Analyzing the brainstem circuits for respiratory chemosensitivity in freely moving mice

Amol Bhandare1, Joseph van de Wiel1, Reno Roberts1, Ingke Braren2, Robert Huckstepp1, Nicholas Dale1*

1School of Life Sciences, University of Warwick, Coventry, United Kingdom; 2University Medical Center Eppendorf, Vector Facility, Institute of Experimental Pharmacology and Toxicology, Hamburg, Germany

Abstract Regulation of systemic PCO₂ is a life-preserving homeostatic mechanism. In the medulla oblongata, the retrotrapezoid nucleus (RTN) and rostral medullary Raphe are proposed as CO₂ chemosensory nuclei mediating adaptive respiratory changes. Hypercapnia also induces active expiration, an adaptive change thought to be controlled by the lateral parafacial region (pFL). Here, we use GCaMP6 expression and head-mounted mini-microscopes to image Ca²⁺ activity in these nuclei in awake adult mice during hypercapnia. Activity in the pFL supports its role as a homogenous neuronal population that drives active expiration. Our data show that chemosensory responses in the RTN and Raphe differ in their temporal characteristics and sensitivity to CO₂, raising the possibility these nuclei act in a coordinated way to generate adaptive ventilatory responses to hypercapnia. Our analysis revises the understanding of chemosensory control in awake adult mouse and paves the way to understanding how breathing is coordinated with complex non-ventilatory behaviours.

Editor's evaluation

This paper presents a novel application of endoscopic imaging with minisscopes in awake-behaving mice to address the important problem of analyzing multicellular activity by calcium activity imaging within circumscribed regions of the medulla oblongata that are proposed to have chemosensory functions for the homeostatic regulation of breathing in response to elevated systemic CO₂ (hypercapnia). The authors importantly demonstrate chemosensory responses of neurons in the retrotrapezoid nucleus-RTN, Raphe magnus and pallidus nuclei, and lateral parafacial region- pFL, and they describe regional heterogeneity of cellular responses to hypercapnia in these regions with important functional implications. Analyzing chemosensory properties of these medullary regions has been the focus of numerous studies, but the problem of analyzing regional multicellular chemosensory responses in the awake freely behaving rodent has not been previously addressed, so this paper represents an advance for the field. The authors present a novel catalog of neuronal responses, and they illustrate the feasibility of this imaging approach, paving the way for further development/application of this approach, while indicating its limitations.

Introduction

The precise control of breathing is fundamental to the survival of all terrestrial vertebrates. Breathing fulfils two essential functions: provision of oxygen to support metabolism; and removal of the metabolic by-product, CO₂. Rapid and regulated removal of CO₂ is essential because its over-accumulation
in blood will result in death from the consequent drop in pH. Understanding of the complexity of the brain microcircuit regulating CO₂ and pH remains incomplete. Whilst the primacy of the ventral medulla surface (VMS) in central chemoreception was described almost 60 years ago, this region contains multiple chemosensory nuclei that are candidates to mediate adaptive control of breathing (Nattie and Li, 1994; Richerson, 1995). This has led to the idea that central chemosensitivity is mediated by a network of chemosensory nuclei distributed throughout the brainstem and extending as far as the limbic system (Huckstepp and Dale, 2011b). Furthermore, there are multiple phenotypes of neurons within single chemosensory nuclei (Huckstepp et al., 2018; Iceman et al., 2014; Iceman and Harris, 2014; Johansen et al., 2015), and even within previously well-defined subpopulations of neurons (Shi et al., 2017; Stornetta et al., 2009).

Recently, brain imaging techniques have been developed to allow recording of activity of defined cell populations in awake, freely moving animals even in deep brain structures such as the hypothalamus (Ziv and Ghosh, 2015). These methods require: the expression of genetically encoded Ca²⁺ indicators such as GCaMP6 in the relevant neurons; the implantation of gradient refractive index (GRIN) lenses at the correct stereotaxic position; and a head-mounted mini-epifluorescence microscope to enable image acquisition during the free behaviour of the mouse. We have now adapted these methods to enable recording of defined neuronal populations in the rostral medulla oblongata of mice to analyze the chemosensory control of breathing.

In this paper, we study the activity of neurons in the retrotrapezoid nucleus (RTN) (Mulkey et al., 2004; Nattie and Li, 1994; Ramanantsoa et al., 2011) and the rostral medullary raphe (Bradley et al., 2002; Ray et al., 2011; Richerson, 1995). Evidence strongly favours their involvement in the central chemosensory response, but thorough understanding as to how these nuclei contribute to central chemosensitivity has been hampered by the inability to record the activity of their constituent cells in awake freely behaving adult rodents. Instead, neuronal recordings have been made from young (<14 d) or adult rodents under anaesthesia. Both of these methods have significant drawbacks -the chemosensory control of breathing matures postnatally; and anaesthesia is known to depress the activity of respiratory neurons and reconfigure the circuit.

We also assess the contribution of the lateral parafacial region (pFL) to an often-overlooked aspect of the hypercapnic ventilatory response, active expiration. During resting eucapnic breathing, there is little active expiration instead the expiratory step involves elastic rebound of the respiratory muscles to push air out of the lungs. However, when the intensity of breathing is increased for example during exercise or hypercapnia, active expiration (recruitment of abdominal muscles to push air out of the lungs) occurs. Mounting evidence suggests the pFL may contain the expiratory oscillator and that neurons in this nucleus are recruited to evoke active expiration (Huckstepp et al., 2015; Huckstepp et al., 2016; Pagliardini et al., 2011). Nevertheless, the key step of recording activity of these neurons in awake behaving animals and linking it to active expiration has not been achieved.

Our data show the pFL did not undergo sustained activation during hypercapnia, but instead contributed to acute transient high amplitude expiratory events. We found that neurons in the RTN and Raphe exhibited a range of responses to hypercapnia. Many RTN neurons exhibited fast adapting responses. This response type was less common in the Raphe, and many Raphe neurons exhibited a slower graded response. Our data are consistent with a tiered chemosensory network, with the RTN and Raphe responsible for detecting different aspects of the hypercapnic stimulus. These novel findings illuminate the fundamental functional components of chemosensory nuclei and show that neuronal responses to hypercapnia are considerably more complex than anticipated.

Results

Optical characteristics of the recording system

The GRIN lens has a diameter of 600 µm with a focal plane ~300 µm below the lens (Jennings et al., 2015; Resendez et al., 2016). However, the focal plane varies depending upon the distance between the camera and GRIN lens, which can be altered via a manual turret adjustment to optimise the focus on fluorescent cells. To document the range of focal plane depth in our system, we imaged fluorescent beads entrapped in agarose under two conditions: the turret at its lowest (i.e. camera is against the end of the GRIN lens, and the focal plane is at its closest to the lens) and its maximum setting (i.e. the camera is as far from the end of the GRIN lens as it can be, and the focal plane is at its deepest...
relative to the lens (Figure 1—figure supplement 1). The focal depth (in which the beads were sharply focused) was 100 µm at any single setting and the full focal plane ranged from 150 to 450 µm below the end of the lens as the turret moved from its lowest to highest setting. The turret setting is given in all figure legends.

**Inclusion/exclusion criteria for cells in the study**

Following assessment for inclusion/exclusion criteria, data from 18 mice comprising recordings from 194 cells were included for analysis (Figure 1A). As the mice were unrestrained and able to move freely during the recordings, to visualize physical movements during Ca²⁺ analysis high-definition videos of mice were synchronized to the simultaneous plethysmograph and Inscopix Ca²⁺ imaging recordings in Inscopix Data Processing Software and Spike2. Our recordings are performed at a single wavelength (Figure 1B). Therefore, it was essential to verify that any signals result from changes in Ca²⁺ rather than from movement artefacts.

We examined the origins of the changes in fluorescence in three ways. Firstly, we transduced RTN neurons of three mice with AAV-syn-GFP and performed imaging during a hypercapnic challenge (Figure 1C–D, Figure 1—video 1). This allowed measurement of comparable levels of fluorescence to GCaMP6 to determine whether movement by itself, in the absence of any Ca²⁺ reporter activity, could generate confounding fluorescent signals. Analysis of changes in fluorescence showed only small amplitude (often negative-going) fluctuations. In no case did we observe large increases in fluorescence of the type we routinely observed when GCaMP6 was expressed (e.g. Figure 1E), suggesting that movement per se cannot give fluorescence signals that resemble Ca²⁺ signals.

Secondly, we analyzed the movement of the mouse simultaneously with the Ca²⁺ recordings (Figure 1E) via two methods. The first was to analyze the headmount movements in the video recordings of the mouse in the plethysmography chamber using AnyMaze tracking software and convert these movements into a colour coded sonogram. The brighter colours in the sonogram indicate head movement. The second method analyzed the large excursions on the whole body plethysmography (WBP) trace. These arise artefactually from movement. Comparison of the sonogram based on the WBP trace (WBP Sonogram) and that based on head movement (Mov Sonogram) showed that the two measures correlated well and that large excursions of the WBP trace often coincided with head movement (compare sonograms in Figure 1E). When we examined the GCaMP6 fluorescence in conjunction with the sonograms of head and body movement, we observed that: (i) large movements of the head/body did not evoke noticeable changes in GCaMP6 fluorescence (Figure 1Ea,b); (ii) GCaMP6 Ca²⁺ signals characterized by a fast rise and exponential decay occurred in the absence of any movement (Figure 1Ec,f); and (iii) similar shaped Ca²⁺ signals occurred during movement (Figure 1Ed,e). Together this suggests that movement of the head or body of the animal does not prevent the recording of genuine changes in GCaMP6 fluorescence related to changes in intracellular Ca²⁺.

Thirdly, we used the same paradigm of stimulation for all experiments as can be seen from the records in Figure 1—figure supplements 2–4. This enabled averaging of the responses aligned either to the onset of hypercapnia, or features of the WBP trace. This averaging brought out consistent features of the presumed Ca²⁺ signals that were temporally related to either the onset of hypercapnia or alterations of breathing. While for the most part we could not record from the same neurons in different recording sessions from the same mouse, we did observe consistent patterns of neuronal firing between recording sessions (Figure 1—figure supplement 5). As movement that could give rise to artefact would not be expected to be similar from recording session to recording session, or from mouse to mouse, the consistency of the patterns of the signals between mice and recording sessions strongly suggests a true biological rather than artefactual origin of the recorded signals.

In some cases, it was possible to identify the same neuron from recording sessions on different days in the same mouse. This was only the case in a minority of sessions perhaps because the brainstem is extracranial and more mobile and thus it is harder to reproduce the same exact focal plane between different recording sessions. When we were able to identify the same neurons their activity patterns were remarkably similar between separate recording sessions (Figure 1—figure supplement 6). This gives further confidence that the observed patterns are a reflection of biological properties.

Finally, we made recordings from anaesthetized mice (Figure 1F and Figure 4—figure supplement 1). In this case movement artefacts cannot be a potential confounding factor. To elicit neural activity,
Figure 1. Experimental approach and movement artefact. (A) A CONSORT flow diagram for inclusion/exclusion of experiments and cells from the study. (B) Representation of GRIN lens (microendoscope), baseplate, and mini epifluorescence camera placement for recording of brainstem nuclei. (C–D) GFP control. RTN neurons were transduced with AAV-Syn-GFP (not Ca²⁺ sensitive) and GRIN lens implanted. Fluorescence was recorded going

*Figure 1 continued on next page*
we induced electrographic seizures via intraperitoneal kainic acid injection in mice in the absence of any behavioural seizure movements such as partial (including forelimb or hindlimb) or whole body continuous clonic seizures (Figure 1—video 2). After ~70 min this resulted in perturbations of the WBP trace and correlated Ca\textsuperscript{2+} activity in the RTN neurons, presumably denoting the invasion of seizures into this nucleus. Detailed examination revealed that the fluorescence changes were of two types: (i) transients with a fast rise and exponential fall; and (ii) slower sustained changes (Figure 1G).

This is an important observation as it shows that we expect to see two types of genuine Ca\textsuperscript{2+} signal in recordings from freely-moving mice: fast transients with an exponential decay phase, that may sum together and slower more gradual rises.

Movements of the visual field, evident during most recordings, were corrected for via the Inscopix motion correction software to allow ROI-based measurement of fluorescence to remain in register with the cells during the recording. Where movement artefacts persisted following motion correction, in some cases it was possible to draw additional ROIs over a high contrast area devoid of fluorescent cells, such as the border of a blood vessel. This allowed for a null signal to be subtracted from the GCaMP signal (Figure 7E) thus providing the neuronal Ca\textsuperscript{2+} transients free of movement artefact. If there was too much uncompensated motion artefact, so that we could not clearly analyze Ca\textsuperscript{2+} signals, we excluded these recordings from quantitative analysis (details given in Figure 1A). GCaMP6 signals were only accepted for categorization if the following criteria were met: (1) the features of the cell (e.g. soma, large processes) could be clearly seen; (2) they occurred in the absence of movement of the mouse or were unaffected by mouse movement; (3) fluorescence changed relative to the background; (4) the focal plane had remained constant as shown by other nonfluorescent landmarks (e.g. blood vessels). Applying these criteria left 144/194 cells eligible for study. All of the recordings that were included are shown in Figure 1—figure supplements 2–4. The activity of the cells fell into the following categories:
Inhibited (I)
Displayed spontaneous Ca\textsuperscript{2+} activity at rest, which was greatly reduced during both 3 and 6% inspired CO\textsubscript{2} and could exhibit rebound activity following the end of the hypercapnic episode.

Excited - adapting (E\textsubscript{A})
Silent or with low level activity at rest and showed the greatest Ca\textsuperscript{2+} activity in response to a change in 3% inspired CO\textsubscript{2}. Following an initial burst of activity, they were either silent or displayed lower level activity throughout the remainder of the hypercapnic episode. These cells often exhibited rebound activity following the end of the hypercapnic episode due to suppression by higher CO\textsubscript{2} levels. These cells essentially encoded the beginning and end of hypercapnia.

Excited - graded (E\textsubscript{G})
Silent or with low level activity at rest and displayed an increase in Ca\textsuperscript{2+} activity at 3% inspired CO\textsubscript{2} with a further increase in activity at 6% that returned to baseline upon removal of the stimulus. These cells encoded the level of inspired CO\textsubscript{2}.

Tonic (T)
Displaying spontaneous Ca\textsuperscript{2+} activity throughout the recording that was unaffected by the hypercapnic episode but could provide tonic drive to the respiratory network.

Sniff-coding (Sn)
Displayed elevated Ca\textsuperscript{2+} signals correlated with exploratory sniffing.

Expiratory (Exp)
Displayed elevated Ca\textsuperscript{2+} signals correlated with large expiratory events.

Non-coding (NC)
Displayed low frequency or sporadic Ca\textsuperscript{2+} activity that was neither tonic in nature nor had any discernable activity that related to the hypercapnic stimulus or any respiratory event.

Non-coding respiratory related (NC-RR)
Displayed Ca\textsuperscript{2+} activity that did not code the hypercapnic stimulus, but it was instead related to variability in breathing frequency.

Our categorisation of neurons into these types was supported by a two-component analysis in which we plotted the change in Ca\textsuperscript{2+} activity (from baseline) elicited by 6% inspired CO\textsubscript{2} versus the change in Ca\textsuperscript{2+} activity elicited by 3% inspired CO\textsubscript{2}. E\textsubscript{A} neurons as they showed more activity at 3% compared to 6% CO\textsubscript{2} should cluster below the line of identity (x=y), whereas E\textsubscript{G} neurons as they respond more strongly to 6% than 3% should cluster above the line of identity. NC neurons as they did not show a response to hypercapnia should be clustered around the origin (x=y=0), and the I neurons should be clustered in the quadrant where the change in Ca\textsuperscript{2+} activity to both stimuli is negative. This analysis shows that the different classes of neurons in both the RTN and Raphe do indeed cluster in the appropriate regions of the graph (Figure 1—figure supplement 7).

Chemosensory responses in the RTN
As the RTN contains chemosensitive glia and neurons (Gourine et al., 2010; Mulkey et al., 2004; Nattie and Li, 1994; Ramanantsoa et al., 2011), and is neuroanatomically diverse (Shi et al., 2017; Stornetta et al., 2009), we began by targeting all neurons of the RTN with a synapsin-GCaMP6s AAV (Figure 2A–C); a necessary step to enable their imaging during free behaviour via a mini-headmounted microscope (Figure 1B). The localization of successful and accurate transduction was confirmed posthoc by using choline acetyltransferase (ChAT) staining to define the facial nucleus and the location of the transduced cells (Figure 2B), with ~7% (9/132) of transduced neurons in the focal plane of the microscope co-labelled with ChAT (Figure 2C). The lens track terminated under the caudal pole of the facial nucleus, containing the highest number of neurons (Figure 2B), with
Figure 2. Excitatory chemosensory responses of RTN neurons in awake mice. (A) AAV9-Syn-GCaMP6s injection into the RTN. (B) Micrograph of lens placement and viral transduction of neurons (green) relative to the facial nucleus (ChAT+ neurons red). (C) Venn diagram of cell counts of GCaMP6s transduced (green), and ChAT+ (red), neurons under the GRIN lens (1 representative section from each of 4 mice). (D) Neurons transduced...
with GCaMP6s also contain NMB confirming their identity as RTN neurons. (E) Venn diagram of cell counts of GCaMP6s transduced (green), and NMB+ (magenta), neurons under the GRIN lens (1 representative section from each of 3 mice). (F) Recording of mouse whole body plethysmography (WBP) in response to hypercapnia time-matched with Inscopix recorded GCaMP6s signals. These show neurons that gave a graded response to CO₂ (Eₐ). (G) Average of the graded neuronal responses aligned to the WBP trace in (F). (H) Examples of neurons that showed an adapting response to a change in inspired CO₂ (Eₐ). WBP trace aligned to the GCaMP6 fluorescence and instantaneous Vₜ (minute ventilation) shown to demonstrate how the signals in the Eₐ neurons closely correspond to Vₜ. (I) Average waveform of all Eₐ neurons in the RTN aligned to 0, 3 and 6% inspired CO₂ (gray bar). (J) A plot of Vₜ versus F/F₀ for the Ca²⁺ signal during the transition from 0 to 3% CO₂ in three individual mice showing positive correlation. Underneath each Vₜ vs F/F₀ graph is a plot of change in Vₜ (grey) and average of Eₐ neuronal Ca²⁺ activity (magenta) from respective mouse during transition from baseline to 3% CO₂. Note that there is a transient increase in breathing at the beginning of hypercapnia that corresponds to the activation of the Eₐ neurons. Abbreviations: 7 N, facial motor nucleus; Py, pyramidal tract; MVe, medial vestibular nucleus; sp5, spinal trigeminal nucleus: RTN, retrotrapezoid nucleus; RMg, raphe magnus; RPa, raphe pallidus; pFL, parafacial lateral region.

The online version of this article includes the following video and figure supplement(s) for figure 2:

**Figure supplement 1.** Validation of NMB immunostaining patterns.

**Figure supplement 2.** The dynamics of individual Ca²⁺ transients from two Eₐ cells and two T cells recorded in the RTN before, during and after hypercapnia.

**Figure 2—video 1.** Excited graded (Eₐ) RTN neuronal response to hypercapnia in synapsin GCaMP6 transfected mice.

https://elifesciences.org/articles/70671/figures#fig2video1

**Figure 2—video 2.** Excited adapting (Eₐ) RTN neuronal response to hypercapnia in synapsin GCaMP6 transfected mice.

https://elifesciences.org/articles/70671/figures#fig2video2

the focal plane 150–450 μm below that, covering the dorso-ventral extent of the RTN (Figure 1—figure supplement 1). Therefore, our viral transduction and lens placement allowed us to record from the RTN. In some mice (n=3), we further checked the identity of transduced neurons by examining whether they expressed neuromedin B (NMB), a marker specific to Phox2b chemosensory neurons in the RTN (Figure 2D, Figure 2—figure supplement 2, Li et al., 2016; Shi et al., 2017). We found 43% (40/93) of NMB+ cells in the RTN were also transduced with GCaMP6, and 20% (40/204) of GCaMP+ neurons in the RTN were NMB+. Thus, we transduced nearly half of the NMB+ neurons, and if the fluorescence imaging sampled transduced cells randomly and without bias to cell type, roughly 1 in 5 of cells recorded would have been of this phenotype.

After assessing the quality of recordings, we retained Ca²⁺ activity traces from 98 RTN neurons in 9 mice (Figure 1A). There was a wide variety of neuronal responses to the hypercapnic challenge (Figure 1—figure supplement 2). While we observed 4/98 neurons exhibited a graded response to hypercapnia (Eₐ, Figure 2F and G, Figure 2—video 1), a much greater number (27/98) were of the adapting subtype (Eₐ, Figure 2H1, Figure 2—video 2). This adapting response may also be physiologically meaningful, as it matched the time course of changes in Vₜ calculated from the WBP records (Figure 2H). In three mice the averaged Ca²⁺ trace of the Eₐ neurons in each mouse matched the changes in Vₜ for the same mouse and a plot of Vₜ versus F/F₀ for the Ca²⁺ signal gave a positive correlation (Figure 2J, and Supplementary file 1). This correspondence between features of the responses in these neurons and the adaptive ventilatory response, supports the hypothesis that these are a physiologically important class of chemosensitive neurons. An alternative explanation that the rates of Ca²⁺ sequestration greatly increased during hypercapnia such that Ca²⁺ levels fell even although firing rates increased can be excluded by examining the dynamics of individual Ca²⁺ transients. The rise time of these transients reflects the rate of Ca²⁺ influx, and the exponentially falling decay phase the rate of Ca²⁺ extrusion or sequestration. Comparison of these transients before, during and after the hypercapnic stimulus shows that these transients do not change in shape (Figure 2H, Figure 2—figure supplement 2).

A further 5/98 neurons were inhibited during hypercapnia (Figure 3A and C, blue traces and Figure 3—video 1). These neurons displayed spontaneous Ca²⁺ activity during normocapnia, but
were abruptly silenced during hypercapnia (Figure 3C). We recorded from sniff-coding (Sn) neurons, 3/98, which showed elevated Ca²⁺ signals correlated with sniffing (Figure 3A and B). In sniff-coding neurons, Ca²⁺ activity coincided with perturbations of the plethysmography traces (increase in respiratory frequency, increase of tidal volume, presence of expiration, Figure 3B and Figure 3—video 1). Analysis of the onset of the Ca²⁺ signal relative to the start of the sniff, showed that, on average,
activity in these neurons preceded the sniff by 0.4–0.8 s (Figure 3D). The temporal correlation between the sniff-coding neurons and the sniff was further confirmed by averaging the Ca²⁺ recordings from multiple sniffs (Figure 3E). Interestingly the different functional types of neuron are clustered adjacent to each other (Figure 3F).

Additionally, 5/98 neurons which were tonically active but did not encode any information about hypercapnia (T, Figure 4A, Figure 4—video 1). Tonically active neurons in the RTN could provide background excitation to the respiratory network. Of 98 neurons, 35 showed sporadic activity that did not correlate with CO₂ (NC, Figure 4B) and a further 19/98 neurons that did not encode CO₂ levels but displayed Ca²⁺ signals that were correlated to changes in respiratory frequency and were generally silent when tidal volume was at its greatest (NC-RR, Figure 4C).

In summary for RTN neurons: ~40% (39/98) had activity patterns that were in some way modulated by CO₂. The majority of these neurons, 69% (27/39), displayed an adaptive response to CO₂, thus marking the onset of hypercapnia. Only a minority of neurons, 10% (4/39), encoded the magnitude of hypercapnia and a similar minority (13%; 5/39) were active and inhibited throughout the hypercapnic episode. Interestingly, we found a small number of sniff-activated neurons, which may be from the most rostral and ventral aspect of the retrofacial nucleus, which overlaps with the most caudal and dorsal aspect of the RTN (Deschênes et al., 2016; Kurnikova et al., 2018).

Effect of anaesthesia on RTN chemosensory responses

Until now most investigation of RTN chemosensory responses at adult life stages has been in anaesthetised preparations. We therefore examined the effect of deep anaesthesia on the activity of RTN neurons before and during chemosensory stimuli (Figure 4—figure supplement 1). As might be expected, compared to the awake state, urethane anaesthesia had a deeply suppressive effect on the spontaneous activity of all RTN neurons. Whereas almost all neurons displayed spontaneous activity, there was hardly any activity under anaesthesia (Figure 4—figure supplement 1A-B). Furthermore, the responses to hypercapnia were greatly blunted, with many neurons simply being unresponsive. When we examined those neurons that retained a chemosensory response to hypercapnia in the anaesthetised state, these responses differed from those that the same neurons showed in the awake state (Figure 4—figure supplement 1C). For example, cells displaying a graded response to hypercapnia under anaesthesia were classified as non-coding respiratory related (NC-RR) or non-coding (NC) in the awake state.

Chemosensory responses in the medullary raphe

As the medullary Raphe contains multiple neuronal types which display functionally different CO₂ responses in vitro (Bradley et al., 2002; Ray et al., 2011; Richerson, 1995) and in vivo (Veasey et al., 1995), we next examined whether the activity of neurons in the rostral Raphe nucleus could be altered by CO₂ (Figure 5). We drove expression of GCaMP6s with a synapsin promoter (n=2 mice; Figure 5A–C). Assessing the ability of this construct to transduce serotonergic neurons in this nucleus, we found that 57/113 (50%) of the transduced neurons in the focal plane of the microscope were TPH+ (tryptophan hydroxylase; a marker of serotonergic neurons) (Figure 5D). As there are also GABAergic neurons (Iceman et al., 2014; Iceman and Harris, 2014), and non-serotonergic NK1R neurons in the Raphe (Hennessy et al., 2017; Iceman and Harris, 2014), we also specifically targeted the serotonergic neurons by driving GCaMP6 expression with a SERT (slc27a4) promoter (n=4 mice, Figure 5E–F). Post-hoc immunocytochemistry showed that all neurons in the optical pathway of the microscope (Figure 5E) which expressed GCaMP6s co-localised with TPH. We recorded 9 synapsin GCaMP6s neurons and 17 SERT GCaMP6s neurons.

As in the RTN, we found there were multiple categories of CO₂-dependent responses (Figure 1—figure supplement 3). A frequently observed class of neurons (8/26) exhibited a graded response to CO₂ (Eₐ, Figure 5G and H and Figure 5—video 1, synapsin). 4/26 neurons showed the adapting response (Eₐ, Figure 5G). The commonest class of neurons in our dataset was inhibited by CO₂ (10/26, all from mice transduced with GCaMP under the SERT promoter, Figure 6A and B, Figure 6—video 1 SERT). The remaining 4/26 neurons displayed activity that was unaffected by hypercapnia (NC, Figure 6C).

In summary, 85% (22/26) of Raphe neurons had Ca²⁺ activity patterns that were modulated by CO₂. Interestingly, unlike the RTN which showed considerable bias toward the Eₐ functional type,
Figure 4. Tonically active, non-coding (NC) and NC-respiratory related (NC-RR) RTN neuronal responses in awake mice. Recording of mouse WBP in response to hypercapnia time-matched with Incsopix recorded GCaMP6 signals (A, B, C). GCaMP fluorescence of RTN (A) tonically active (T; violet blue) (B) non-coding (NC; green) and (C) non-coding respiratory related (NC-RR; shamrock green) neurons. Average of NC-RR neuronal activity (dotted box) expanded under it displayed Ca²⁺ signals that were correlated to changes in respiratory frequency (FR) and were generally silent when tidal volume (WBP) was at its greatest.

The online version of this article includes the following video, source data, and figure supplement(s) for figure 4:

Figure supplement 1. Hypercapnia induced RTN neuronal responses in awake and anaesthesized mice.

Figure 4 continued on next page
EA neurons were much less prevalent in the Raphe (only 15%), with \( E_g \) (31%), and I (38%) being the most common types of response we observed. In the Raphe, there were no tonically active neurons or neurons that were co-active with any non-respiratory related orofacial movements such as sniffing.

**Expiratory activity of neurons in the pFL**

Neurons of the pFL, lateral and adjacent to the RTN, have been proposed as the expiratory oscillator (Huckstepp et al., 2015; Huckstepp et al., 2016). Under resting conditions, eupneic breathing, with the exception of during REM sleep (Andrews and Pagliardini, 2015), does not involve active expiration, so the neurons of the pFL are largely silent. A hypercapnic stimulus causes an increase in respiratory frequency, tidal volume and also the onset of active expiration (Huckstepp et al., 2015). We therefore transduced neurons of the pFL in 3 mice with the synapsin-GCaMP6f construct (Figure 7A–D) and observed their responses during a hypercapnic stimulus (Figure 7F, Figure 1—figure supplement 4). We found that the \( \text{Ca}^{2+} \) activity reflected changes in respiratory frequency and tidal volume with surprising fidelity (compare grey trace (\( f_R \)) with GCaMP traces in Figure 7F). Closer inspection revealed the temporal relationship between \( \text{Ca}^{2+} \) activity and changes in the plethysmographic traces (Figure 7G). Whilst \( \text{Ca}^{2+} \) activity in the pFL neurons on average preceded an increase in tidal volume and respiratory frequency by ~0.4 s, some neurons showed \( \text{Ca}^{2+} \) activity only after changes in tidal volume and respiratory frequency had occurred (Figure 7H–J). This time separation was independent of the level of inspired \( \text{CO}_2 \) (Figure 7I and J). These characteristics are more compatible with a correlative rather than a causal relationship between the activation of pFL neurons and changes in tidal volume and respiratory frequency.

When we specifically studied the incidence of active expiration, we found that \( \text{Ca}^{2+} \) activity in pFL neurons always preceded a change in the expiratory activity (Figure 7H–M). Importantly, the time delay between activity in the pFL neurons and active expiration was sensitive to inspired \( \text{CO}_2 \) (Figure 7K–M). At 6% inspired \( \text{CO}_2 \) this time difference was ~0.5 s. (Figure 7K and L), while at 9% inspired \( \text{CO}_2 \) the time interval shortened to ~0.3 s (Figure 7K and M). In all 20 neurons in the pFL that exhibited activity, this activity preceded active expiration. This timing is consistent with a causal rather than a correlative link between neuronal activity in the pFL and induction of active expiration. Post-hoc immunocytochemistry showed that a small population of neurons which expressed GCaMP6 co-localised with ChAT (2/71, 1.5%, Figure 7B and D) and were located in the focal plane of the microscope (Figure 7B). These neurons either displayed expiratory-linked activity or more likely were silent and would have been excluded from our dataset, given no other responses other than expiratory activity were recorded in this region.

**Discussion**

**Limitations**

We have recorded the intracellular \( \text{Ca}^{2+} \) signal from neurons present in the RTN and Raphe. The summation and temporal variation of the signal will depend on the \( \text{Ca}^{2+} \) buffering/extrusion systems within the cell, the density and activation properties of \( \text{Ca}^{2+} \) permeable channels, and whether \( \text{Ca}^{2+} \) may additionally be released from intracellular stores. Therefore, this \( \text{Ca}^{2+} \) signal cannot tell us the precise firing rates or dynamics of the neuronal activity, and so is an imprecise proxy of neuronal activity. Despite this imperfection, \( \text{Ca}^{2+} \) activity of neurons is a widely used to assess their electrical activity (Chen et al., 2013; Dana et al., 2019; Huang et al., 2021). We verified that the rates of \( \text{Ca}^{2+} \) influx and extrusion or sequestration do not change with the hypercapnic stimulus by examining individual \( \text{Ca}^{2+} \) transients before, during and after hypercapnia to show that their rise times and decay
**Figure 5.** CO₂-excitated medullary raphe neurons in awake mice. (A) AAV injection into the Raphe. (B) Micrograph of lens placement and AAV9-Syn-GCaMP6s viral transduction of neurons (green) relative to the Raphe (TPH+neurons red). (C) GCaMP6s fluorescence signal from AAV9-Syn-GCaMP6s transduced raphe neurons in freely behaving mice (Scale bar- 50 μm). (D) Venn diagram of cell counts of AAV9-Syn-GCaMP6s transduced neurons (green) under the GRIN lens with TPH+neurons (red) in the raphe (1 representative section from each of 4 mice). (E) Micrograph of lens placement and AAV9-SERT-GCaMP6s viral transduction of neurons (green) relative to the Raphe (TPH+neurons red). (F) GCaMP6s fluorescence signal from AAV9-SERT-GCaMP6s transduced raphe neurons in freely behaving mice (Scale bar- 50 μm). (G) WBP recording in response to hypercapnia time-matched with inscopix recorded GCaMP6 signals. GCaMP fluorescence of raphe excitatory graded (Eₕ), and excitatory adapting (Eₐ) neurons in response to hypercapnia. (H–I) Average waveform of raphe Eₕ neuronal responses to hypercapnia aligned to the WBP trace in G. Abbreviations defined in Figure 2.

The online version of this article includes the following video for figure 5:

**Figure 5—video 1.** Excited graded (Eₕ) and adapting (Eₐ) Raphe neuronal responses to hypercapnia in synapsin GCaMP6 transfected mice.

[https://elifesciences.org/articles/70671/figures#fig5video1](https://elifesciences.org/articles/70671/figures#fig5video1)
rates remain unaffected (Figure 2—figure supplement 2). This gives further confidence that the pattern of Ca\textsuperscript{2+} activity reflects the pattern of neuronal firing.

We used GCaMP6s for recording from the RTN and Raphe as this is the most sensitive Ca\textsuperscript{2+} sensor and we did not expect to need to resolve rapidly changing Ca\textsuperscript{2+} signals. For the pF\textsubscript{L} we used faster responding, but less sensitive, GCaMP6f to attempt to document cycle by cycle changes in fluorescence, but these were not resolved in the current experiments.

We made our recordings at a single wavelength as this was the capability of the minimicroscopes available at the time of the study. Dual channel versions are now available and these would assist with the treatment and exclusion of movement artefacts e.g. through use of ratiometric imaging at two wavelengths. Nevertheless, our extensive controls for movement artefacts, and data analysis, show that we have resolved real Ca\textsuperscript{2+} signals that reflect different patterns of neuronal firing that are reproducible between across the imaged neuronal population in different recording sessions from a single mouse and recordings between multiple mice. These signals are characterised by the well-documented pattern for true Ca\textsuperscript{2+} signals of a fast onset and a slower exponentially-falling offset.

While we can be confident of the location of the GRIN lens and the volume of tissue in which labelled neurons could have contributed to the observed signals from posthoc analysis, it is important to note that we cannot identify the actual neurons that were recorded in each session. Related to this, we were unable, for the most part, to make consecutive recordings from the same neurons from session to session. We used a genetic targeting strategy that would give broad coverage of all...
Figure 7. pF₁ neurons drive active expiration. (A) AAV9-Syn-GCaMP6f injection into the pF₁. (B) Micrograph of lens placement and viral transduction of pF₁ neurons (green) relative to the facial nucleus (ChAT + neurons red). (C) GCaMP6f fluorescence signal from transduced pF₁ neurons in freely behaving mice (Scale bar- 50 μm). (D) Venn diagram of cell counts of GCaMP6f transduced (green) and ChAT+ (red) neurons under the GRIN lens (1,000 μm).
neuronal types within the nuclei. This presents an overview of the different activity patterns that occur during responses to hypercapnia, however it does not allow identification of the responses of specific neuronal subtypes. Now that we have a library of all activity patterns in these regions, more precise genetic targeting of neurons is required to relate phenotype to firing pattern. Given that no single unique genetic marker has yet been identified to mark chemosensory neurons, such an approach would have to utilize intersectional genetics to achieve the necessary level of precision.

Our observations document the different types of neuronal responses to hypercapnia in awake mice. However, we cannot be sure whether these responses were a direct consequence of the chemosensory stimulus, i.e. a direct action of CO₂ or pH on the recorded neurons, or an indirect secondary outcome shaped by the synaptic networks present within the medulla. Additionally, recordings from the same neurons in response to repeated periods of hypercapnia would give further assurance that the activity patterns observed are reproducible responses to hypercapnia at a single cell as opposed to population level.

**Chemosensory activity in the rostral medulla of adult mice**

For many years the RTN was considered to be a homogeneous population of chemosensory neurons (Guyenet et al., 2010; Nattie and Li, 1994). However, recent neuroanatomical (Li et al., 2016; Shi et al., 2017; Stornetta et al., 2009) and pharmacological (Huckstepp et al., 2018; Li et al., 2016) evidence suggests the region may be more heterogenous than first thought and that functional subdivisions of the RTN may exist in adult rodents. In accordance with this, we found 7 functional subpopulations of neurons. There were two classes of neurons that were excited by CO₂ and indicated an aspect of the hypercapnic stimulus (Figure 2): E_a (the start and end of hypercapnia), and E_o (the presence and magnitude of the hypercapnic stimulus). In addition, there were neurons that were inhibited by hypercapnia (I). Inhibition of RTN neuronal firing by hypercapnia has been previously observed (Cleary et al., 2021; Nattie et al., 1993; Ott et al., 2011).

We found the majority of neurons (from five different mice) in our recordings from the RTN were of the E_a subtype that is they responded to the initial increase in inspired CO₂ but did not maintain their activation. In some of these neurons there could be a further small increase in activity at the transition of inspired CO₂ from 3 to 6%. This suggests that these neurons some aspect of the sensory response adapts or fatigues, or they are subject to delayed CO₂-dependent feedback inhibition that depresses their activity. It is notable that in many of these neurons, activity increased following removal of the hypercapnic stimulus. This activation during transitions at the beginning and end of a stimulus is reminiscent of a multitude of rapidly adapting sensory neurons (e.g. rapidly adapting pulmonary receptors Widdicombe, 1954, Pacinian and Meissner corpuscles Vallbo and Johansson, 1984). This could be due to rapid removal of sensory adaptation followed reactivation and rebound activity as the arterial CO₂ levels moves back to resting levels.

While adapting responses to hypercapnia have not been recognised before, the Phox2b+neurons of the RTN have been subdivided into types 1 and 2 on the basis of their pH sensitivity (Lazarenko...
Neuroscience

A second possibility is that much of prior evidence for RTN chemosensory responses depends heavily on recordings from anaesthetized animals and that anaesthesia alters the dynamics of neuronal responses to CO$_2$. When we compared RTN responses in awake and anaesthetized animals we found that deep urethane anaesthesia dramatically changed neuronal firing patterns before and during hypercapnia (Figure 4—figure supplement 1). While the choice of anaesthetic agent and depth of anaesthesia is likely to alter the degree to which RTN neurons retain their natural activity, our recordings suggest that studies of chemosensory responses in anaesthetised preparations should be interpreted with caution.

These possibilities are not mutually exclusive and it is likely that they may all contribute to the relatively low proportion of neurons of the E$_G$ subtype in our dataset. Unexpectedly, whilst we recorded from sniff activated neurons, we did not find any neurons with sigh related activity, which may be due to their sparsity in number (Li et al., 2016). Therefore, more recordings may be necessary to uncover further functional subpopulations within the RTN region.

Several lines of evidence have suggested the importance of medullary Raphe serotonergic neurons in mediating respiratory chemosensitivity: the proximity of raphe neuron processes to blood vessels...
the correspondence of the location of CO_{2}-dependent ATP release at the medullary surface and the raphe neurons (Bradley et al., 2002; Gourine et al., 2005); suppression of the medullary raphe by isofluorane (Johansen et al., 2015; Ray et al., 2011) which matches the reduction in the overall hypercapnic ventilatory response; and most compellingly the observation that inhibition of their activity via inhibitory DREADD receptors removes ~40% of the total adaptive ventilatory response in awake mice (Ray et al., 2011). There were two classes of neuron excited by CO_{2} and these marked the presence and magnitude of the hypercapnic stimulus (E_{z}) and the start of the hypercapnic episode (E_{s}), supports the hypothesis that the Raphe acts as a primary chemosensory nucleus.

Of the 2 known serotonergic subtypes in the Raphe, NK1R-negative serotonergic neurons project to areas responsible for CO_{2} integration (Brust et al., 2014), whereas NK1R-positive serotonergic neurons project to motor nuclei responsible for airway patency and co-ordination, and diaphragmatic movements (Hennessy et al., 2017). Interestingly both subtypes project to the preBötC (Brust et al., 2014; Hennessy et al., 2017). Furthermore, both serotonergic and GABAergic raphe neurons project to the pF_{r} (Silva et al., 2020), with serotonergic neurons also innervating areas that influence expiration, namely the C1 (Malheiros-Lima et al., 2020), and NTS (Silva et al., 2019). Therefore, Raphe neurons are well placed to have far-reaching effects on breathing.

While both the RTN and Raphe have a high proportion of neurons that can be activated by CO_{2}, there are interesting differences in the nature of the responses that we observed (Figure 8A). The majority of RTN neurons that we observed were of the E_{z} subtype, thus they will encode a change in inspired CO_{2} rather than the presence of the entire stimulus. Even the E_{s} neurons responded to a change in inspired CO_{2} with a transient change in Ca^{2+} activity in addition to the graded response. By contrast the situations was reversed in the Raphe: we observed a greater number of Raphe neurons were of the E_{s} subtype than the E_{z} subtype. This may suggest a complementarity between the RTN and Raphe, such that the more superficially located RTN rapidly encodes changes in PCO_{2}, with the deeper sitting Raphe providing a more sustained and graded response to encode the magnitude. It is noteworthy that plethysmographic changes in breathing are well-known to partially adapt to step changes in inspired CO_{2} (illustrated in Figure 2H and J, see also Bravo et al., 2016) suggesting that the E_{s} subtype is physiologically important. The rapid response to a sudden change in inspired CO_{2} could plausibly be regarded as a trigger to quick behavioural adaptation that could remove the animal from the source of hypercapnia, or trigger a rapid adjustment in breathing that is sufficient to restore normocapnia.

CO_{2}-inhibited neurons in the RTN and Raphe have been previously described (Iceman et al., 2014; Nattie et al., 1993). Our recordings give further evidence for these subtypes in both nuclei. In the Raphe, CO_{2} inhibited neurons were thought to be exclusively GABAergic. Our finding that serotonergic Raphe neurons can be inhibited by CO_{2} is unexpected. The mechanism of CO_{2}-dependent inhibition is unclear. One possibility is that a CO_{2} activated K’ channel is present in these cells. An inward rectifier K’ channel that is activated by CO_{2} has been described in HeLa cells (Huckstepp and Dale, 2011a), indicating that this mechanism of intrinsic sensitivity is possible. However, a likelier alternative hypothesis is that the inhibitory action of CO_{2} on the serotonergic and GABAergic neurons is indirect and depends upon synaptic connectivity -for example via CO_{2}-dependent activation of local GABAergic interneurons that are present in the medullary Raphe (Figure 8B).

While the emphasis of chemosensory mechanisms has been on the CO_{2}/pH-dependent activation of excitatory neurons, CO_{2}/pH-dependent inhibition of neurons could also be a powerful contributor to the hypercapnic ventilatory reflex (Figure 8A). While we have not demonstrated synaptic connections from any of the classes of neurons we describe, if CO_{2}-inhibited neurons were to tonically excite neurons that normally inhibit the preBötzinger complex, a process of disinhibition during hypercapnia could result in greater excitation of preBötzinger complex neurons (Figure 8A). RTN and Raphe neurons are known to project to the Bötzinger complex (Morinaga et al., 2019; Rosin et al., 2006), which contains inhibitory neurons (Ezure and Manabe, 1988), whilst RTN neurons are also known to project to inhibitory cells of the preBötC (Yang et al., 2019), which regulate the synchronisation of excitatory bursting and the emergence of the inspiratory rhythm (Ashhad and Feldman, 2020). The CO_{2}-dependent disinhibition of the inhibitory interneurons in the preBötC could contribute to the emergence of more powerful inspiratory bursts and hence an increase in tidal volume (Figure 8A).
Figure 8. Summary diagram of findings in the paper showing contribution of the RTN, Raphe and pFl neurons to respiratory chemosensitivity and active expiration. (A) Block diagram showing the types of activity pattern observed in neurons of the RTN, pFl, and Raphe. Proposed potential interconnections to the preBötzing and Bötzing complexes are speculative and based on regional interactions reported in the literature. (B) Hypothesis for generating CO2 dependent inhibition of serotonergic neurons. CO2 is proposed to activate GABAergic neurons that inhibit tonically active serotonergic neurons which are not directly CO2 sensitive. If these were to activate a separate population of non-CO2 sensitive GABAergic neurons, these could normally silence the CO2 sensitive neurons until an episode of hypercapnia. This mechanism could explain an indirect inhibitory action of CO2 on both serotonergic and GABAergic neurons. (C) Averages of the different types of neuronal activity seen in the RTN, Raphe and pFl during hypercapnia aligned to an average of the WBP trace.
Non-chemosensory mechanisms in the RTN

The RTN showed several forms of Ca\textsuperscript{2+} activity that did not respond to hypercapnia. In support of previous findings (Nattie et al., 1993), we saw a small population of tonically firing neurons that were unaffected by elevating inspired CO\textsubscript{2}. These neurons might provide tonic drive to the respiratory network that drives tidal volume, which is known to originate at least in part from the RTN (Huckstepp et al., 2015). However, in 67/98 of the RTN neurons, we observed some tonic activity during room air breathing. Therefore, about 2/3 of the RTN neurons we imaged could plausibly contribute to tonic drive at rest. In addition to providing drive to basal breathing, tonic activity in neurons may also provide a level of baseline excitation that permits for the system to be more easily manipulated, allowing other respiratory features to be expressed. For example there is an absolute requirement for network wide activity to enable expiratory motor output to occur (Huckstepp et al., 2016). Furthermore, it is well documented that as respiratory network excitation decreases during sleep, sensitivity to CO\textsubscript{2} diminishes leading to lower minute ventilation, a higher resting PaCO\textsubscript{2} (Douglas et al., 1982a), and reduced hypercapnic ventilatory responses (Douglas et al., 1982b). Thus, tonic activity in the RTN may be vital to allow a greater sensitivity to CO\textsubscript{2} during wakefulness.

We also found non-coding neurons in the RTN. These neurons could be subdivided into those that displayed Ca\textsuperscript{2+} activity not related to the level of CO\textsubscript{2}, nor any abstractable feature on the respiratory recording (NC), and those that had Ca\textsuperscript{2+} activity that matched variations in respiratory output (NC-RR). This latter population were largely silent when V\textsubscript{T} was at its greatest but displayed activity when the respiratory frequency was high and the faster ventilatory cycle meant that V\textsubscript{T} was smaller. The NC-RR neurons might therefore provide excitation to increase respiratory frequency. The NC sub-population could represent a novel neuronal type in the RTN, or could be a known non-CO\textsubscript{2} coding subpopulation that are likely to have other functions such as those that express high levels neuromedin B (Shi et al., 2017).

Sniffing is a mechanism that allows for olfactory sampling of the air to discern location of smell and to test for irritants before air is taken into the lung. Sniffing is obligatory for odor perception (Mainland and Sobel, 2005), and thus activates the piriform cortex (Sobel et al., 1998a). However, sniffing also causes neuronal activity in the absence of odorants (Sobel et al., 1998a). It activates the hippocampus (Vanderwolf, 2001), and the cerebellum (Sobel et al., 1998b), respectively, likely to prime odour-related memory recall, and co-ordination of movement toward or away from specific odours. Whilst the activation of these brain regions during sniffing has been well documented, the pattern generating microcircuit for sniffing has only recently been identified. Sniffing requires the activation of two sets of muscles (i) the nasal muscles to open the airway and decrease resistance, allowing more rapid movement of air through the nose, and (ii) the respiratory (inspiratory and expiratory) muscles, to draw air in and out of the nasal cavity. The RTN is located adjacent to, and innervates, the facial motor nucleus (Deschênes et al., 2016; Kurnikova et al., 2018). These RTN projections to the facial are directly involved in sniffing (Deschênes et al., 2016) and control of nasal direction during the odour response (Kurnikova et al., 2018). In conjunction the RTN provides significant drive to the preBötC (Rosin et al., 2006), and the pF\textsubscript{L} (Zoccal et al., 2018), both of which are integral parts of the sniffing microcircuit (Deschênes et al., 2016; Moore et al., 2013). Therefore, it is not surprising that the sniff pathway originates in, or passes through, the RTN.

Active expiration

The pF\textsubscript{L} is thought to be a subsidiary conditional oscillator for expiration that is normally suppressed at rest (Huckstepp et al., 2016). That all pF\textsubscript{L} neurons showed the same discharge pattern, supports the hypothesis that the pF\textsubscript{L} is comprised of a single homogenous group of neurons acting with a single purpose (Huckstepp et al., 2018). Even though the nucleus appears to integrate information from many nuclei -RTN (Zoccal et al., 2018), C1, (Malheiros-Lima et al., 2020), NTS, rostral pedunculo-pontine tegmental (Silva et al., 2019) and the preBötC (Huckstepp et al., 2016) - the pF\textsubscript{L} appears to be a simple on-off switch, rather than forming complex outputs as we describe in the RTN.

Surprisingly, we found that pF\textsubscript{L} neurons were only transiently active during large expiratory efforts, (such as sniffing, sighing, or other forms of brief deep breathing), in contrast to the sustained active expiration previously reported (Huckstepp et al., 2015; Leirão et al., 2018). This may be due to our use of brief periods of graded CO\textsubscript{2} in conscious animals, rather than sustained CO\textsubscript{2} (~1 hr) (Leirão et al., 2018) or use of the vagotomised anaesthetised preparation in prior studies (Huckstepp et al., 2015).
Importantly, in the un-anaesthetised, vagi intact, in situ preparation of young adult rats, pF_L neurons only displayed a small number of action potentials (~10) during the latter portion of expiratory phase 2 (approximately the final 20% of the expiratory period) (Magalhães et al., 2021). In our recordings the expiratory period was ~100ms at the highest level of CO₂, allowing for 20ms of pF_L neuronal discharge during times of active expiration. Thus Ca²⁺ signals in pF_L neurons were likely too small to elicit a recordable change in GCaMP fluorescence, except when expiratory efforts were notably large in terms of both duration and amplitude. Furthermore, in contrast to our expectation that pF_L neurons would be phasically active between inspiratory bursts, pF_L neurons exhibited elevated intracellular calcium beginning before, and spanning, the expiratory period.

Concluding remarks

Endoscopic imaging of the activity of the brainstem neurons involved in the control of breathing provides a new way to investigate the neural circuitry for the chemosensory control of breathing in awake adult mice, and to understand how breathing is coordinated with complex non-ventilatory behaviours. Some components, such as expiratory activity of the pF_L or sniff activity in the RTN, are easy to interpret, whilst others are more complicated. The responses to CO₂ were more heterogeneous in both hSyn+ RTN and hSyn+ and serotonergic Raphe neurons than would be expected from the prior literature. Notably, our dataset of RTN chemosensitive neurons contained a preponderance of the E_A subtype. The E_A neuronal responses are potentially well suited to detect small increases of inspired CO₂ outside the normal range that can occur during normal behavioural conditions. In contrast, the Raphe neurons tended to be active during the entire CO₂ stimulus and conceivably these neurons could take over from RTN neurons during more sustained episodes of hypercapnia. These data provide an intriguing possibility that the chemosensory network is arranged in a hierarchy with each layer conveying a unique range of CO₂.

Materials and methods

Continued on next page

| Key resources table |
|---------------------|
| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
| Gene (Mus musculus) | Promoter of mouse Slc6a4, Gene Accession: NM_010484 | Genecopoiea | MPRM41232-PG02 |
| Strain, strain background (Mus musculus, male) | wild-type | Jackson Laboratories | C57BL/6 J background |
| Cell line (Homo sapiens) | 293AAV Cell Line | CellBioLabs | AAV-100 | HEK293-T |
| Antibody | (Goat polyclonal) anti-cholineacetyl transferase antibody | Millipore | RRID:AB_262156 (1:100 dilution) |
| Antibody | (Rabbit polyclonal) anti-Tryptophan hydroxylase 2 antibody | Millipore Sigma | RRID:AB_10806898 (1:500 dilution) |
| Antibody | (Rabbit polyclonal) anti- NMB (CENTER) antibody | Sigma-Aldrich | RRID:AB_2619620 (1:1000 dilution) |
| Antibody | (donkey polyclonal) anti-rabbit IgG (H+L) Alexa Fluor 680 | Jackson Immunoresearch | RRID:AB_2340627 (1:250 dilution) |
| Antibody | (donkey polyclonal) anti-rabbit IgG (H+L) Alexa Fluor 594 | Jackson Immunoresearch | RRID:AB_2340621 (1:250 dilution) |
| Antibody | (donkey polyclonal) anti-goat IgG (H+L) Alexa Fluor 568 | Abcam | RRID:AB_2636995 (1:250 dilution) |

Continued on next page
| Reagent type (species or resource) | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|---------------------|-------------|------------------------|
| Antibody                         | (donkey polyclonal) anti-goat IgG (H+L) Alexa Fluor 680 | Jackson Immunoresearch | RRID:AB_2340432 | (1:250 dilution) |
| Recombinant DNA reagent          | AAV-9: pGP-AAV-syn-GCaMP6s-WPRE.4.641 | Addgene, Watertown, MA, USA | BS1-NOSAAV9 | Dilution 1:10 |
| Recombinant DNA reagent          | AAV-9: pGP-AAV-syn-GCaMP6f-WPRE.24.693 | Addgene, Watertown, MA, USA | BS3-NXFAAV9 | Dilution 1:10 |
| Recombinant DNA reagent          | pAAVSyn.GCaMP6s.WPRE.SV40 | Addgene, Watertown, MA, USA | Plasmid#100843 |
| Recombinant DNA reagent          | AAV packaging plasmid pAAV-DJ, AAV-DJ Helper Free Expression System | Cellbiolabs | VPK-410-DJ |
| Recombinant DNA reagent          | AAV packaging plasmid pHHelper, AAV-DJ Helper Free Expression System | Cellbiolabs | VPK-410-DJ |
| Sequence-based reagent           | VC327-4 6 s HindIII for | This paper | PCR primers | 5'-TTGACTGCTTAAGCTTgccaccatgcactatcatatcatctcg 300 nM |
| Sequence-based reagent           | VC327-4 6 s Afe rev | This paper | PCR primers | 5’-GATCTCTGAGCAGCGCTcactctgcgtctatcttgataaat 300 nM |
| Peptide, recombinant protein     | PrimeSTAR GXL DNA Polymerase | Takara Bio Europe SAS | R050A | 1 U µg⁻¹ |
| Commercial assay, kit            | In-Fusion HD Cloning Kit | Takara Bio Europe SAS | 639642 |
| Chemical compound, drug          | OptiPrep | PROGEN Biotechnik GmbH | 1114542 | Iodixanol |
| Commercial assay, kit            | 2 x qPCRBIO SyGreen Mix Hi-ROX | PCR Biosystems | PB20.12 |
| Chemical compound, drug          | kainic acid | Fisher Scientific | 15467999 | 8 mg kg⁻¹ IP |
| Chemical compound, drug          | atropine | Westward Pharmaceutical Co. | 0641-6006-10 | 120 µg kg⁻¹ |
| Chemical compound, drug          | meloxicam | Norbrook Inc. | 2 mg kg⁻¹ |
| Chemical compound, drug          | buprenorphine | Reckitt Benckiser | 100 µg kg⁻¹ |
| Chemical compound, drug          | PEI MAX Hydrochloride Transfection Grade Linear 40,000 mwco | Polysciences Europe GmbH | 24765-1 | 1 µg µL⁻¹ |

Continued on next page
Experiments were performed in accordance with the European Commission Directive 2010/63/EU (European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes) and the United Kingdom Home Office (Scientific Procedures) Act (1986) with project approval from the University of Warwick’s AWERB.

**Cell lines**

To produce AAV particles, we obtained a proprietary cell line, HEK293AAV cells, directly from CellBioLabs for this study. The certification from CellBioLabs stated the cells’ identity and that they were free of microbial contamination. These cells were only used to produce the AAV particles and were not used by themselves to generate any of the data in this study.

**AAV-DJ: pAAV-SERT-GCaMP6s vector particle production and purification**

The CMV promoter region was excised from pAAV-GFP (Cellbiolabs) by restriction digest with MluI and CiaI, blunting with Klenow Fragment (ThermoScientific) and re-ligation with T4 DNA ligase (ThermoScientific) to generate pAAV-GFP without promoter. A SERT promoter clone was purchased from Genecopiae (Product ID: MPRM41232-PG02, Symbol: Slc6a4, Species: Mouse, Target Gene Accession: NM_010484, Alias: S-HTT, AI323329, Htt, SertA). The promoter region was cut out with EcoRI and HndIII and introduced by T4 DNA ligation into the previously modified pAAV-GFP to generate pAAV-SERT. Subsequently, a PCR performed using PrimeStar GLX Polymerase (Takara Clontech) with pAAV.Syn.GCaMP6s.WPRE.SV40 (Addgene Plasmid#100843) as a template (forward primer 5´- TTGA CTGC CTAA GCTT gcca ccat gcat catc atca tcat catg  and reverse primer 5´- GATC TCTC GAGC AGCG CTtc actt cgct gtca tcat ttgt acaa act) to amplify the GCaMP6s fragment. pAAV- SERT was digested with HindIII and AfeI and the PCR product was inserted by InFusion Cloning (Takara Clontech) to generate pAAV- SERT His- GCaMP6s. Accuracy of all cloning steps was verified by PCR, restriction digests and DNA sequencing analysis.

AAV-DJ pseudotyped vectors were generated by co-transfection of HEK293- AAV cells (Cellbiolabs) with the AAV transfer plasmid pAAV-SERT His-GCaMP6s and the AAV packaging plasmid pAAV-DJ and pHelper (both Cellbiolabs). HEK293AAV cells (Cellbiolabs) were cultivated in Dulbecco’s modified Eagle’s medium (DMEM, High Glucose, Glutamax) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 0.1 mM MEM Non-Essential Amino Acids (NEAA), 100 U/ml penicillin and 100 μg/ml streptomycin. Tissue culture reagents were obtained from Life Technologies. Briefly, 1x10^7 HEK293- AAV cells were seeded one day before transfection on 15 cm culture dishes and transfected with 7.5 μg pAAV-DJ, 10 μg pHelper and 6.5 μg pAAV plasmid per plate complexed with Max-polyethylenimine (PEI, Polysciences) at a PEI:DNA ratio (w/w) of 3:1. After 72 hr cells were harvested and resuspended in 5 ml lysis buffer (50 mM Tris base, 150 mM NaCl, 5 mM MgCl2, pH 8.5). After three freeze-thaw cycles, benzonase (Merk; final concentration 50 U/ml) was added and the lysates were incubated for 1 hr at 37 °C. Cell debris was pelleted and vector containing lysates were purified using iodixanol step gradients. Finally, iodixanol was removed by ultrafiltration using Amicon Ultra Cartridges (50 mwco) and three washes with DPBS.

The genomic titers of DNase-resistant recombinant AAV particles were determined after alkaline treatment of virus particles and subsequent neutralization by qPCR using the qPCRBIO SY Green Mix Hi-Rox (Nippon Genetics Europe GmbH) and an ABI PRISM 7900HT cycler (Applied Biosystems).
Vectors were quantified using primers specific for the GCaMP6s sequence (5'- CACA GAAG CAGA GCTGAG and 5'- acctgaggaggtcagac). Real-time PCR was performed in a total volume of 10 µl with 0.3 µM for each primer. The corresponding pAAV transfer plasmid was used as a copy number standard. A standard curve for quantification was generated by serial dilutions of the respective plasmid DNA. The cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 35 cycles of 95 °C for 15 s and 60 °C for 60 s. Calculations were done using the SDS 2.4 software (Applied Biosystems).

Virus handling

Raphe and RTN neurons - AAV-9: pGP-AAV-syn-GCaMP6s-WPRE.4.641 at a titre of 1x10^{13} GC·ml^{-1} (Addgene, Watertown, MA, USA); Raphe: AAV-DJ: pAAV-SERT-GCaMP6s at a titre of 1.8x10^{13}GC·ml^{-1} (University hospital Hamburg-Eppendorf, Hamburg, Germany). pFl - AAV-9: pGP-AAV-syn-GCaMP6f-WPRE.24.693 at a titre of 1x10^{13} GC·ml^{-1} (Addgene, Watertown, MA, USA).

Viruses were aliquoted and stored at –80 °C. On the day of injection, viruses were removed and held at 4 °C, loaded into graduated glass pipettes (Drummond Scientific Company, Broomall, PA, USA), and placed into an electrode holder for pressure injection. The AAV-syn-GCaMP6s and AAV-syn-GCaMP6f vectors use the synapsin promoter, and therefore transduced neurons showing higher tropism for the AAV 2/9 subtype, and to a much lesser extent neurons that show low tropism for AAV 2/9 within the injection site, e.g. facial motoneurons, they do not transduce non-neuronal cells. Vector AAV SERT-GCaMP6s was specific for serotonergic neurons.

Viral transfection of RTN, Raphe and pFl neurons

Adult male C57BL/6 mice (20–30 g) were anaesthetized with isofluorane (4%; Piramal Healthcare Ltd, Mumbai, India) in pure oxygen (4 L·min^{-1}). Adequate anaesthesia was maintained with 0.5–2% Isoflurane injection of atropine (120 µg·kg^{-1}; Westward Pharmaceutical Co., Eatontown, NJ, USA) and meloxicam (2 mg·kg^{-1}; Norbrook Inc, Lenexa, KS, USA). Mice were placed in a prone position into a digital stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA) on a heating pad (TCAT 2-LV: Physitemp, Clifton, NJ, USA) and body temperature was maintained at a minimum of 33°C via a thermocouple. The head was levelled at bregma, and 2 mm caudal to bregma, and graduated glass pipettes containing the virus were placed stereotaxically into either the RTN, rostral medullary raphe or pFL (Figures 2A, 5A and 7A). The RTN was defined as the area ventral to the caudal half of the facial nucleus, bound medi ally and laterally by the edges of the facial nucleus (coordinates with a 9° injection arm angle: −1.0 mm lateral and −5.6 mm caudal from Bregma, and −5.5 mm ventral from the surface of the cerebellum; Figure 2A). The Raphe was defined as the medial, TPH containing regions level with the caudal face of the facial nucleus: the Raphe Magnus (RMg) was directly above the pyramidal tracts (Py), and the Raphe Pallidus (RPa) was bound laterally by the Py (coordinates with a 9° injection arm angle: 0 mm lateral and −5.8 mm caudal from Bregma, and −5.2 mm ventral from the surface of the cerebellum; Figure 5A). The pFl was defined as the neurons bound laterally by the spinothalmic tract and medially by the lateral edge of the facial motor nucleus (coordinates with a 9° injection arm angle: −1.8 mm lateral and −5.55 mm caudal from Bregma, and −4.7 mm ventral from the surface of the cerebellum; Figure 7A). The virus solution was pressure injected (<300 nL) unilaterally. Pipettes were left in place for 3–5 min to prevent back flow of the virus solution up the pipette track. Postoperatively, mice received intraperitoneal (IP) injections of buprenorphine (100 µg·kg^{-1}; Reckitt Benckiser, Slough, UK). Mice were allowed 2 weeks for recovery and viral expression, with food and water ad libitum.

GRIN lens implantation

Mice expressing GCaMP6 were anesthetized with isofluorane, given pre-surgical drugs, placed into a stereotax, and the head was levelled as described above. To widen the lens path whilst producing the least amount of deformation of tissue, a graduated approach was taken; firstly a glass pipette was inserted down the GRIN lens path to a depth 200 µm above where the lens would terminated and left in place for 3 min; this procedure was then repeated with a blunted hypodermic needle. The GRIN lens (600 µm diameter, 7.3 mm length; Inscopix, Palo Alto, CA, USA) was then slowly inserted at a rate of 100 µm·min^{-1} to a depth ~1300 µm above the target site, then lowered at a rate of 50 µm·min^{-1} to a depth ~300 µm above the RTN, Raphe or pFl (coordinates with a 9° injection arm angle: RTN – 1.1 mm
lateral and –5.75 mm caudal from Bregma, and –5.3 mm ventral from the surface of the cerebellum; Raphe – 0 mm lateral and –5.95 mm caudal from Bregma, and –5.1 mm ventral from the surface of the cerebellum; pFt – 1.7 mm lateral and –5.7 mm caudal from Bregma, and –4.6 mm ventral from the surface of the cerebellum). The lens was then secured in place with SuperBond (Prestige Dental, Bradford, UK). Postoperatively, mice received buprenorphine, and were allowed 2 weeks for recovery, with food and water ad libitum.

**Baseplate installation**

Mice expressing GCaMP6 and implanted with GRIN lens were anesthetized with isofluorane, given pre-surgical drugs, and placed into a stereotax as described above. To hold the miniaturized microscope during recordings, a baseplate was positioned over the lens and adjusted until the cells under the GRIN lens were in focus. The baseplate was then secured with superbond, and coated in black dental cement (Vertex Dental, Soesterberg, the Netherlands) to stop interference of the recording from ambient light. Mice were allowed 1 week for recovery, with food and water ad libitum.

**Ca²⁺ imaging in freely moving mice**

All mice were trained with dummy camera and habituated to plethysmography chamber at least twice before imaging. The miniature microscope with integrated 475 nm LED (Inscopix, Palo Alto, CA, USA) was secured to the baseplate. GCaMP6 fluorescence was visualised through the GRIN lens, using nVista 2 HD acquisition software (Inscopix, Palo Alto, CA, USA). Calcium fluorescence was optimised for each experiment so that the histogram range was ~150–600, with average recording parameters set at 10–20 frames/sec with the LED power set to 10–20 mW of light and a digital gain of 1.0–4.0. A TTL pulse was used to synchronize the calcium signalling to the plethysmography trace. All images were processed using Inscopix data processing software (Inscopix, Palo Alto, CA, USA). GCaMP6 movies were run through: preprocessing algorithm (with temporal downsampling), crop, spatial filter algorithm (0.005–0.5 Hz), motion correction and cell identification through manual regions of interest (ROIs) operation to generate the identified cell sets. Cell sets were imported into Spike2 software for processing.

**Plethysmography**

Mice were placed into a custom-made 0.5 L plethysmography chamber, with an airflow rate of 1 l·min⁻¹. The plethysmography chamber was heated to 31 °C (thermoneutral for C57/BL6 mice). CO₂ concentrations were sampled via a Hitech Intruments (Luton, UK) GIR250 Dual Sensor Gas analyzer or ML206 gas analyzer (ADinstruments, Sydney, Australia) connected to the inflow immediately before entering the chamber. The analyser had a delay of ~15–20 sec to read-out the digital output of gas mixture. Pressure transducer signals and CO₂ measurements were amplified and filtered using the NeuroLog system (Digitimer, Welwyn Garden City, UK) connected to a 1401 interface and acquired on a computer using Spike2 software (Cambridge Electronic Design, Cambridge, UK). Video data was recorded with Spike2 software and was synchronised with the breathing trace. Airflow measurements were used to calculate: tidal volume (Vₜ: signal trough at the end of expiration subtracted from the peak signal during inspiration, converted to mL following calibration and normalized to body weight), and respiratory frequency (fR: breaths per minute). Minute ventilation (Vₑ) was calculated as Vₜ x fR.

**Hypercapnia in freely behaving mice**

Instrumented mice were allowed ~30 mins to acclimate to the plethysmograph. The LED was activated through a TTL pulse synchronised with the Spike2 recording and 1 or 3 min of baseline recordings were taken (gas mixture: 0% CO₂ 21% O₂ 79% N₂). The mice were then exposed to 3 min epochs of hypercapnic gas mixture at different concentrations of CO₂: RTN and Raphe transduced mice were exposed to 3% followed by 6% CO₂, and pFt transduced mice were exposed to 6% followed by 9% CO₂. All gas mixtures contained 21% O₂ balanced N₂. Following exposure to the hypercapnic gas mixtures, CO₂ levels were reduced back to 0% and calcium signals were recorded for a further 3–4 minutes recovery period.
Hypercapnia and seizure induction in urethane anaesthetised mice

Instrumented mice were anaesthetised with an IP injection of 1.2–1.5 g·kg⁻¹ urethane (Sigma-Aldrich, St Louis, MO, USA) and placed into the plethysmograph. For recording responses of RTN neurons to hypercapnia, the LED was activated and 1 or 3 minute of baseline recording was taken. The mice were then exposed to 2 or 3 minute epochs of hypercapnic gas mixture 3%, 6% and 9% CO₂ (in 21% O₂ balanced N₂) sequentially, as experiments into the chemosensitivity of RTN neurons in mice often use 6% CO₂ in freely behaving experiments but 9% CO₂ in anaesthetised animals. Following exposure to the hypercapnic gas mixtures, CO₂ levels were reduced back to 0% and calcium signals were recorded for a further 3 minute recovery period.

For neurons recorded in the RTN, after completion of hypercapnic responses and a period of rest, baseline Ca²⁺ activity was recorded for 5 min. The mice were then injected with a dose of kainic acid (8 mg·kg⁻¹ IP) sufficient to induce an electrographic seizures in the absence of any movement from behavioural seizures. Following injection of kainic acid, calcium activity was recorded every alternate 5 min to avoid the fluorophore bleaching and concurrently record the calcium activity for a long enough to evidence the effect of induction of seizure on the activity of RTN neurons (at least for 90 min post kainic acid injection).

Preparation of fixed brain slices

Mice were humanely killed by pentobarbital overdose (>100 mg·kg⁻¹) and transcardially perfused with paraformaldehyde solution (4% PFA; Sigma-Aldrich, St Louis, MO, USA). The head was removed and postfixed in PFA (4 °C) for 3 days to preserve the lens tract. The brains were removed and postfixed in PFA (4 °C) overnight. Brainstems were serially sectioned at 50–70 μm.

Immunohistochemistry

Free-floating sections were incubated for 1 hr in a blocking solution (PBS containing 0.1% Triton X-100 and 5% BSA). The tissue was then incubated overnight at room temperature in primary antibodies: goat anti-choline acetyl transferase [ChAT; 1:100; Millipore, Burlington, MA, USA] alone for co-labelling with GCaMP6, or rabbit anti-tryptophan hydroxylase [TPH; 1:500; Sigma-Aldrich, St Louis, MO, USA] alone for co-labelling with GCaMP6.

Slices were washed in PBS (6 × 5 min) and then incubated in a blocking solution to which secondary antibodies were added; donkey anti-rabbit Alexa Fluor 594 (1:250; Jackson Laboratory, Bar Harbor, ME, USA) for co-labelling with GCaMP6, or donkey anti-goat Alexa Fluor 594 (1:250; Jackson Laboratory, Bar Harbor, ME, USA; RTN) for co-labelling with GCaMP6 in RTN tissue or donkey anti-goat Alexa Fluor 568 (1:250; Jackson Laboratory, Bar Harbor, ME, USA) for co-labelling with GCaMP6 in pFL tissue. The tissue was then incubated 2–4 hr at room temperature. Tissue was washed in PBS (6 × 5 min). Slices were mounted on polylysine adhesion slides and were coverslipped with Vectashield Antifade Mounting Medium with DAPI (Vectorlabs, Burlingame, CA, United States).

Heat-induced epitope retrieval and immunohistochemistry for NMB

Slices were mounted onto poly-lysine coated microscope slides and allowed to dry and adhere. Mounted brain sections were added to the pre-heated sodium citrate antigen retrieval buffer (10 mM sodium citrate, 0.05% Tween 20, pH 9.0) and incubated for 15 min. The mounted brain sections were then removed and washed in PBS.

The tissue was then incubated for 1 hr in a blocking solution. Without washing, the tissue was then incubated overnight at room temperature in blocking solution containing primary antibodies: goat anti-ChAT [same as above] alone for co-labelling with GCaMP6, or in conjunction with rabbit anti-Neuromedin-B [NMB; 1:100; SAB1301059; Sigma-Aldrich, St Louis, MO, USA] antibody for co-labelling of ChAT and NMB.

The tissue was then washed in PBS (6 × 5 min) and incubated for 1 hr in a blocking solution. Without washing, the tissue was then incubated 2 hr at room temperature in blocking solution containing secondary antibodies: donkey anti-rabbit Alexa Fluor 568 (1:250; Invitrogen, Waltham, MA, United States) for co-labelling with GCaMP6, or donkey anti-rabbit Alexa Fluor 488 (1:250; Invitrogen, Waltham, MA, United States) and donkey anti-goat Alexa Fluor 594 (1:250; Invitrogen, Waltham, MA, United States) for co-labelling of ChAT and NMB. Tissue was washed in PBS (6 × 5 min).
Slides were examined using a Zeiss 880 confocal microscope with ZEN acquisition software (Zeiss, Oberkochen, Germany).

**Antibody specificity**

The antibodies that we used have been independently validated by others in the field: anti-ChAT ([Dempsey et al., 2015; Saunders et al., 2015; Zhang et al., 2020](https://doi.org/10.7554/eLife.70671.sa1)); anti-TPH ([Pitzer et al., 2015; Quina et al., 2020; Zhong et al., 2017](https://doi.org/10.7554/eLife.70671.sa2)); and anti-NMB ([Li et al., 2016](https://doi.org/10.7554/eLife.70671.sa3)). The staining patterns we report are highly consistent with these prior studies indicating that these antibodies are specifically recognising their targets with the tissue we have examined. Additionally, we compared NMB immunostaining with in situ hybridization patterns for NMB documented in the Allen Brain Atlas and found it to be very similar (Figure 2—figure supplement 1).

**Acknowledgements**

This work was supported by an MRC Discovery Award MC_PC_15070. ND is a Royal Society Wolfson Research Merit Award Holder. We thank Tamara Sotelo-Hitschfeld for help in the early stages of developing the method.

**Additional information**

**Funding**

| Funder                  | Grant reference number | Author          |
|-------------------------|------------------------|-----------------|
| Medical Research Council| MC_PC_15070            | Nicholas Dale   |
| Royal Society           |                        | Nicholas Dale   |

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

**Author contributions**

Amol Bhandare, Data curation, Formal analysis, Investigation, Methodology, Writing - review and editing; Joseph van de Wiel, Investigation, Methodology; Reno Roberts, Investigation; Ingke Braren, Resources, Methodology; Robert Huckstepp, Formal analysis, Supervision, Investigation, Methodology, Writing - review and editing; Nicholas Dale, Conceptualization, Supervision, Funding acquisition, Writing - original draft, Project administration, Writing - review and editing

**Author ORCIDs**

Amol Bhandare [http://orcid.org/0000-0002-5214-9355](http://orcid.org/0000-0002-5214-9355)

Robert Huckstepp [http://orcid.org/0000-0003-4410-3397](http://orcid.org/0000-0003-4410-3397)

Nicholas Dale [http://orcid.org/0000-0003-2196-2949](http://orcid.org/0000-0003-2196-2949)

**Ethics**

Experiments were performed in accordance with the European Commission Directive 2010/63/EU (European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes) and the United Kingdom Home Office (Scientific Procedures) Act (1986) with project approval from the University of Warwick’s AWERB.

**Decision letter and Author response**

Decision letter [https://doi.org/10.7554/eLife.70671.sa1](https://doi.org/10.7554/eLife.70671.sa1)

Author response [https://doi.org/10.7554/eLife.70671.sa2](https://doi.org/10.7554/eLife.70671.sa2)

**Additional files**

Supplementary files

- Supplementary file 1. A user-modifiable worksheet that uses simple mathematical functions to explore the predicted correlation between V<sub>T</sub> and F/F<sub>0</sub> for E<sub>A</sub> neurons under two assumptions. (i) no
adapting component in the Vt response to hypercapnia; and (ii) an adapting Vt component in the response to hypercapnia is present. This should be compared to the data shown in Figure 2J.

- Transparent reporting form

Data availability

All data generated or analysed during this study are included in the MS and supporting files. Source data files have been provided for Figure 3D, Figure 7I–K, Figure 1—figure supplement 7, and Figure 4—figure supplement 2.

References

Andrews CG, Pagliardini S. 2015. Expiratory activation of abdominal muscle is associated with improved respiratory stability and an increase in minute ventilation in REM epochs of adult rats. Journal of Applied Physiology 119:968–974. DOI: https://doi.org/10.1152/japphysiol.00420.2015, PMID: 26338455

Ashlad S, Feldman JL. 2020. Emergent elements of inspiratory rhythmogenesis: network synchronization and synchrony propagation. Neuron 106:482–497. DOI: https://doi.org/10.1016/j.neuron.2020.02.005, PMID: 32130872

Bradley SR, Pieribone VA, Wang W, Severson CA, Jacobs RA, Richerson GB. 2002. Chemosensitive serotonergic neurons are closely associated with large medullary arteries. Nature Neuroscience 5:401–402. DOI: https://doi.org/10.1038/nn848, PMID: 11967547

Bravo K, Eugenin JL, Llona I. 2016. Perinatal fluoxetine exposure impairs the CO2 chemoreflex. Implications for sudden infant death syndrome. American Journal of Respiratory Cell and Molecular Biology 55:368–376. DOI: https://doi.org/10.1165/rcmb.2015-0384OC, PMID: 27018763

Brust RD, Corcoran AE, Richerson GB, Nattie E, Dymecki SM. 2014. Functional and developmental identification of a molecular subtype of brain serotoninergic neuron specialized to regulate breathing dynamics. Cell Reports 9:2152–2165. DOI: https://doi.org/10.1016/j.celrep.2014.11.027, PMID: 25497093

Chen T-W, Wardill TJ, Sun Y, Pulver SR, Renninger SL, Baohan A, Schreiter ER, Kerr RA, Orger MB, Jayaraman V, Looger LL, Svoboda K, Kim DS. 2013. Ultrasensitive fluorescent proteins for imaging neuronal activity. Nature 499:295–300. DOI: https://doi.org/10.1038/nature12354, PMID: 23868258

Cleary CM, Milla BM, Kuo FS, James S, Flynn WF, Robson P, Mulkey DK. 2021. Somatostatin-expressing parafacial neurons are co2/H+ sensitive and regulate baseline breathing. eLife 10:e06317. DOI: https://doi.org/10.7554/eLife.60317

Dana H, Sun Y, Mohar B, Hulse BK, Kerlin AM, Hassman JP, Tsegaye G, Tsang A, Wong A, Patel R, Macklin JJ, Chen Y, Konnerth A, Jayaraman V, Looger LL, Schreiter ER, Svoboda K, Kim DS. 2019. High-Performance calcium sensors for imaging activity in neuronal populations and microcompartments. Nature Methods 16:649–657. DOI: https://doi.org/10.1038/s41999-019-0435-6, PMID: 31209382

Dempsey B, Turner AJ, Le S, Sun QJ, Bou Farah L, Allen AM, Goodchild AK, McMullan S. 2015. Recording, labeling, and transfection of single neurons in deep brain structures. Physiological Reports 3:e12246. DOI: https://doi.org/10.14814/phy2.12246, PMID: 25602013

Deschênes M, Kurnikova A, Elbaz M, Kleinfeld D. 2016. Circuits in the ventral medulla that phase-lock motorneurons for coordinated sniffing and whisking. Neural Plasticity 2016:7493048. DOI: https://doi.org/10.1155/2016/7493048, PMID: 27293905

Douglas NJ, White DP, Pickett CK, Weil JV, Zwillich CW. 1982a. Respiration during sleep in normal man. Thorax 37:840–844. DOI: https://doi.org/10.1136/thx.37.11.840, PMID: 7164002

Douglas NJ, White DP, Weil JV, Pickett CK, Zwillich CW. 1982b. Hypercapnic ventilatory response in sleeping adults. American Review of Respiratory Disease 126:758–762. DOI: https://doi.org/10.1164/arrd.1982.126.5.758

Ezure K, Manabe M. 1988. Decrementing expiratory neurons of the Bötzinger complex. II. direct inhibitory synaptic linkage with ventral respiratory group neurons. Experimental Brain Research 72:159–166. DOI: https://doi.org/10.1007/BF00248511, PMID: 3169183

Gourine AV, Kasymov V, Marina N, Tang F, Figueiredo MF, Lane S, Teschemacher AG, Spyer KM, Deisseroth K, Gourine AV, Llaudet E, Dale N, Spyer KM. 2005. Atp is a mediator of chemosensory transduction in the central nervous system. Nature 436:108–111. DOI: https://doi.org/10.1038/nature03690, PMID: 16001070

Gourine AV, Kasymov V, Marina N, Tang F, Figueiredo MF, Lane S, Teschemacher AG, Spyer KM, Deisseroth K, Kasparov S. 2010. Astrocytes control breathing through pH-dependent release of ATP. Science 329:571–575. DOI: https://doi.org/10.1126/science.1190721, PMID: 20647426

Guyenet PG, Stornetta RL, Bayliss DA. 2010. Central respiratory chemoreception. The Journal of Comparative Neurology 518:3883–3906. DOI: https://doi.org/10.1002/cne.22435, PMID: 20737591

Hennessy ML, Corcoran AE, Brust RD, Chang Y, Nattie EE, Dymecki SM. 2017. Activity of tachykinin1-expressing pet1 raphe neurons modulates the respiratory chemoreflex. The Journal of Neuroscience 37:2016. DOI: https://doi.org/10.1523/JNEUROSCI.2316-16.2016

Hérent C, Diem S, Fortin G, Bouvier J. 2021. Upregulation of breathing rate during running exercise by central locomotor circuits. bioRxiv. DOI: https://doi.org/10.1101/2021.07.28.453983

Huang L, Ledochowitsch P, Knoblisch U, Lecoq J, Murphy GJ, Reid RC, de Vries SE, Koch C, Zeng H, Buice MA, Waters J, Li L. 2021. Relationship between simultaneously recorded spiking activity and fluorescence signal in gcamp6 transgenic mice. eLife 10:e51675. DOI: https://doi.org/10.7554/eLife.51675, PMID: 33683198
acetylcholineergic neurons in the ventral medullary raphe. Front. Cell. Neurosci. 8:209. DOI: https://doi.org/10.3389/fncel.2014.00209, PMID: 25222428

Huckstepp RTR, Cardoza KP, Henderson LE, Feldman JL. 2015. Role of parafacial nuclei in control of breathing in adult rats. The Journal of Neuroscience 35:1052–1067. DOI: https://doi.org/10.1523/JNEUROSCI.2953-14.2015, PMID: 26209622

Huckstepp RT, Henderson LE, Cardoza KP, Feldman JL. 2016. Interactions between respiratory oscillators in adult rats. eLife 5:e14203. DOI: https://doi.org/10.7554/eLife.14203, PMID: 27300271

Huckstepp RTR, Cardoza KP, Henderson LE, Feldman JL. 2018. Distinct parafacial regions in control of breathing in adult rats. PLOS ONE 13:e0201485. DOI: https://doi.org/10.1371/journal.pone.0201485, PMID: 30096151

Iceman KE, Corcoran AE, Taylor BE, Harris MB. 2014. CO2-inhibited neurons in the medullary raphe are GABAergic. Respiratory Physiology & Neurobiology 203:28–34. DOI: https://doi.org/10.1016/j.resp.2014.07.016, PMID: 25087734

Jennings JH, Jung RL, Resendez SL, Stamatikis AM, Taylor JG, Huang J, Veleta K, Kantak PA, Aita M, Shilling-Scrivo K, Ramakrishnan C, Deisseroth K, Otte S, Stuber GD. 2015. Visualizing hypothalamic network dynamics for appetitive and consummatory behaviors. Cell 160:516–527. DOI: https://doi.org/10.1016/j.cell.2014.12.026, PMID: 25635459

Johnsen SL, Iceman KE, Iceman CR, Taylor BE, Harris MB. 2015. Isoflurane causes concentration-dependent inhibition of medullary raphe 5-HT neurons in situ. Autonomic Neuroscience 193:51–56. DOI: https://doi.org/10.1016/j.autneu.2015.07.002, PMID: 26213357

Kurnikova A, Deschénes M, Kleinfeld D. 2018. Functional brain stem circuits for control of nose motion. Journal of Neurophysiology 121:205–217. DOI: https://doi.org/10.1152/jn.00608.2018, PMID: 30461370

Lazarenko RM, Milner TA, Depuy SD, Stornetta RL, West GH, Kievits JA, Bayliss DA, Guyenet PG. 2009. Acid sensitivity and ultrastructure of the retrotrapezoid nucleus in phox2b-EGFP transgenic mice. The Journal of Comparative Neurology 517:69–86. DOI: https://doi.org/10.1002/cne.22136, PMID: 19711410

Leirão IP, Silva CA Jr, Gargaglioni LH, da Silva GSF. 2018. Hypercapnia-induced active expiration increases in sleep and enhances ventilation in unanesthetized rats. The Journal of Physiology 596:3271–3283. DOI: https://doi.org/10.1113/JP274726, PMID: 28776683

Li P, Janczewski WA, Yackle K, Kam K, Pagliardini S, Krasnow MA, Feldman JL. 2016. The peptidergic control circuit for sighing. Nature 530:293–297. DOI: https://doi.org/10.1038/nature16946, PMID: 26855425

Magalhães KS, da Silva MP, Mecawi AS, Paton JFR, Machado BH, Moreira DJA. 2021. Intrinsic and synaptic mechanisms controlling the expiratory activity of excitatory lateral parafacial neurones of rats. The Journal of Physiology 599:4925–4948. DOI: https://doi.org/10.1113/jp.2021.552572, PMID: 34510468

Mainland J, Sobel N. 2005. The sniff is part of the olfactory percept. Chemical Senses 31:181–196. DOI: https://doi.org/10.1093/chemse/bj012, PMID: 16339268

Malheiros-Lima MR, Silva JN, Souza FC, Takakura AC, Moreira TS. 2020. C1 neurons are part of the circuitry that recruits active expiration in response to the activation of peripheral chemoreceptors. eLife 9:e52572. DOI: https://doi.org/10.7554/eLife.52572, PMID: 31971507

Moore JD, Deschénes M, Furuta T, Huber D, Smear MC, Demers M, Kleinfeld D. 2013. Hierarchy of orofacial rhythms revealed through whisking and breathing. Nature 497:205–210. DOI: https://doi.org/10.1038/497205a, PMID: 23624373

Moriyama R, Nakamura N, Yamamoto Y. 2019. Serotonergic projections to the ventral respiratory column from retrotrapezoid nucleus in rats. Neuroscience Research 143:20–30. DOI: https://doi.org/10.1016/j.neures.2018.05.004, PMID: 29803764

Mulkey DK, Stornetta RL, Weston MC, Simmons JR, Parker A, Bayliss DA, Guyenet PG. 2004. Respiratory control by ventral surface chemoreceptor neurons in rats. Nature Neuroscience 7:1360–1369. DOI: https://doi.org/10.1038/nn1357, PMID: 15558061

Nattie EE, Fung ML, Li A, St John WM. 1993. Responses of respiratory modulated and tonic units in the retrotrapezoid nucleus to CO2. Respiratory Physiology 94:35–50. DOI: https://doi.org/10.1016/0034-5687(93)90055-f, PMID: 8272580

Nattie EE, Li A. 1994. Retrotrapezoid nucleus lesions decrease phrenic activity and CO2 sensitivity in rats. Respiratory Physiology 97:63–77. DOI: https://doi.org/10.1016/0034-5687(94)90012-4, PMID: 8091025

Ott MM, Nuding SC, Segers LS, Lindsey BG, Morris KF. 2011. Ventrolateral medullary functional connectivity and the respiratory and central chemoreceptor-evoked modulation of retrotrapezoid-parafacial neurons. Journal of Neurophysiology 105:2960–2975. DOI: https://doi.org/10.1152/jn.00262.2010, PMID: 21389310

Pagliardini S, Janczewski WA, Tan W, Dickson CT, Deisseroth K, Feldman JL. 2011. Active expiration induced by excitation of ventral medulla in adult anesthetized rats. The Journal of Neuroscience 31:2895–2905. DOI: https://doi.org/10.1523/JNEUROSCI.5338-10.2011, PMID: 21419411

Pitzer M, Lueras J, Warden A, Weber S, McBride J. 2015. Viral vector mediated expression of mutant huntingtin in the dorsal raphe produces disease-related neuropathology but not depressive-like behaviors in wildtype mice. Brain Research 1608:177–190. DOI: https://doi.org/10.1016/j.brainres.2015.02.027, PMID: 25732261
Quina LA, Walker A, Morton G, Han V, Turner EE. 2020. GAD2 expression defines a class of excitatory lateral habenula neurons in mice that project to the raphe and pontine tegmentum. Eneuro 7:Eneuro. DOI: https://doi.org/10.1523/ENEURO.0527-19.2020

Ramanantsoa N, Hirsch MR, Thoby-Brisson M, Dubreuil V, Bouvier J, Ruffault PL, Matrot B, Fortin G, Brunet JF, Gallego J, Goridis C. 2011. Breathing without CO (2) chemosensitivity in conditional PHOX2B mutants. The Journal of Neuroscience 31:12880–12888. DOI: https://doi.org/10.1523/JNEUROSCI.1721-11.2011, PMID: 21900568

Ray RS, Corcoran AE, Brust RD, Kim JC, Richerson GB, Nattie E, Dymecki SM. 2011. Impaired respiratory and body temperature control upon acute serotonergic neuron inhibition. Science 333:637–642. DOI: https://doi.org/10.1126/science.1205295, PMID: 21798952

Resende SL, Jennings JH, Ung RL, Nanmoddi VMK, Zhou ZC, Otis JM, Nomura H, McHenry JA, Kosyk O, Stuber GD. 2016. Visualization of cortical, subcortical and deep brain neural circuit dynamics during naturalistic mammalian behavior with head-mounted microscopes and chronically implanted lenses. Nature Protocols 11:556–597. DOI: https://doi.org/10.1038/nprot.2016.021, PMID: 26914316

Richerson GB. 1995. Response to CO2 of neurons in the rostral ventral medulla in vitro. Journal of Neurophysiology 73:933–944. DOI: https://doi.org/10.1152/jn.1995.73.3.933, PMID: 7608778

Rosin DL, Chang DA, Guyenet PG. 2006. Afferent and efferent connections of the rat retrotrapezoid nucleus. The Journal of Comparative Neurology 499:64–89. DOI: https://doi.org/10.1002/cne.21105, PMID: 16958085

Saunders A, Granger AJ, Sabatini BL. 2015. Corelease of acetylcholine and GABA from cholinergic forebrain neurons. eLife 4:e06412. DOI: https://doi.org/10.7554/eLife.06412

Shi Y, Stornetta RL, Stornetta DS, Onengut-Gumuscu S, Farber EA, Turner SD, Guyenet PG, Bayliss DA. 2017. Neuromedin B expression defines the mouse retrotrapezoid nucleus. eLife 4:e06412. DOI: https://doi.org/10.7554/eLife.06412

Silva JN, Oliveira LM, Souza FC, Moreira TS, Takakura AC. 2019. Distinct pathways to the parafacial respiratory group to trigger active expiration in adult rats. American Journal of Physiology-Lung Cellular and Molecular Physiology 317:L402–L413. DOI: https://doi.org/10.1152/ajplung.00467.2018, PMID: 31242022

Silva JN, Oliveira LM, Souza FC, Moreira TS, Takakura AC. 2020. GABAergic neurons of the medullary raphe regulate active expiration during hypercapnia. Journal of Neurophysiology 123:1933–1943. DOI: https://doi.org/10.1152/jn.00698.2019, PMID: 32267190

Sobel N, Prabhakaran V, Desmond JE, Glover GH, Goode RL, Sullivan EV, Gabrieli JDE. 1998a. Sniffing and smelling: separate subsystems in the human olfactory cortex. Nature 392:282–286. DOI: https://doi.org/10.1038/32654, PMID: 9521322

Sobel N, Prabhakaran V, Hartley CA, Desmond JE, Zhao Z, Glover GH, Gabrieli JDE, Sullivan EV. 1998b. Odorant-induced and sniff-induced activation in the cerebellum of the human. The Journal of Neuroscience 18:8990–9001. DOI: https://doi.org/10.1523/JNEUROSCI.18-21-08990.1998

Souza GMPR, Kanbar R, Stornetta DS, Abbott SBG, Stornetta RL, Guyenet PG. 2018. Breathing regulation and blood gas homeostasis after near complete lesions of the retrotrapezoid nucleus in adult rats. The Journal of Physiology 596:2521–2545. DOI: https://doi.org/10.1113/jphysiol.2017.157886, PMID: 29667182

Stornetta RL, Moreira TS, Takakura AC, Kang BJ, Chang DA, West GH, Brunet JF, Mulkey DK, Bayliss DA, Guyenet PG. 2006. Expression of PHOX2B by brainstem neurons involved in chemosensory integration in the adult rat. The Journal of Neuroscience 26:10305–10314. DOI: https://doi.org/10.1523/JNEUROSCI.2917-06.2006, PMID: 17021186

Stornetta RL, Spirovski D, Moreira TS, Takakura AC, West GH, Gwilt JM, Pilowsky PM, Guyenet PG. 2009. Galanin is a selective marker of the retrotrapezoid nucleus in rats. The Journal of Comparative Neurology 512:373–383. DOI: https://doi.org/10.1002/cne.21897, PMID: 1906184

Vallejo AB, Johansson RS. 1984. Properties of cutaneous mechanoreceptors in the human hand related to touch sensation. Human Neurobiology 3:3–14 PMID: 6330008.

Vanderwolf CH. 2001. The hippocampus as an olfacto-motor mechanism: were the classical anatomists right after all? Behavioural Brain Research 127:25–47. DOI: https://doi.org/10.1016/s0166-4328(01)00354-0, PMID: 11718883

Veasey SC, Fornal CA, Metzler CW, Jacobs BL. 1995. Response of serotonergic caudal raphe neurons in relation to specific motor activities in freely moving cats. The Journal of Neuroscience 15:5346–5359. DOI: https://doi.org/10.1523/JNEUROSCI.15-07-05346.1995, PMID: 7623157

Wang S, Shi Y, Shu S, Guyenet PG, Bayliss DA. 2013. Phox2b-expressing retrotrapezoid neurons are intrinsically responsive to H+ and CO2. The Journal of Neuroscience 33:7756–7761. DOI: https://doi.org/10.1523/JNEUROSCI.5550-12.2013, PMID: 23637167

Widdicombe JG. 1954. Receptors in the trachea and bronchi of the cat. The Journal of Physiology 123:71–104. DOI: https://doi.org/10.1113/jphysiol.1954.sp005034, PMID: 13131247

Yang CF, Kim EJ, Callaway EM, Feldman JL. 2019. Monosynaptic Projections to Excitatory and Inhibitory PreBötzingler Complex Neurons. bioRxiv. DOI: https://doi.org/10.1101/694711

Zhang JY, Dou YN, Yuan L, Li Q, Zhu YJ, Wang M, Sun YG. 2020. Different neuronal populations mediate inflammatory pain analgesia by exogenous and endogenous opioids. eLife 9:e55289. DOI: https://doi.org/10.7554/eLife.55289, PMID: 32519950

Zhong W, Li Y, Feng Q, Luo M. 2017. Learning and stress shape the reward response patterns of serotonin neurons. The Journal of Neuroscience 37:8863–8875. DOI: https://doi.org/10.1523/JNEUROSCI.1181-17.2017, PMID: 28821671
Ziv Y, Ghosh KK. 2015. Miniature microscopes for large-scale imaging of neuronal activity in freely behaving rodents. *Current Opinion in Neurobiology* **32**:141–147. DOI: https://doi.org/10.1016/j.conb.2015.04.001, PMID: 25951292

Zoccal DB, Silva JN, Barnett WH, Lemes EV, Falquetto B, Colombari E, Molkov YI, Moreira TS, Takakura AC. 2018. Interaction between the retrotrapezoid nucleus and the parafacial respiratory group to regulate active expiration and sympathetic activity in rats. *American Journal of Physiology. Lung Cellular and Molecular Physiology* **315**:L891–L909. DOI: https://doi.org/10.1152/ajplung.00011.2018, PMID: 30188747