainstem
regions is essential for understanding neural control of breathing. Since the
identification of the parafacial RTN (Smith et al., 1989; Ellenberger and Feldman, 1990; Li et al., 1999; Mulkey et al., 2004), increasing attention has been focused on understanding its role and, as
more data became available (Onimaru and Homma, 2003; Janczewski and Feldman, 2006; Feldman et al., 2009; Pagliardini et al.,
2011; Tupal et al., 2014), e.g., the role of other parafacial regions. In adult rats, we found that two anatomically separate
parafacial regions, the pFV and pFV, perform very distinct roles in control of
breathing, with an overlapping role in central chemoreception.

pFV and pFV have different active states
at rest
At rest the lack of significant expiratory
pumping by abdominal muscles (Izuka and Fregosi, 2007) can be transformed into active expiration by disinhibition or
photoactivation of the presumptive pFV
(Pagliardini et al., 2011). This suggests
that the pFV is silent at rest due to tonic suppression by inhibitory
neurons, and that once disinhibited and/or excited, the pFV drives
active expiration (Pagliardini et al., 2011). We predicted that hy-
perpolarizing pFV:HM4DR neurons should not alter basal venti-
lation, and that was the case (Fig. 4).

Disinhibition in the pFV, i.e., B + SAlst, produces expiratory-
related AbdEMG activity (Pagliardini et al., 2011), a reduction in f
with a concomitant increase in Vt, and an increase in inspiratory-
related DIaEMG and GGEMG. In pFV:HM4DR-transfected rats,
these effects were significantly reduced following application of
CNO (Fig. 5). That CNO did not completely abolish the effects of
B + SAlst is likely due to incomplete transfection of pFV neurons
(~76%; Fig. 2) in the effective B + Sap injection site and/or B + SAp
produced sufficient disinhibitory depolarization to over-
come the hyperpolarizing effects of CNO on HM4DR-transfected neurons. We conclude that the pFV is silent at rest due, at least in part, to postsynaptic inhibition mediated by GABAergic and/or glycine-ergic receptors, but once sufficiently excited, triggers active expiration; this is consistent with our hypothesis that the pFV is a conditional expiratory oscillator (Fig. 14; Mellen et al., 2003; Janczewski and Feldman, 2006; Pagliardini et al., 2011).

The area ventral to the facial nucleus is generally accepted to process and integrate multiple sensory inputs related to ventilation and provide a facilitatory drive to breathe (Nattie, 2000; Li et al., 2006; Moreira et al., 2007a; Mulkey et al., 2007). Consistent with previous results (Nattie and Li, 2002; Marina et al., 2010), we found that hyperpolarizing pFV:AlstR neurons reduced basal ventilation, via a reduction in VT due, at least in part, to attenuated DiaEMG activity with no change in f (Fig. 8). We conclude that at rest the pFV provides an excitatory drive to breathe (Fig. 14).

**pFV and pFL have different roles in the response to both hypoxia and hypercapnia**

In vagotomized urethane-anesthetized rats at rest, both the pFV and pFL are involved in responses to hypoxia and hypercapnia. The pFV and pFL were distinguished by their particular roles in these responses. Hyperpolarizing pFL neurons only attenuated hypcapnia-induced (Fig. 6) and hypoxia-induced (Fig. 7) expiratory-related AbdEMG, suggesting that this change is not related to the modality of the perturbation, but was specific to the initiation and maintenance of active expiration. Hyperpolarizing pFV neurons attenuated increases in both hypercapnia-induced (Figs. 10, 12) and hypoxia-induced (Figs. 11, 13) expiratory-related AbdEMG and inspiratory-related GGEMG, suggesting that these changes are not related to the modality of the perturbation, but were specific to the loss of facilitatory drive affecting respiratory muscle activity. Thus when ventilatory demand increases due to changes in blood gases, the pFV drives active expiration and the pFL decreases away resistance during inspiration along with a concomitant facilitatory drive that in-creases expiratory-related AbdEMG activity (Fig. 14).

**Further evidence the pFV is a conditional expiratory oscillator**

The pFV contains glutamatergic neurons (Nattie and Li, 2002; Mulkey et al., 2004; Stornetta et al., 2006), lacks glyciner-ergic neurons (Tanaka et al., 2003a; Fortuna et al., 2008; Abbott et al., 2009), and is almost completely devoid of GABAergic neurons (Ellenberger, 1999; Stornetta and Guyenet, 1999; Tanaka et al., 2003). The pFL also contains glutamatergic neurons (Onimaru et al., 2003) and lacks inhibitory neurons (Ellenberger, 1999; Stornetta and Guyenet, 1999; Tanaka et al., 2003). Thus for this discussion we consider neurons in these parafacial regions to be exclusively excitatory.

Disinhibition of the pFV (with B + SppFV) reduces f (Fig. 5), either directly through activation of preBoTC inhibitory neurons or indirectly through activation of BoTC inhibitory neurons (Fig. 14). That activation of the pFV also led to expiratory-related activity on both the AbdEMG and GGEMG (Fig. 5) suggests that it projects, directly or indirectly, to premotor neurons controlling the abdominal muscles (Janczewski et al., 2002) and the tongue (Fig. 14). Finally, hyperpolarizing pFV neurons following hypoxia or hypercapnia only reduced expiratory activity, but did not affect any inspiratory parameters (Figs. 6, 7). Collectively these data are consistent with our hypothesis that the pFV gates active expiration, most likely as a conditional expiratory oscillator (Pagliardini et al., 2011).
\( \text{pF}_V \) provides inspiratory drive to breathe

Hyperpolarizing either \( \text{pF}_V \) or \( \text{pF}_L \) neurons attenuated expiratory-related \( \text{AbdEMG} \), suggesting either a direct interaction between these nuclei or a downstream convergence of their projections onto abdominal (pre)motoneurons (Janczewski et al., 2002). Due to the apparent lack of inhibitory neurons in the \( \text{pF}_L \) and \( \text{pF}_V \) (see above), if \( \text{pF}_V \) neurons project to the \( \text{pF}_L \), hyperpolarizing \( \text{pF}_V \) neurons should attenuate all \( \text{B} + \text{SpFL} \)-induced changes in respiration; yet, hyperpolarizing \( \text{pF}_V \) neurons only reduced \( \text{B} + \text{SpFL} \)-induced expiratory-related \( \text{AbdEMG} \), but not \( \text{f}, \text{Ti}_L, \text{T}_F, \text{Vp}, \text{GGEMG}, \text{or DiaEMG} \) (Fig. 9), supporting the idea that the \( \text{pF}_V \) modulates expiratory-related abdominal activity at the (pre)motoneuronal level, i.e., expiratory bulbospinal neurons (Janczewski et al., 2002). Importantly, the \( \text{pF}_V \) projects directly (Núñez-Abades et al., 1993; Gerrits and Holstege, 1996; Rosin et al., 2006) and indirectly, via the \( \text{Bo}\text{tC} \) and the parabrachial/Kölliker-fuse nuclei (Núñez-Abades et al., 1993; Rosin et al., 2006), to the caudal ventral respiratory group (cVRG; Fig. 14), which contains bulbospinal neurons projecting to spinal interneurons that in turn project to abdominal motoneurons (Iscoe, 1998; Cinelli et al., 2012), providing a pathway by which the \( \text{pF}_V \) can influence expiratory-related \( \text{AbdEMG} \) activity (Fig. 14).

\( \text{pF}_V \) provides inspiratory drive

Interestingly, hyperpolarizing \( \text{pF}_V \) neurons had differential effects on the amplitude of inspiratory-related \( \text{GGEMG} \) and \( \text{DiaEMG} \) activities, dependent on respiratory drive, i.e., hyperpolarizing \( \text{pF}_V \) neurons at rest attenuated \( \text{DiaEMG} \) (Fig. 8), whereas hyperpolarizing \( \text{pF}_V \) neurons during hypoxia or hypercapnia reduced \( \text{GGEMG} \) (Figs. 9–12). This suggests that \( \text{pF}_V \) neurons that drive the diaphragm are active at rest, but that no additional \( \text{pF}_V \) neurons are recruited to drive the diaphragm during active expiration as elicited here. Conversely, neurons that drive the genioglossus are silent at rest but are recruited during active expiration. Thus different \( \text{pF}_V \) populations may separately innervate hypoglossal and phrenic premotoneurons. The \( \text{pF}_V \) (Rosin et al., 2006) and \( \text{preBo} \text{tC} \) (Tan et al., 2010) both innervate a parahypoglossal region (pXII) that is most likely the premotor relay for inspiratory drive to the XII nucleus (Chamberlin et al., 2007). This provides a neural pathway by which the \( \text{pF}_V \) may alter the amplitude of \( \text{GGEMG} \) independent of the actions of the \( \text{preBo} \text{tC} \) (Fig. 14). Similarly, both the \( \text{pF}_V \) (Núñez-Abades et al., 1993; Rosin et al., 2006) and \( \text{preBo} \text{tC} \) (Tan et al., 2010) project to the rostral VRG (rVRG), the premotor bulbospinal relay for inspiratory drive to the phrenic nucleus (Portillo and Núñez-Abades, 1992). This provides a neural pathway by which the \( \text{pF}_V \) can affect \( \text{DiaEMG} \) independent of the actions of the \( \text{preBo} \text{tC} \) (Fig. 14). Therefore we propose that the \( \text{pF}_V \) can influence inspiratory-related \( \text{DiaEMG} \) and \( \text{GGEMG} \) activity at the premotoneuronal level, i.e., pXII and rVRG, in addition to any effects mediated by projections to the \( \text{preBo} \text{tC} \) (Smith et al., 1989; Ellenberger and Feldman, 1990; Rosin et al., 2006; Abbott et al., 2011; Takakura et al., 2014; Fig. 14).

Summary

\( \text{pF}_L \) neurons have characteristics consistent with their acting as an expiratory oscillator. At rest, in the absence of active expiration (Sherrey et al., 1988; Iizuka and Fregosi, 2007; Marina et al., 2010; Pagliardini et al., 2011), the activity of \( \text{pF}_L \) neurons is not inspiratory-modulated (Pagliardini et al., 2011); when disinhibited, or excited, active expiration is induced and many \( \text{pF}_L \) neurons become expiratory-modulated (Pagliardini et al., 2011). Consistent with these observations, hyperpolarizing \( \text{pF}_L \) neurons affects expiratory-related activity, but not inspiratory-related activity. \( \text{pF}_V \) neurons are different. They are active at rest (Mulkey et al., 2004; Moreira et al., 2007b; Stornetta et al., 2009), where they provide excitatory input to the phrenic nerve (Marina et al., 2010) that affects \( \text{VT} \) (Nattie and Li, 2002; Li et al., 2006). Consistent with this observation is that hyperpolarizing \( \text{pF}_V \) neurons reduced \( \text{DiaEMG} \) amplitude; excitation of \( \text{pF}_V \) neurons sufficient to increase inspiratory activity does not induce active expiration.

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**Figure 14.** Schematic of minimal respiratory central pattern generator, which at its core consists of three essential components: (1) an inspiratory oscillator in \( \text{preBo} \text{tC} \) that drives inspiration by exciting inspiratory premotor neuronal populations, e.g., rVRG and parahypoglossal region (pXII), and inhibits \( \text{pF}_V \); (2) a (conditional) expiratory oscillator in \( \text{pF}_V \) that gates and drives expiration by exciting expiratory premotor neuronal populations, i.e., cVRG (Janczewski et al., 2002) and pXII, and assures alteration of phases by exciting neurons that inhibit \( \text{preBo} \text{tC} \), e.g., inhibitory neurons in either the \( \text{preBo} \text{tC} \) or \( \text{Bo} \text{tC} \); and (3) a source of tonic drive in \( \text{pF}_V \) that is responsive to CO\(_2\)/pH and integrates other sensory afferents affecting respiratory drive, via excitatory connections to \( \text{preBo} \text{tC} \), \( \text{Bo} \text{tC} \), and respiratory premotor neurons, e.g., rVRG, cVRG, and pXII.
(Pagliardini et al., 2011, their Fig. 5). When the drive to breathe increases, i.e., during hypoxia and hypercapnia, hyperpolarizing pFV neurons affect both inspiratory-related GGEMG and expiratory-related AbdEMG amplitude. Hyperpolarizing pFV neurons did not affect t During hypoxia and hypercapnia, similar to previous reports involving silencing, or destruction, of presumptive pFV neurons (Nattie and Li, 2002; Marina et al., 2010). Thus, the pFV appears to provide a generic facilitatory drive to respiratory-related (pre)motoneurons that does not affect frequency.

In conclusion, by differentially decreasing the excitability of neighboring parafacial regions, we identify two distinct functional parafacial regions: the pFV is a conditional expiratory oscillator, and the pFV provides a generic facilitatory drive to respiration. We postulate that central pattern generation for breathing has (at least) three essential functional components associated with specific sites in the brainstem: (1) an inspiratory oscillator in the preBoTc, (2) a (conditional) expiratory oscillator in the pFV, and (3) a source of respiratory drive related to chemosensory and likely descending inputs (such as related to exercise) in the pFV (Fig. 14).

Notes
Supplemental material for this article is available at http://feldmanlab.neurobio.ucla.edu. Supplemental information contains figures and legends for control experiments involving CNO and Alt. This material has not been peer reviewed.

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