Locus Coeruleus as a vigilance centre for active inspiration and expiration in rats

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At rest, inspiration is an active process while expiration is passive. However, high chemical drive (hypercapnia or hypoxia) activates central and peripheral chemoreceptors triggering reflex increases in inspiration and active expiration. The Locus Coeruleus contains noradrenergic neurons (A6 neurons) that increase their firing frequency when exposed to hypercapnia and hypoxia. Using recently developed neuronal hyperpolarising technology in conscious rats, we tested the hypothesis that A6 neurons are a part of a vigilance centre for controlling breathing under high chemical drive and that this includes recruitment of active inspiration and expiration in readiness for flight or fight. Pharmacogenetic inhibition of A6 neurons was without effect on resting and on peripheral chemoreceptors-evoked inspiratory, expiratory and ventilatory responses. On the other hand, the number of sighs evoked by systemic hypoxia was reduced. In the absence of peripheral chemoreceptors, inhibition of A6 neurons during hypercapnia did not affect sighing, but reduced both the magnitude and incidence of active expiration, and the frequency and amplitude of inspiration. These changes reduced pulmonary ventilation. Our data indicated that A6 neurons exert a CO2-dependent modulation of expiratory drive. The data also demonstrate that A6 neurons contribute to the CO2-evoked increases in the inspiratory motor output and hypoxia-evoked sighing.

The Locus Coeruleus (LC) is the principal noradrenergic nucleus (A6 neurons) in the CNS. These neurons send extensive projections throughout the neuraxis and are implicated in the control of many homeostatic functions, including chemosensory control of breathing. In this regard, A6 neurons of rats respond to hypercapnic stimulation of central chemoreceptors in vivo and a large proportion of them are found to be intrinsically sensitive to CO2/[H+] in vitro. In conscious rats, a permanent chemical lesion of A6 neurons significantly reduced the CO2-induced ventilatory response. A6 neurons also respond to activation of peripheral chemoreceptors using hypoxia. However, a permanent chemical lesion of A6 neurons did not affect the ventilatory response to systemic hypoxia in conscious rats, suggesting that such neurons play a major role in the respiratory responses evoked by activation of peripheral chemoreceptors. However, experimentally determining a causal role for A6 neurons in promoting and maintaining chemosensory control of breathing has remained elusive using traditional chemical ablation approaches, since a permanent loss of neuronal populations may be compensated for by other parts of the neuronal circuitry.

Breathing at rest is characterized by three phases: inspiration, post-inspiration and passive expiration. The pre-Bötzinger Complex (pre-BötC) is responsible for generating inspiratory rhythm. The neural mechanisms underlying post-inspiration are attributed to a distributed network involving lung mechanoreceptors, pontine circuits and a medullary conditional oscillator so-called "post-inspiratory complex". Under conditions of high chemical drive (activation of central and/or peripheral chemoreceptors), abdominal (Abd) motor activity is recruited at the end of expiration (active expiration). This is a mechanism to contract Abd muscles phasically to force exhalation, which assists in reducing expiratory time to promote enhanced pulmonary ventilation. Active expiration depends on a conditional active expiratory oscillator within the ventral medullary parafacial Respiratory Group (pFRG). High chemical drive also produces differing degrees of both alertness and...
arousal25, which is highly associated with A6 neuronal activation5. It has been proposed that the pons modulates central generation of Abd active expiration under high chemical drive20. A6 neurons project to the ventral medulla26, which we propose to modulate the activity of expiratory neurons located in the pFRG or even those determining the activity of spinal Abd motoneurons in the caudal Ventral Respiratory Group (cVRG). Given their role in mediating arousal5, we hypothesise that A6 neurons are a part of a vigilance centre (LC) for controlling breathing under high chemical drive and that this will include recruitment of both active inspiration and expiration in readiness for flight or fight.

The development of pharmacogenetic tools provides a neuronal phenotype specific way to acutely and reversibly inhibit the activity of selected neuronal populations27. Therefore, to determine the role of the LC-noradrenaline (NA) system for both resting, inspiratory and the expiratory (active expiration) responses to activation of central and peripheral chemoreceptors in conscious adult rats, we studied the effects of inhibiting A6 neurons using the insect peptide allatostatin (Alst) following their transduction with a lentiviral construct to express the G-protein-coupled Drosophila allatostatin receptor (AlstR). Activation of AlstR produces a strong sustained neuronal hyperpolarisation via the opening of inwardly rectifying K+ channels28,29, rendering them inactive.

**Results**

**A6 transduction efficacy.** Noradrenergic brainstem neurons express the transcriptional factor Phox2 and can be targeted using lentiviral vectors (LVV) to express gene of interest under the control of an artificial promotor - PRSx830,31. Direct bilateral stereotaxic injection of PRSx8-AlstR-GFP-LVV into the LC produced selective expression of the fluorophore-AlstR construct within immunocytochemically characterized LC neurons (Fig. 1A). These transduced neurons showed characteristic membrane-delimitied fluorescence with strong signal from the dendrites and axonal processes, as expected of the receptor-fluorophore construct. The expression was limited to neurons that were identified as noradrenergic (A6 neurons) on the basis of their tyrosine hydroxylase
(TH) immunofluorescence (Fig. 1A). Fluorophore-AlstR construct was expressed in 91 ± 1.7% of A6 neurons at the entire LC (9.16 to 10.32 mm caudal to the bregma; n = 32), demonstrating the specificity of viral targeting of the A6 neurons.

**Single A6 Unit Recording.** The effect of AlstR activation on spontaneous electrical activity of A6 neurons was tested in PRSx8-AlstR-GFP-LVV anaesthetised rats (Fig. 1B,C). A6 neurons, expressing GFP, had a mean firing frequency of 3.22 ± 0.41 Hz (n = 4), which was similar to that observed in previous studies. Acute activation of peripheral (intravenous injection of potassium cyanide - KCN; p < 0.0001) or central chemoreceptors (7% CO₂; p = 0.003) significantly augmented their basal firing frequency (26.76 ± 2.57 and 8.8 ± 0.81 Hz, respectively). These neurons were then recorded during application of Alst into the lateral ventricle, which reversibly abolished their firing frequency within ~1 min of delivery in every cell tested (Fig. 1C).

**Respiratory responses to Alst-induced inhibition of A6 neurons.** The effect of AlstR activation on spontaneous electrical activity of A6 neurons was tested in PRSx8-AlstR-GFP-LVV anaesthetised rats (Fig. 1B,C). A6 neurons, expressing GFP, had a mean firing frequency of 3.22 ± 0.41 Hz (n = 4), which was similar to that observed in previous studies. Acute activation of peripheral (intravenous injection of potassium cyanide - KCN; p < 0.0001) or central chemoreceptors (7% CO₂; p = 0.003) significantly augmented their basal firing frequency (26.76 ± 2.57 and 8.8 ± 0.81 Hz, respectively). These neurons were then recorded during application of Alst into the lateral ventricle, which reversibly abolished their firing frequency within ~1 min of delivery in every cell tested (Fig. 1C).

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Hypoxic stimulation of peripheral chemoreceptors. In order to investigate whether A6 neurons affect breathing under conditions of high chemical drive, we first activated the peripheral chemoreceptors using intravenous injection of KCN to mimic cytotoxic hypoxia (n = 6). Figure 3A shows the peripheral chemoreflex-induced inspiratory and expiratory responses from one rat transduced with PRSx8-AlstR-GFP-LVV into LC. Acute activation of peripheral chemoreceptors increased fR and DiaEMG amplitude, as well as evoked active expiration in AbdEMG (Fig. 3A-Bi). Following inhibition of A6 neurons these responses remained unchanged [(inspiratory = ΔfR: 99.25 ± 5.24 vs 98.54 ± 6.16 cpm; ΔDiaEMG amplitude: 136.4 ± 9.76 vs 140.8 ± 9.81%) (expiratory = ΔAbdEMG: 402.2 ± 10 vs 401.5 ± 9.6%); Fig. 3B–E]. However, the sighing rate (red lines of Fig. 4A) was reduced to 7.85 ± 1.06 from 15.57 ± 1.51 (p < 0.0001) during 20 min of systemic hypoxic hypoxia (7% O2; n = 7), but sigh amplitude was unchanged (15.18 ± 2.08 vs 15.37 ± 1.44 ml/Kg; Figs 4A and 5A,B). Systemic hypoxic hypoxia also increased fR, DiaEMG amplitude, Vt, and V̇l, but did not evoke active expiration (data not shown). Alst application did not modify these peripheral chemoreceptor-evoked reflex responses [at 20 minutes; (fR: 153.89 ± 4.11 vs 155.34 ± 4.23 cpm) (DiaEMG amplitude: 147.4 ± 7.4 vs 142.9 ± 7.59%) (Vt: 9.41 ± 0.54 vs 9.33 ± 0.77 ml/Kg) (V̇l: 1428.49 ± 50.89 vs 1400.1 ± 51.24 ml/Kg/min); Figs 4A,B and 5C–F]. Thus, A6 neurons are involved in hypoxia-induced sighing, but not in other to systemic hypoxic hypoxia-evoked respiratory responses in conscious adult rats. Alst produced no effect on sighing rate (15.5 ± 2.08 vs 14.75 ± 3.8; n = 4) in rats transduced with PRSx8-GFP-LVV (control virus) during systemic hypoxic hypoxia.
Hypercapnic stimulation of central chemoreceptors. Hypercapnia (20 min of 7% CO₂; n = 7) increased DiaEMG amplitude, the number of sighs, fR (by reducing DI and DE), V̇ₚ, V̇ₑ and evoked active expiration in AbdEMG of PRSx8-AlstR-GFP-LVV transduced rats, in which carotid peripheral chemoreceptors had been denervated (Figs 6A,B and 7A–H). Active expiration was prevalent as noted by the high values of expiratory AbdEMG incidence (Fig. 7H) in response to activation of central chemoreceptors. Alst application reduced the central chemoreceptors-induced responses of fR (at 20 minutes: 122.56 ± 2.31 vs 141.67 ± 2.78 cpm; p < 0.0001), VT (at 20 minutes: 7.18 ± 0.39 vs 8.67 ± 0.53 ml/Kg; p < 0.0001), V̇ₑ (at 20 minutes: 856.79 ± 43.79 vs 1210.68 ± 53.56 ml/Kg/min; p < 0.0001) and DiaEMG amplitude (at 20 minutes: 83.42 ± 9.3 vs 120.6 ± 3.73%; p < 0.0001), but did not change either the number of sighs (15.86 ± 1.21 vs 16.43 ± 0.58) or their amplitude (16.05 ± 1.64 vs 15.45 ± 2.11 ml/Kg; Figs 6A,B and 7A–F). Alst also reduced both the incidence (0.52 ± 0.09 vs 0.81 ± 0.05; p < 0.0001) and magnitude of AbdEMG active expiration (375.3 ± 13.64 vs 427.8 ± 14.76%; p < 0.0001; Figs 6A,B and 7G,H). On the other hand, Alst application increased duration of inspiration (DI; at 20 minutes: 0.20 ± 0.01 vs 0.18 ± 0.02; p = 0.03; Fig. 6A,B). Alst produced no effect on fR (at 20 minutes: 142.5 ± 3.33 vs 146.44 ± 3.59 cpm), V̇ₚ (at 20 minutes: 8.89 ± 0.77 vs 8.19 ± 0.86 ml/Kg), V̇ₑ (at 20 minutes: 1200.56 ± 67.55 vs 1248.44 ± 59.44 ml/Kg/min) or AbdEMG active expiration magnitude (419.1 ± 15.44 vs 437.55 ± 22.44%) in rats transduced with PRSx8-GFP-LVV (n = 4) during hypercapnia.

To determine the contribution of the reduction in active expiration after acute inhibition of A6 neurons on V̇ₑ, we evaluated the ventilatory parameters at different times after Alst application during hypercapnia in carotid body denervated rats, i.e. in the presence (red arrow - Fig. 6B) and absence (green arrow - Fig. 6B) of AbdEMG active expiration. Alst application reduced fR, V̇ₚ and V̇ₑ, but increased DI and DE, regardless of whether AbdEMG active expiration was present. However, reductions in fR, V̇ₚ and V̇ₑ, and the increase in DE, were greater in the absence of active expiration than during its presence [(fR: −12.79 ± 3.21 vs −9.48 ± 1.32%; p = 0.02) (DE: 11.25 ± 2.39 vs 8.2 ± 2.11%; p = 0.02) (V̇ₑ: −15.34 ± 3.61 vs −10.54 ± 2.34%; p = 0.01) (V̇ₚ: −26.39 ± 4.11 vs −18.67 ± 3.99%; p = 0.003), suggesting that the reduction in V̇ₑ is due to reductions in both active inspiratory and expiration responses.

Figure 4. Acute inhibition of A6 neurons and effect on the inspiratory and ventilatory responses to systemic hypoxic hypoxia of conscious adult rats. (A) Raw and integrated (∫) records of DiaEMG activity from a rat in which the LC was transduced with PRSx8-AlstR-GFP-LVV, illustrating the changes in inspiration and in the number of sighs (red lines) induced by systemic hypoxic hypoxia (7% O₂) before and after Alst application into lateral ventricle. (B) Magnification of baseline and reflex inspiratory and ventilatory (barometric respiratory movements) responses from the same rat before and after Alst application. Note the absence of changes in the hypoxia-induced inspiratory and ventilatory responses, but the significant reduction in the number of sighs, after acute inhibition of A6 neurons.
Discussion

This report reveals a novel and essential functional role of A6 neurons in the chemosensory control of inspiration and expiration in adult conscious rats. In the absence of peripheral chemoreceptors, inhibition of A6 neurons reduced the magnitude and incidence of both the CO₂-evoked AbdEMG active expiration and DiaEMG inspiratory activity (frequency and amplitude). These changes in both inspiratory and expiratory activities were responsible for the observed reduction in \( \dot{V_E} \) during hypercapnia when A6 neuronal activity was pharmacologically inhibited. However, inhibition of A6 neurons had no effect on resting or peripheral chemoreflex-evoked respiratory activities and \( \dot{V_E} \), although the number of sighs evoked by systemic hypoxic hypoxia was reduced. Together, these data indicate that A6 neurons are a part of a vigilance centre for controlling breathing under high chemical drive and that this includes a CO₂-dependent augmentation of inspiratory and active expiratory drive and hypoxia-evoked sighing.

Rapid inhibitory and reversible effects of Alst were reported previously to be selective for AlstR-transduced neuron\(^28,29\). In the present study, we confirmed effective silencing of AlstR-expressing A6 neurons by Alst during single unit extracellular recording in anaesthetised rats. Thus, the physiological data on perturbations of respiratory pattern reported herein, together with our cellular-level electrophysiological evidence provided, suggest that Alst application effectively inhibits a significant proportion of transduced A6 neurons in vivo.

The present study also demonstrated that inspiratory and expiratory activities, and \( \dot{V_E} \) were unaffected by Alst under eupnoic breathing, suggesting that A6 neurons play no role in the control of breathing in conscious rats at rest while breathing room air. Similar results regarding ventilatory parameters have been demonstrated in previous studies using permanent chemical lesions of A6 neurons in rats\(^13,15\). Likewise, acute silencing of these neurons...
did not affect the respiratory responses to activation of peripheral chemoreceptors using either cytotoxic or hypoxic hypoxia. Although previous studies indicate that A6 neurons: (i) receive excitatory synaptic drive from the central respiratory network, (ii) are responsive to activation of peripheral chemoreceptors and (iii) are intrinsically sensitive to low levels of O$_2$\textsuperscript{10,14,34,35}, the present study ruled out a possible contribution of these neurons for the inspiratory and expiratory responses to activation of carotid body chemoreceptors. We propose that A6 neurons are involved in both the behavioural arousal and respiratory-modulated response of the sympathetic outflow to hypoxia, since these neurons are excited, along with sympathetic outflow, during peripheral chemoreceptor stimulation, and exhibit a pronounced respiratory pattern under conditions of strong entrainment of the sympathetic vasomotor outflow by the central respiratory network\textsuperscript{14}.

Sighing during normal breathing has been hypothesised to prevent lung atelectasis, maintaining lung compliance and improving alveolar oxygenation\textsuperscript{36}. Sighing increases in response to hypoxia and hypercapnia\textsuperscript{15,37}. The data of the present study demonstrate the crucial role of A6 neurons for driving sighs induced by systemic hypoxic hypoxia, but not during hypercapnia or eucapnic breathing. Recent studies have shown that sighing is controlled by two bombesin-like neuropeptide pathways, neuromedin B and gastrin-releasing peptide, expressed in the pFRG neurons, which mediate signalling with the pre-BötC\textsuperscript{38}. A6 neurons receive a dense network of

Figure 6. Acute inhibition of A6 neurons and effect on the inspiratory, active expiratory and ventilatory responses to stimulation of central chemoreceptors in conscious adult rats. (A) Raw and integrated (∫) records of Dia$_{EMG}$ and Abd$_{EMG}$ activities from one animal in which the LC was transduced with PRSx8-AlstR-GFP-LVV. Note the changes in inspiration, expiration and in the number of sighs (red lines) induced by activation of the central chemoreceptor (7% CO$_2$), before and after Alst application into lateral ventricle. (B) Magnification of baseline and reflex inspiratory, expiratory and ventilatory (barometric respiratory movements) responses from the same rat before and after Alst application. Note that Alst application reduced significantly the fR, Dia$_{EMG}$ amplitude, the Abd$_{EMG}$ active expiration incidence (green arrow: absence of active expiration; red arrow: active expiration) and magnitude, as well as the ventilatory parameters.
immunoreactive fibres expressing bombesin-like neuropeptides. Therefore, it is reasonable to suggest that A6 neurons might be activated by bombesin-like neuropeptides from pFRG during hypoxia to increase the frequency of sighing (Fig. 8A). A6 neurons may also be activated by other sigh-promoting regions responsive to hypoxia.
such as the ventral medullary C1 catecholaminergic neurons\(^40\) (Fig. 8A). This is a group of neurons with dual catecholaminergic/glutamatergic phenotype\(^41\) that modulate the activity of inspiratory pre-Bötzinger neurons during hypoxia by local release of NA. Adrenergic receptor activation of the pre-Bötzinger Complex (pre-BötC, inspiratory neurons) and parafacial Respiratory Group (pFRG, for active expiration and sigh), as well as the expiratory Bötzinger Complex (BötC), rostral Ventral Respiratory Group (rVRG; containing inspiratory bulbo-spinal premotor neurons) and caudal Ventral Respiratory Group (cVRG; containing expiratory bulbo-spinal premotor neurons) are also shown. (A) Sigh response during systemic hypoxia: A6 neurons might be directly activated by systemic hypoxia (\(\downarrow O_2\))\(^{34,35}\) or by sigh-promoting bombesin-like pFRG\(^{58}\) and C1 catecholaminergic neurons\(^{40}\) to increase the frequency of sighing through adrenergic receptor activation of pre-BötC\(^5\). (B) Active inspiratory and expiratory responses during hypercapnia: A6 neurons, activated by CO\(_2\) or C1 catecholaminergic neurons\(^{59}\), enhance inspiration and \(V_E\) through adrenergic receptor activation of pre-BötC\(^{52,53}\). A6 neurons might also provide either tonic or expiratory-related excitatory input to the conditional expiratory oscillator located in the pFRG or directly to the cVRG, for onward relay to expiratory spinal motoneurons, enhancing active expiration.

Figure 8. Schematic depicting proposed neural mechanisms by which A6 neurons regulate inspiratory and expiratory responses to high chemical drive. Parasagittal views of the brainstem showing the location of the medullary ventral respiratory group and C1 catecholaminergic region, as well as the pontine LC A6 neurons (red) and Facial Motor Nucleus (VII). The respiratory rhythmogenic sites of the pre-Bötzinger Complex (pre-BötC, inspiratory neurons) and parafacial Respiratory Group (pFRG, for active expiration and sigh), as well as the expiratory Bötzinger Complex (BötC), rostral Ventral Respiratory Group (rVRG; containing inspiratory bulbo-spinal premotor neurons) and caudal Ventral Respiratory Group (cVRG; containing expiratory bulbo-spinal premotor neurons) are also shown. (A) Sigh response during systemic hypoxia: A6 neurons might be directly activated by systemic hypoxia (\(\downarrow O_2\))\(^{34,35}\) or by sigh-promoting bombesin-like pFRG\(^{58}\) and C1 catecholaminergic neurons\(^{40}\) to increase the frequency of sighing through adrenergic receptor activation of pre-BötC\(^5\). (B) Active inspiratory and expiratory responses during hypercapnia: A6 neurons, activated by CO\(_2\) or C1 catecholaminergic neurons\(^{59}\), enhance inspiration and \(V_E\) through adrenergic receptor activation of pre-BötC\(^{52,53}\). A6 neurons might also provide either tonic or expiratory-related excitatory input to the conditional expiratory oscillator located in the pFRG or directly to the cVRG, for onward relay to expiratory spinal motoneurons, enhancing active expiration.

It is well established that LC participates in central chemoreception to CO\(_2[/H^+]/\). Hypercapnia/acidosis increases the firing frequency of these neurons both in vivo as well as in *in vitro* preparations of adult rats\(^9\). Previous studies have employed subtractive methods to study the role of the A6 neurons in CO\(_2\) regulation of breathing. These methods using toxins to inhibit the noradrenergic system showed different magnitudes of inhibition of central chemoreception\(^{13,56,51}\). This may, in part, be ascribed to the long-term adaptation to the loss of a population of neurons. Our findings clearly show a crucial involvement of A6 neurons in the control of inspiration (IR and DiaEMG amplitude) and in the generation of AbdEMG active expiration (its magnitude and incidence)
during stimulation of central chemoreceptors. A6 neurons project to the pre-BötC and regulate breathing. It is possible that hypercapnia evokes A6 neurons to activate α1-adrenoceptors in pre-BötC, thereby enhancing inspiration and VE (Fig. 8B). Several observations also suggest that this metabolic challenge could activate the A6 via C1 neurons (Fig. 8B). The C1 neurons densely innervate the LC and optogenetic stimulation of the C1 activates A6 neurons and optogenetic stimulation of the C1 activates A6 neurons. Hypercapnia also produce differing degrees of alertness and arousal and increased alertness is highly associated with A6 and C1 neuronal activation. Therefore, excitatory activation transmitted from C1 to A6 neurons under hypercapnia is not hard to rationalize and this neural pathway could facilitate arousal and therefore contribute to the airway defensive responses to hypercapnia, an issue that requires further study.

Our study unearths for the first time that A6 neurons play a major role in central generation of Abd EMG active expiration to enhance VE during hypercapnia. We observed that reductions in active expiration incidence led to decreases in VE. These data agree with the proposal that active expiration enhances VE by increases in both VT (recruiting expiratory reserve volume) and FR, but decreases in DE and total time of the respiratory cycle. Active expiration may be state-dependent being more predominant in sleep than in wakefulness during hypercapnia. Although their inhibition does not affect the duration of sleep, A6 neurons are necessary for long-term wakefulness and arousal. Therefore, we propose that A6 neurons’ role in generation of active expiration is in part important for maintaining vigilance whether this is to expiratory hypercapnic responses or a threatening environment. Some evidence suggest that the posture and proprioceptive inputs from the abdominal wall may also affect active expiration. Considering that the LC presents specialized subgroups of noradrenergic neurons, projecting to prefrontal cortex, that are involved in the anxiogenic behaviours and that inhalation of CO2-enriched air can produce anxiety and fear-like behaviours, we cannot rule out that changes in the behavioural responses to hypercapnia, after acute A6 inhibition, may contribute to the reductions in both active expiration magnitude and incidence through postural changes and even by Abd proprioceptive components.

Chemoreceptive neurons sensitive to CO2/HI and identified in the LC in vivo are either tonically active over a wide range of arterial CO2 levels or show different patterns of respiratory modulation in their discharge, including expiratory modulation. Thus, A6 expiratory-modulated neurons may mediate the CO2-evoked activity recorded from AbdEMG. Alternatively, when arterial CO2 increases, A6 neurons might provide tonic excitatory input to the conditional expiratory oscillator located in the pFRG or directly to the cvRG of the medulla for onward relay to expiratory spinal Abd motoneurons (Fig. 8B); this remains to be determined. There is a dense catecholaminergic innervation in the pFRG partially overlapping, adjacent and more medial chemosensitive region (Retrotrapezoid Nucleus- RTN). Despite the recent data showing that NA in the RTN does not contribute to the respiratory response evoked by activation of central chemoreceptors in anaesthetized rats, additional experiments are needed to evaluate the role of adrenergic receptors in the pFRG for hypercapnia-evoked active expiration in conscious rats. NA may affect membrane potential subthreshold oscillations of the presumable intrinsic bursting pFRG expiratory neurons during hypercapnia, thereby regulating both the incidence and magnitude of AbdEMG activity. Therefore, new electrophysiological experiments are needed to evaluate the synaptic mechanisms and the role of neuromodulators determining the excitability of pFRG expiratory neurons during hypercapnia.

In conclusion, the present study in conscious adult rats reveals a powerful modulatory role of A6 neurons in the CO2-evoked increases in active expiration, the inspiratory motor output and VE, as well as hypoxia-evoked sighing. The present study prompts interesting questions as to whether A6 neurons have anatomical and cellular specialisation acting on different regions of the CNS for the control of sighing, inspiration and active expiration that are independently selected and regulated by hypoxia and hypercapnia, and whether these specialized subgroups of noradrenergic neurons share their involvement with other behavioural functions (e.g. arousal and anxiety).

Methods

Animals. The experiments were performed on male Wistar rats provided by the Animal Care Facility at the Ribeirão Preto campus of the University of São Paulo, Brazil. All experimental protocols were approved by the Institutional Ethics Committee for Animal Experimentation at the School of Medicine of Ribeirão Preto, University of São Paulo (protocols #093/2009 and #122/2016). All methods were carried out in accordance with The Principles of Laboratory Animal Care (NIH publication no. 85/23, revised 1996). Animals were housed with a 12 h light/dark cycle at a constant temperature (22 ± 1°C) with ad libitum access to standard rat chow and water.

Viral vectors. The LVV system used here was HIV-1-derived and pseudo-typed with the VSV-G envelope. The plasmids pTYF-PR5x8-AlstR-IRES2-GFP and pTYF-PR5x8-IRES2-GFP were cloned into the LVV. Titres of PR5x8-AlstR-GFP-LVV and the control virus (PR5x8-GFP-LVV) were between 1 × 109 and 1 × 1010 pfu. Viral concentration and titration were performed as described in detail previously.

In vivo gene transfer. Male rats (225–250 g) were anaesthetised with a ketamine (75 mg kg⁻¹ i.p.)/xylazine (5 mg kg⁻¹ i.p.) mixture. The depth of anaesthesia was checked at regular intervals (15–20 min) by assessing the withdrawal reflex response to noxious pinching of the tail or hind paw. Animals were placed in a stereotaxic frame (David Kopf, Tujunga, USA) and four microinjections (each 250 μm apart in the dorsoventral axis) per side of PR5x8-AlstR-GFP-LVV (250 nl each, over 2 min) were delivered into LC bilaterally (Picospritzer II; Parker Instruments, Cleveland, USA). The microinjections were made 2 mm caudal to bregma, ±1.2 mm lateral from the midline and 5.5–6.0 mm below the brain surface with a 10° rostral angulation. A stainless-steel guide cannula (13 mm long, 0.6 mm o.d., 0.4 mm i.d.) was implanted into the lateral cerebral ventricle (−0.6 mm to Bregma, 1.5 mm lateral to the midline and −3.6 mm ventral to dura mater). The guide cannula was fixed to the cranium using dental acrylic resin. Post-surgery, rats were treated with one prophylactic dose of analgesic and antiptyretic...
In vivo experiments. Anaesthetised studies. Experiments in anaesthetised PRSx8-AltR-GFP-LVV rats were performed to evaluate the single cellular response of A6 neurons to activation of AltR fifteen days after the LVV injections. General anaesthesia was induced with 5% halothane (AstraZeneca do Brasil Ltda, Cotia, Brazil) in 100% O₂. A tracheostomy was performed and the halothane concentration was reduced to a level of 1.4–1.5%, which was sufficient to abolish the corneal reflex and the retraction of distal phalanges to strong nociceptive stimulation of the hindpaw until the end of surgery. A polyethylene catheter (PE-10 connected to PE-50; Clay Adams, Parsippany, USA) was inserted into the femoral vein for systemic administration of fluids and drug. Rats were placed onto a stereotaxic apparatus (David Kopf) on a heating pad (ALB 200 RA; Bonther, Ribeirão Preto, Brazil) and core body temperature was monitored and maintained at a minimum of 37°C via a thermocouple (MLT1403; Harvard Apparatus, Holliston, USA). All rats were ventilated (Small animal ventilator 683; Harvard Apparatus) with 100% O₂ throughout the experiment. End tidal-CO₂ was monitored throughout the experiment with a capnometer (Columbus Instruments, Ohio, USA). The discharges of neurons in the LC were recorded extracellularly (Duo 773 Electrometer; World Precision Instruments, Sarasota, USA) using glass electrodes (30–50 MΩ) filled with 1.5% biocytin (Molecular Probes, Grand Island, USA) in 0.5 M sodium acetate. Effects produced by: (i) activation of peripheral chemoreceptors using an intravenous injection of KCN (50 μl; 40 μg/Kg; Merck, Darmstadt, Germany), (ii) activation of central chemoreceptors stepping end-expiratory CO₂ from 5% to 7% and (iii) activation of AltR on the discharge of LC neurons were evaluated. AltR (2 mM, 10μl; Phoenix Pharmaceuticals, Inc., Burlingame, USA) was administered intracerebroventricularly (125 μl syringe; Hamilton Company, Reno, USA) (needle 33-gauge; Small Parts, Miami Lakes, USA). The correct placement of the guide cannula was confirmed at the end of the experiment by injection of Evans Blue (2% in 10μl; Sigma-Aldrich, St. Louis, USA) and its visible presence in the intracerebroventricular system. After electrophysiological experiments, LC neurons were filled with biocytin using the previously described juxtacellular labelling method (200 μm pulses of 1.0–4.0 nA at 2.5 Hz for 1–3 min)20. All signals were acquired by a data acquisition system (5 KHz; CED 1401; Cambridge Electronic Design, UK) controlled by a computer running Spike 2 software (Cambridge Electronic Design).

Conscious studies. EMG of respiratory muscles was used to evaluate inspiratory and expiratory indexes in conscious rats71. Ten days after the microinjections, PRSx8-AltR-GFP-LVV rats were re-anaesthetised (ketamine/xylazine mixture; i.p.). The depth of anaesthesia was also checked at regular intervals by assessing the withdrawal reflex response to noxious pinching of the tail or hind paw. Bipolar tellon-coated stainless steel EMG electrodes were implanted in the Dia and Abd oblique muscles, a temperature datalogger (SubCue, Calgary, Canada) was implanted within the abdominal cavity for body temperature (Tb) measurements and a polyethylene catheter was inserted into the femoral vein for systemic administration of drug. Wires were attached to an electrical socket, tunneled under the skin and positioned on the back of the rat’s neck together with the distal end of the catheter. In the experiments involving activation of central chemoreceptors, the carotid artery bifurcations were exposed, inserted into the femoral vein for systemic administration of fluids and drug. Rats were treated with analgesic, antipyretic and veterinary antibiotic as above. Five days later, the electrical socket of the EMG was connected to an amplifier (1700 amplifier, A-M Systems, Sequim, USA) and animals placed in a cylindrical plethysmograph chamber (5 liters). The chamber was flushed with room air at a flow rate of 1000 ml/min during baseline condition. The gas was then switched to a hypercapnic (7% CO₂, 21% O₂, N₂ balance) or hypoxic (7% O₂, N₂ balance) mixtures for 20 minutes. Gases were mixed with a gas mixer (Pegas 4000, Columbus Instruments, Columbus, USA) using an equivalent flow rate. \( \dot{V}_E \), a product of \( V_T \) and \( f_R \), was obtained by using whole body plethysmography79. A volume calibration was performed by injecting a known volume of air (1 ml) inside the chamber. \( V_T \) was calculated using the formula described by Bartlett & Tenney74 and Tb was recorded using the temperature datalogger programmed to acquire every 5 min. AltR was also administered intracerebroventricularly. The EMG (0.3–5 KHz of bandpass) and breathing-related pressure oscillations (MLT141 spirometer, ADInstruments, Bella Vista, Australia) were acquired by a data acquisition system (5 KHz; PowerLab, ADInstruments) controlled by a computer running LabChart software (ADInstruments).

Data analysis. EMGs were recorded in absolute units (μV) and analyses were performed off-line from rectified and integrated (\( \int \)) signals (time constant: 50 ms). \( \Delta V_{EMG} \) burst activity was assessed as \( \Delta R \). DI and DE were calculated from the \( V_T \) trace. A sigh was defined as a high-amplitude inspiratory breath in the barometric respiratory movements and in the \( \Delta V_{EMG} \) activity of at least 100% larger in amplitude than the mean amplitude of five breaths preceding each sigh. Sigh frequency was expressed as the number of sighs per hour or per protocol (systemic hypoxia or hypercapnia - 20 minutes) and sigh amplitude was expressed as changes in \( V_T \). Changes in the \( \Delta V_{EMG} \) (amplitude) and AbdEMG activities during baseline conditions (room air exposure) after AltR application were expressed in μV. Based upon absolute values of \( \Delta V_{EMG} \) and AbdEMG (μV), we determined percentage changes in order to compare their activities in each animal during different experimental conditions (systemic hypoxic hypoxia and hypercapnia), before and after AltR applications. Changes in the \( \Delta V_{EMG} \) and AbdEMG amplitude (percentage values) in response to acute activation of peripheral chemoreceptors using KCN were assessed by the difference between baseline and the peak of response observed after the stimulus (Δ fR and \( \Delta V_{EMG} \) amplitude). The AbdEMG respiratory responses to acute peripheral chemoreflex activation was assessed by the measurement of the area under the curve, in a time window of ≤ 20 s after the stimulus, and expressed as percentage values (\( \Delta Abd_{EMG} \) in percentage) in relation to EMG activity before the stimulus. Active expiration was defined by the presence of AbdEMG expiratory activity, i.e. rhythmic burst of activity (between inspiratory \( \Delta V_{EMG} \) activities) above tonic...
levels. Event detection and the measurement of the amplitude of Abd$_{EMG}$ active expiration was performed on LabChart software. This allowed an assessment of the incidence of active expiration. All data were normalized to their largest integrated (∫) peak amplitude of Abd$_{EMG}$ expiratory activity obtained. The normalized values were used for comparisons of active expiration incidence during the activation of central chemoreceptors, before and after Alst application across animals (i.e number 1 being Abd$_{EMG}$ active expiration at its maximal value observed in each animal).

**Statistical analyses.** Results are expressed as mean ± SD. Data were compared using Student’s paired t test or Two-way ANOVA with Bonferroni post hoc test (GraphPad Prism 4, La Jolla, USA) in accordance with the experimental protocol. Differences were considered significant at p < 0.05.

**Histology and immunocytochemistry.** Rats were killed with an overdose of anaesthetics (ketamine/xylazine mixture; i.p.) and perfused transcardially first with phosphate buffered saline (PBS; 0.1 M) and then with 4% paraformaldehyde (PFA) and brains removed and post fixed in PFA for 2–5 days. Transverse sections (40 μm thick) were cut through the LC with a cryostat (Leica CM1800; Buffalo Grove, USA) and collected into a cryoprotectant solution and stored at −20°C before further processing. The immunofluorescence was performed with free-floating sections. Sections were blocked and permeabilized in PBS containing 10% normal horse serum and 0.5% Triton X-100 for 1 h at room temperature. After three PBS washes, the sections were incubated in primary antibodies against TH (mouse - 1:1000; Millipore, Billerica, USA) and GFP (chicken - 1:1000; AVES Labs, Tigard, USA) for 24 hrs at 4°C. After three PBS washes, the sections were incubated in secondary antibodies Alexa 405-conjugated streptavidin (1:1000; Molecular Probes), Alexa 647-conjugated donkey anti-mouse (1:500; Molecular Probes) and Alexa 488-conjugated donkey anti-chicken (1:500; Molecular Probes) for 1 h at room temperature. Sections were washed in PBS and mounted in Fluoromount (Sigma-Aldrich). Images were collected on a Leica TCS SP5 confocal microscope equipped with 405, 488 and 633 nm laser lines and tunable emission wavelength detection. For each preparation, biocytin- and GFP-expressing neurons were identified and confocal z stacks collected sequentially for the other channel to detect the TH-expressing neurons and verify colocalization of fluorophores. GFP- and TH-expressing neurons were counted over the entire length of the LC from each rat (~ 9.16 to ~ 10.32 mm from bregma). Analyses were performed using a computerized image analysis system (Image J) developed at the National Institutes of Health (https://imagej.nih.gov/ij/).

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Additional Information
Competing Interests: The authors declare no competing interests.

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