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Contributions of carotid bodies, retrotrapezoid nucleus neurons and preBötzinger complex astrocytes to the CO_2-sensitive drive for breathing

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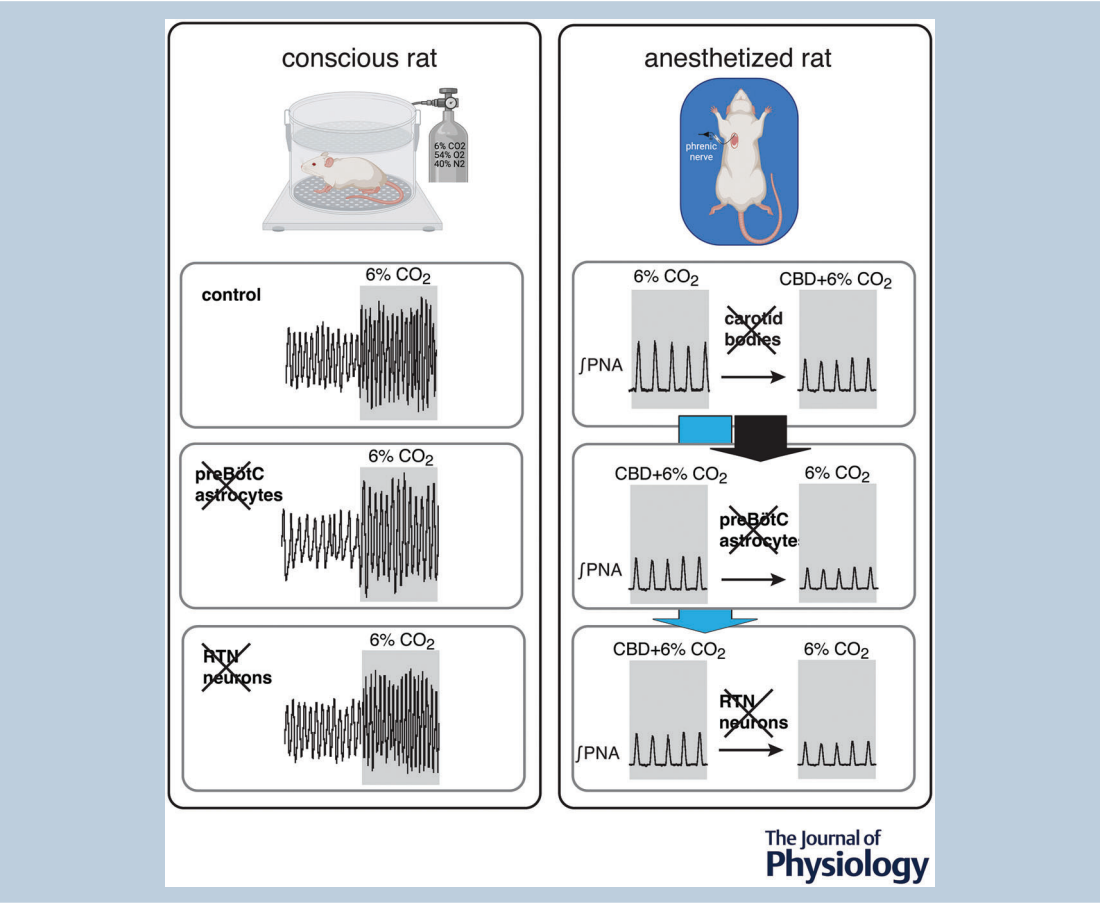

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Abstract Current models of respiratory CO_2 chemosensitivity are centred around the function of a specific population of neurons residing in the medullary retrotrapezoid nucleus (RTN). However, there is significant evidence suggesting that chemosensitive neurons exist in other brainstem areas, including the rhythm-generating region of the medulla oblongata – the preBötzinger complex (preBötC). There is also evidence that astrocytes, non-neuronal brain cells, contribute to central CO_2 chemosensitivity. In this study, we reevaluated the relative contributions of the RTN neurons, the preBötC astrocytes, and the carotid body chemoreceptors in mediating the respiratory responses to CO_2 in experimental animals (adult laboratory rats). To block astroglial signalling via exocytotic release of transmitters, preBötC astrocytes were targeted to express the tetanus toxin light chain (TeLC). Bilateral expression of TeLC in preBötC astrocytes was associated with ∼20% and ∼30% reduction of the respiratory response to CO_2 in conscious and anaesthetized animals, respectively. Carotid body denervation reduced the CO_2 respiratory response by ∼25%. Bilateral inhibition of RTN neurons transduced to express Gi-coupled designer receptors exclusively activated by designer drug ($DREADD_{Gi}$) by application of clozapine-*N*-oxide reduced the CO_2 response by ∼20% and ∼40% in conscious and anaesthetized rats, respectively. Combined blockade of astroglial signalling in the preBötC, inhibition of RTN neurons and carotid body denervation reduced the CO_2-induced respiratory response by ∼70%. These data further support the hypothesis that the CO_2-sensitive drive to breathe requires inputs from the peripheral chemoreceptors and several central chemoreceptor sites. At the preBötC level, astrocytes modulate the activity of the respiratory network in response to CO_2, either by relaying chemosensory information (i.e. they act as CO_2 sensors) or by enhancing the preBötC network excitability to chemosensory inputs.

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Abstract figure legend This study reevaluated the roles played by the carotid bodies, neurons of the retrotrapezoid nucleus and astrocytes of the preBötC in mediating the CO_2-sensitive drive to breathe. The data obtained show that the disruption of preBötC astroglial signalling, blockade of inputs from the peripheral chemoreceptors or inhibition of RTN neurons similarly reduce the respiratory response to hypercapnia. These data provide further support for the hypothesis that the CO_2-sensitive drive to breathe is mediated by the inputs from the peripheral chemoreceptors and several central chemoreceptor sites.

Key points

- This study reevaluated the roles played by the carotid bodies, neurons of the retrotrapezoid nucleus (RTN) and astrocytes of the preBötC in mediating the CO_2-sensitive drive to breathe.
- The data obtained show that disruption of preBötC astroglial signalling, blockade of inputs from the peripheral chemoreceptors or inhibition of RTN neurons similarly reduce the respiratory response to hypercapnia.
- These data provide further support for the hypothesis that the CO_2-sensitive drive to breathe is mediated by the inputs from the peripheral chemoreceptors and several central chemoreceptor sites.

Shahriar SheikhBahaei received his Ph.D. from the University College London (UCL)-National Institutes of Health (NIH) joint doctoral program in neuroscience. His doctoral research evaluated the role of astrocytes in the modulation of respiratory motor circuits. Currently, he is an Independent Research Scholar at the Intramural Research Program (IRP) of the National Institute of Neurological Disorders and Stroke (NINDS), USA. As the head of the Neuron-Glia Signalling and Circuits Unit, he leads a research group that studies the development of motor circuits and investigates motor control disorders, including speech disorders.

Introduction

Respiratory CO_2 chemosensitivity is fundamentally important for the control of breathing. Under resting conditions, the central respiratory network that generates the rhythm of breathing requires a certain level of CO_2 to be active (Nielsen & Smith, 1952). Indeed, under most physiological conditions, lung ventilation is directly proportional to the amount of CO_2 produced during metabolism (Phillipson et al., 1981). Specialized chemoreceptors located in the carotid bodies (and aortic bodies in some species) and in the central nervous system are responsible for respiratory CO_2/pH chemosensitivity (Heymans & Bouckaert, 1930; Heymans & Neil, 1958; O'Regan & Majcherczyk, 1982). There is evidence that approximately one-third of the ventilatory response to CO_2 is mediated by the peripheral chemoreceptors, whereas the rest of the response is attributed to the actions of CO_2/H^+ on central respiratory chemoreceptors residing in the brainstem (Bisgard et al., 1976; Dahan et al., 2008; Forster et al., 2008; Guyenet, 2014; Heeringa et al., 1979; Mitchell et al., 1963, 2006; Pan et al., 1998; Richerson, 1995; Takakura et al., 2011).

Current models of central respiratory chemosensitivity (the mechanism that controls breathing in accord with brain parenchymal P_{CO2}/pH) propose that specialized neurons in the retrotrapezoid nucleus (RTN) and medullary raphé are primarily responsible for central CO_2 sensing, which is detected via proxy of extracellular pH changes (Guyenet et al., 2019; Kumar et al., 2015; Teran et al., 2014). An alternative hypothesis of 'distributed central chemosensitivity' suggests that central respiratory sensitivity to CO_2 is mediated by inputs from several brainstem sites (including the RTN and raphé), with each site providing a fraction of the tonic drive to breathe in eucapnia and a proportion of the overall response to hypercapnia (Forster et al., 1997; Nattie, 1999, 2000). The RTN may act not only as a CO_2 sensor but also as an upstream integration centre for CO_2-sensitive inputs to the preBötzinger complex (preBötC), the key site of inspiratory rhythm generation (Del Negro et al., 2018; Smith et al., 1991). The preBötC is also capable of increasing lung ventilation in response to acidification (Feldman et al., 2003; Koizumi et al., 2010; Krause et al., 2009; Solomon, 2003a; Solomon et al., 2000). Experimental studies in rats involving localized acidification or lesions of the preBötC neurons suggested that the preBötC contributes ∼20%–25% of the ventilatory response to CO_2 (Grey et al., 2001; Nattie, 2000). Impairment of the signalling function of preBötC astrocytes by interfering with the exocytotic release of astroglial signalling molecules results in a similar 20%−25% reduction of the CO_2-induced ventilatory response (Sheikhbahaei, Turovsky et al., 2018).

This study was designed to define the role of the preBötC mechanism(s), focusing specifically on preBötC astrocytes, in central respiratory CO_2 chemosensitivity. Using two experimental models – conscious and anaesthetized adult rats – we compared the respiratory responses to the increases in inspired CO_2 before and after a series of interventions (applied separately or in combination) that included (i) the blockade of exocytotic release mechanisms in preBötC astrocytes by virally driven expression of tetanus toxin light chain (TeLC) (Angelova et al., 2015; Rajani et al., 2018; Sheikhbahaei, Turovsky et al., 2018), (ii) inhibition of RTN neurons transduced to express G_i-coupled designer receptors exclusively activated by designer drug ($DREADD_{Gi}$) by application of clozapine-*N*-oxide (Basting et al., 2015; Huckstepp et al., 2015; Korsak et al., 2018; Marina et al., 2010), and (iii) surgical denervation of the carotid bodies bilaterally (Sheikhbahaei et al., 2017; Smith et al., 2015). We show that in the anaesthetized state, disruption of preBötC astroglial signalling reduced the respiratory response to hypercapnia by ∼30%; this effect was similar to that produced by the removal of inputs from the peripheral chemoreceptors or inhibition of RTN neurons. In conscious animals under hyperoxic conditions (to minimize the activity of the carotid bodies), blockade of preBötC astroglial signalling reduced the CO_2-induced increases in the respiratory frequency by ∼20%, whereas inhibition of RTN neurons reduced the increases in tidal volume by the same degree, ∼20%. These data support the hypothesis of distributed central CO_2 chemosensitivity. At the preBötC level, astrocytes contribute to the CO_2-sensitive drive to breathe, either by relaying chemosensory information (i.e. acting as CO_2 sensors) or by enhancing preBötC network excitability to chemosensory inputs.

Materials and methods

Ethical approval

All the experiments were performed in adult Sprague–Dawley rats 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), the UK Home Office (Scientific Procedures) Act (1986) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the respective Institutional Animal Care and Use Committees. The rats were housed in a temperature-controlled facility (22–25°C) with a 12 h light–dark cycle (lights switched on at 7:00 AM). Water and laboratory rodent food chow were provided *ad libitum*. This research was conducted in accordance with the animal ethics checklist outlined in the Journal's instructions for authors.

Viral vectors

Adenoviral vector for the expression of TeLC in astrocytes. To block the mechanisms of vesicular exocytosis, pre-BötC astrocytes were targeted by an adenoviral vector (AVV) designed to drive the expression of TeLC under the transcriptional control of the glial fibrillary acidic protein (GFAP) promoter (AVV-sGFAP-EGFP-TeLC; 2.1×10^8 unit ml^{-1}) (Angelova et al., 2015; Dutta et al., 2018; Rajani et al., 2018; Sheikhbahaei, Turovsky et al., 2018). TeLC blocks exocytosis in astrocytes by proteolytic degradation of SNARE proteins (Coco et al., 2003). The design of the vector and validation of TeLC efficacy in blocking vesicular release in brainstem astrocytes have been described in detail previously (Angelova et al., 2015). AVV-driven expression of a calcium-translocating channel rhodopsin variant (CatCh) fused with EGFP (AVV-sGFAP-CatCh-EGFP; 2.1×10^9 unit ml^{-1}) was used as a control (Angelova et al., 2015; Sheikhbahaei, Turovsky et al., 2018).

Adeno-associated viral vector for the expression of DREADD$_{Gi}$ in RTN neurons. RTN neurons were transduced to express DREADD$_{Gi}$ under the transcriptional control of human synapsin (hSyn) promoter (AAV2-hSyn-DREADD$_{Gi}$-mCitrine; 5.6×10^{12} unit ml^{-1}; UNC Vector Core, Chapel Hill, NC, USA). Acute inhibition of RTN was then achieved by application of the DREADD ligand CNO (Armbruster et al., 2007; Gomez et al., 2017; Rogan & Roth, 2011). hSyn promoter was used; therefore, all neurons at the site of the injection were expected to express the transgene. The efficacy and specificity of this approach in inhibiting RTN neuronal activity have been demonstrated previously (Huckstepp et al., 2015; Korsak et al., 2018). Adeno-associated viral vector (AAV)-induced expression of ChR2-EGFP (4×10^{12} unit ml^{-1}, UNC Vector Core) in RTN neurons was used in the control group of animals.

Viral gene transfer

Young male rats (60−80 g) were anaesthetized with an intramuscular injection of ketamine (60 mg kg^{-1}) and medetomidine (250 μg kg^{-1}). Supplemental anaesthesia was administered, and adequate anaesthesia was ensured throughout the surgery through the absence of a withdrawal response to a paw pinch. Animals were placed in a stereotaxic frame (tooth bar −18 mm below the interaural line), and the dorsal surface of the brainstem was exposed by separating the neck muscles at the mid-line followed by an incision of the atlanto-occipital membrane. The RTN was targeted bilaterally with two microinjections per side (100–120 nl each, ∼50 nl min^{-1}) of DREADD$_{Gi}$-mCitrine or ChR2-EGFP-expressing AAVs using the following coordinates from *calamus scriptorius*: 1.7 mm lateral from the mid-line and 3.7 mm ventral, with the micropipette holder arm positioned at 24° and 16° for each injection (Korsak et al., 2018; Marina et al., 2010). Anaesthesia was reversed with atipamezole (1 mg kg^{-1}, I.M.). No complications were observed after the surgery, and the animals gained weight normally. Carprofen (5 mg kg^{-1} per day subcutaneously) was given for postsurgical analgesia for 3 days, and the animals were allowed to recover for 7 days. After a recovery period of 3−4 weeks (the animals reaching the body weights of ∼200−250 g), AVV constructs designed to induce the expression of EGFP-TeLC or CatCh-EGFP in astrocytes were microinjected bilaterally into the preBötC following the same anaesthetic and surgical protocol. The microinjections were centred at the preBötC region and delivered using the following coordinates: 2 mm lateral, 0.9 mm rostral and 2.7 mm ventral from the *calamus scriptorius* (Rajani et al., 2018; Sheikhbahaei, Turovsky et al., 2018). Experiments were conducted 5−7 days after the injections (Fig. 1).

Experimental groups

(1) The control group of rats was transduced to express CatCh-EGFP in preBötC astrocytes and ChR2-EGFP in RTN neurons (control, $n = 5$).
(2) The first experimental group of rats was transduced to express CatCh-EGFP in preBötC astrocytes and DREADD$_{Gi}$ in RTN neurons (RTN-DREADD$_{GI}$, $n = 5$). CNO was then administered to inhibit RTN neurons transduced to express DREADD$_{Gi}$ to determine RTN contribution to the hypercapnic ventilatory response. CO_2 challenges were applied before and after denervation of the carotid bodies. The hypercapnic ventilatory response remaining after the inhibition of RTN and denervation of the carotid body was interpreted to represent the response mediated by other central CO_2-chemosensitive sites, including the preBötC.
(3) The second experimental group of rats transduced to express TeLC in preBötC astrocytes and DREADD$_{Gi}$ in RTN neurons (preBötC-TeLC/RTN-DREADD$_{Gi}$, $n = 5$). A contribution of preBötC astrocytes to the hypercapnic ventilatory response was suggested by the data reported previously (Beltrán-Castillo et al., 2017; Sheikhbahaei, Turovsky et al., 2018). In this group of animals CO_2 challenges were applied before and after denervation of the carotid bodies and subsequently after RTN inhibition following CNO application. The hypercapnic ventilatory response remaining in these animals after RTN inhibition and carotid body denervation was interpreted to represent the response mediated by central CO_2-chemosensitive sites, other than RTN and the preBötC; that is, the difference in the ventilatory

response to CO_2 between the experimental animals in groups 2 and 3 reflected the contribution of the preBötC astrocytes.

Experimental design

All experiments and data analyses were conducted by investigators who were blinded to the identity of the experimental groups.

Experiment 1. The aim was to determine the relative contribution of RTN neurons and preBötC astrocytes to the hypercapnic ventilatory response in conscious rats.

Respiratory activity in conscious rats was assessed using whole-body plethysmography (Enhorning et al., 1998; Mortola & Frappell, 1998; Sheikhbahaei et al., 2017). Animals were placed in a Plexiglass recording chamber (~1 l, temperature: 22–24°C) that was flushed continuously (1.2 l min^{-1}) with humidified gas mixture containing >60% O_2 (balanced with N_2) to reduce the drive from the peripheral chemoreceptors (Chavez-Valdez et al., 2012; Gonzalez et al., 1994). To reduce the potentially confounding influence of circadian variation in physiological responses, all experiments were conducted at the same time of the day (between 11:00 AM and 3:00 PM). Concentrations of O_2 and CO_2 in the chamber were monitored online using a fast-response O_2/CO_2 analyser (ML206, AD Instruments, Colorado Springs, CO, USA). To determine the control response to CO_2, all animals received an injection of saline (0.3–0.4 ml, I.P.). The animals were allowed to acclimatize to the chamber environment for at least 1 h before baseline respiratory activity was measured. Hypercapnia was then induced by stepwise increases in CO_2 concentration in the respiratory gas mixture from <0.3% to 3% and 6% (since 3% CO_2 did not trigger a significant effect on ventilation in these experiments, we only report the responses evoked by 6% CO_2). Each CO_2 concentration was maintained for 5 min. Animals were left to recover for 2 days, after

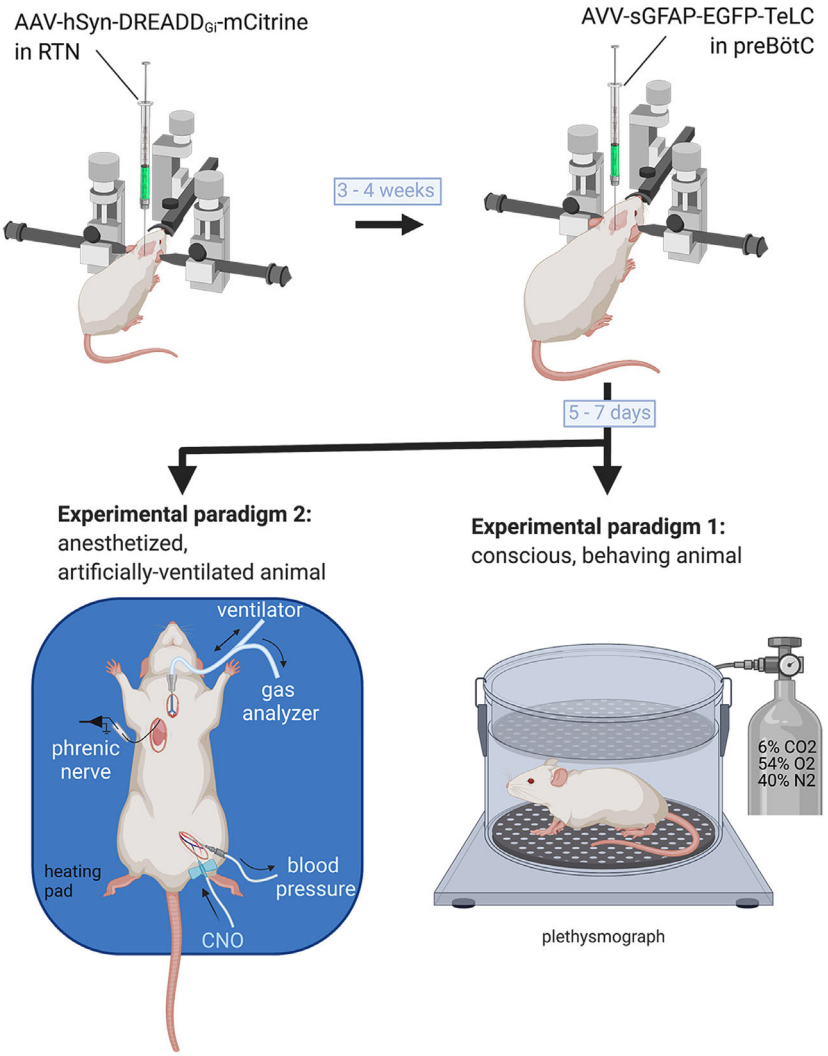

Figure 1. Experimental paradigm
After stereotaxic injections of AAV2-hSyn-DREADD$_{Gi}$-mCitrine (or control vector in RTN) and AVV-sGFAP-EGFP-TeLC (or control vector in preBötC), hypercapnic ventilatory responses in each rat were recorded in conscious and anaesthetized states.

Table 1. Blood gas analysis at baseline, after CBD, and after CBD +CNO

Blood gas analysis			
Baseline	Blood pH	P_{CO2} (mmHg)	P_{O2} (mmHg)
Control (n = 5)	7.39 ± 0.03	34.6 ± 1.1	98.2 ± 9.0
RTN-DREADD$_{Gi}$ (n = 5)	7.38 ± 0.04	35.0 ± 4.5	103.0 ± 6.2
preBötC-TeLC/RTN-DREADD$_{Gi}$ (n = 5)	7.38 ± 0.03	36.6 ± 1.6	99.8 ± 3.5
After CBD			
Control	7.32 ± 0.05	40.5 ± 4.2	163.8 ± 6.4
RTN-DREADD$_{Gi}$	7.37 ± 0.04	38.7 ± 4.6	150.4 ± 32.5
preBötC-TeLC/RTN-DREADD$_{Gi}$	7.36 ± 0.04	40.1 ± 3.7	144.4 ± 20.1
After CBD + CNO			
Control	7.33 ± 0.06	38.0 ± 5.4	143.8 ± 6.8
RTN-DREADD$_{Gi}$	7.37 ± 0.05	35.2 ± 4.1	148.1 ± 26.3
preBötC-TeLC/RTN-DREADD$_{Gi}$	7.33 ± 0.02	36.4 ± 5.2	147.0 ± 23.0

Abbreviations: CBD, carotid body denervation; CNO; DREADD$_{Gi}$, Gi-coupled designer receptors exclusively activated by designer drug; preBötC, preBötzinger complex; RTN, retrotrapezoid nucleus; TeLC, tetanus toxin light chain.

which the CO_2 challenge was repeated after the systemic administration of CNO (2 mg kg^{-1}, I.P.) to inhibit RTN neurons transduced to express DREADD$_{Gi}$. Treatment of preBötC-TeLC/RTN-DREADD$_{Gi}$ rats with CNO allowed us to assess the combined contribution of RTN neurons and preBötC astrocytes to the hypercapnic ventilatory response. The contribution of preBötC astrocytes was determined by comparing the responses recorded in preBötC-TeLC/RTN-DREADD$_{Gi}$ and RTN-DREADD$_{Gi}$ rats prior to CNO administration (Fig. 1).

Experiment 2. The aim was to determine the relative contribution of the carotid body chemoreceptors, RTN neurons, and preBötC astrocytes to the hypercapnic ventilatory response in anaesthetized rats.

Animals were anaesthetized with urethane (1.3 g kg^{-1}, I.V.) after cannulation of the femoral artery and vein under 3% isoflurane. Adequate anaesthesia was ensured throughout the experiment through continuous monitoring of the arterial blood pressure stability and the absence of a withdrawal response to a paw pinch. Supplemental anaesthesia was administered as needed. Blood gases were monitored (Table 1), the trachea was cannulated, and the animal was mechanically ventilated with a gas mixture containing ∼30% O_2 and ∼70% N_2. End-tidal levels of O_2 and CO_2 were monitored using a fast-response O_2/CO_2 analyser (ML206, AD Instruments). Phrenic nerve activity (PNA) and EMG$_{ABD}$ were recorded as measures of central inspiratory and expiratory activities, respectively (Huckstepp et al., 2015; Marina et al., 2010). The carotid sinus nerves (CSN) were dissected for subsequent bilateral sectioning. Core body temperature was kept at ∼37°C using a servo-controlled heating blanket. P_{O2}, P_{CO2}, and pH of the arterial blood were measured prior to each CO_2 challenge. After surgical preparation, each animal was allowed to stabilize for ∼30 min. To determine the baseline inspiratory and expiratory responses to CO_2, phrenic nerve and EMG$_{ABD}$ activities were recorded for 5 min followed by the application of the first hypercapnic challenge (10%–12% CO_2 in the inspired gas mixture for 5 min; Fig. 2). The contribution of preBötC astrocytes to the hypercapnic ventilatory response was determined by comparing the peaks of CO_2-evoked PNA and EMG$_{ABD}$ responses between the preBötC-TeLC/RTN-DREADD$_{Gi}$ and RTN-DREADD$_{Gi}$ experimental animals prior to the administration of CNO.

After ∼20 min recovery from the first CO_2 challenge (to allow blood gases to return to normal levels), the inputs from the carotid bodies were removed by bilateral sectioning of the CSNs. CO_2-evoked PNA and EMG$_{ABD}$ responses were determined 20 min after carotid body denervation (CBD). The differences in CO_2-evoked PNA and EMG$_{ABD}$ responses before and after CBD revealed the contribution of the peripheral chemoreceptors to the hypercapnic respiratory response in this preparation. Shortly after the blood gases returned to baseline levels (∼20 min), the rats were given CNO (Tocris Bioscience, Minneapolis, USA, 2 mg kg^{-1}, I.V.) followed by a third hypercapnic challenge 20 min later. The contribution of RTN neurons to the hypercapnic ventilatory response was determined by assessing the differences in CO_2-evoked PNA and EMG$_{ABD}$ responses between the control and RTN-DREADD$_{Gi}$ experimental animals after the administration of CNO.

Immunohistochemistry

At the end of the experiments, the animals were killed by an overdose of pentobarbitone sodium (200 mg kg^{-1}, I.P.). The animals were perfused transcardially with 0.9% NaCl solution followed by ice-cold 4% paraformaldehyde (PFA). Brains were removed and postfixed overnight in 4% PFA at 4°C. Brainstems were isolated and cryoprotected in 30% sucrose, and serial coronal sections (50 μm) were cut using a freezing microtome. One series of sections from each animal was incubated overnight at 4°C in the following antibodies: chicken anti-GFP (1:250, Aves Labs, Davis, USA category GFP-1020, RRID: AB_10000240), mouse anti-tyrosine hydroxylase (TH) (1:1000, MilliporeSigma, Rockville, USA, category T8700, RRID: AB_1080430), rabbit anti-GFAP (1:1000, Agilent, Carpinteria, USA, category z-0334, RRID: AB_10013382) and/or goat anti-choline acetyltransferase (ChAT) (1:200, EMD Millipore, category AB144P, RRID: AB_11212843). The sections were subsequently incubated with secondary antibodies conjugated to the fluorescent probes (each 1:250, Life Science Technologies, Leawood, USA) for 1 h at room temperature as described before (Sheikhbahaei, Morris et al., 2018). Slices were mounted, cover slipped and examined using a confocal microscope (LSM 510, Zeiss, Hebron. USA). To determine the spread of viral transduction and the expression of $DREADD_{Gi}$ in the RTN, the signal (mCitrine or EGFP expression) was amplified by anti-GFP immunostaining (Korsak et al., 2018). To estimate the extent of transgene expression, transduced neurons were counted in a single section from each animal containing the largest area of expression, as described in detail previously (Korsak et al., 2018).

Data analysis

Analysis of whole-body plethysmography recordings (experiment 1). The duration (T_{TOT}) and amplitude (tidal volume, V_T) of the respiratory cycles were determined after the animals had habituated to the plethysmography chamber environment for at least 1 h. The average baseline T_{TOT} was calculated for the respiratory cycles recorded during periods of calm wakefulness and/or quiet sleep recorded over a 30-min period and was used to determine the respiratory frequency (number of breaths per minute, f_R) as described (Sheikhbahaei et al., 2017; Sheikhbahaei, Turovsky et al., 2018). V_T (normalized to the body weight) was determined by measuring the amplitude of

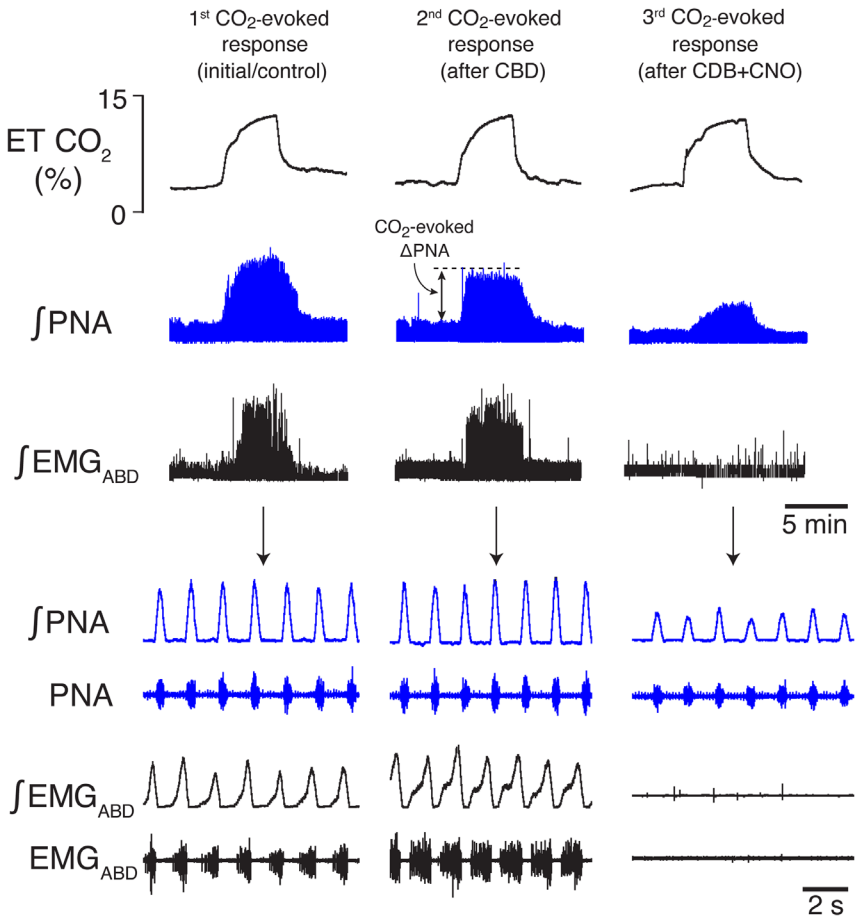

Figure 2. Hypercapnic respiratory responses in anaesthetized and artificially ventilated rats
Time-condensed records illustrating changes in the amplitude of the integrated phrenic nerve activity (∫PNA) and abdominal EMG ($\int EMG_{ABD}$) in response to sequential *hypercapnic* challenges in rats transduced to express $DREADD_{Gi}$ in RTN (retrotrapezoid nucleus) neurons (RTN-$DREADD_{Gi}$). The initial response to the hypercapnic challenge (5 min, 10%–12% CO_2) was induced in rats with intact carotid bodies. The carotid body nerves were then bilaterally sectioned [CBD (carotid body denervation)], and the rat was exposed to the second hypercapnic challenge. Shortly after blood gas levels returned to normal physiological ranges, CNO was applied systemically (2 mg kg^{-1}, I.V.), followed by the third hypercapnic challenge. Animals were left to recover for at least 20 min between each experimental manipulation. Changes in PNA recorded during hypercapnia before (initial) and after each treatment (CBD and application of CNO) were normalized with respect to resting activity (100%) and expressed as the percentage increase in PNA (CO_2-evoked ΔPNA). Expanded recordings of PNA and EMG_{ABD} are shown, illustrating late-expiratory abdominal activity evoked by CO_2 that was blocked by CNO application.

pressure changes in the chamber. Minute ventilation (V_E) was calculated using the following formula: $V_E = f_R \times V_T$.

Analysis of data obtained in anaesthetized preparations (experiment 2). Electrophysiological data were digitized (3 kHz sampling rate) and analysed offline (*Spike2*, Cambridge Electronic Design, Cambridge, UK). Peak PNA responses to CO_2 (Fig. 2) before and after CBD, and then after CNO administration in conditions of CBD, were compared and expressed as percentage changes from the control CO_2 response recorded at baseline (Fig. 2). Peak increases in PNA were also analysed and expressed as percentage increases in the inspiratory burst amplitude relative to the resting activity. Phrenic nerve discharge frequency was not analysed as it was entrained by the ventilator frequency.

Statistical analysis

The data were reported as mean (SD) and compared by paired *t* test, unpaired *t* test or one-way ANOVA using GraphPad Prism 9 (RRID:SCR_002798). Each data point on figures represents data obtained from one animal.

Results

Targeting the RTN neurons to express DREADD$_{Gi}$

The extent of bilateral DREADD$_{Gi}$ or control transgene expression in the RTN was examined in all the experimental animals. To aid the identification of transduced cells, anti-GFP immunostaining was performed (Huckstepp et al., 2015; Korsak et al., 2018). Transduced neurons were identified within the targeted RTN region, extending in the rostrocaudal direction between −10.8 and −12.1 mm from bregma (Fig. 3*A*). Fig 3*B* shows a representative image taken from the centre of the RTN. Transduced cells were counted in a section with maximum expression (Korsak et al., 2018), which revealed 61 (28) transduced neurons per section at this level. No staining was observed in the neighbouring Bötzinger complex (BötC), and no co-localization with TH immunoreactivity was observed (Fig. 3*C*), suggesting that the brainstem catecholaminergic neurons were not transduced or were expressing the transgenes at a very low level.

Targeting preBötC astrocytes to express TeLC

The nucleus ambiguus (NA, immunostained for ChAT) was used as an anatomical landmark to aid in the identification of the preBötC region, which is located ventral to the semi-compact division of the nucleus (NAsc; Fig. 3*D*). The extent of the bilateral TeLC expression in preBötC astrocytes of all the experimental animals was histologically reconstructed from serial coronal sections after immunohistochemical detection of EGFP (Fig. 3*D*). Transduced astrocytes were found rostrocaudally from −12.1 to −13.4 mm relative to bregma, with the peak density of expression observed at the preBötC level (Fig. 3*D*). A representative high-magnification confocal image illustrating TeLC expression in preBötC astrocytes (−12.8 mm relative to bregma) is shown in Fig. 3*E*. TeLC expression driven by this vector was observed exclusively in astrocytes, as reported previously (Angelova et al., 2015; Dutta et al., 2018; Rajani et al., 2018; Sheikhbahaei, Turovsky et al., 2018)

The contribution of RTN neurons and preBötC astrocytes to hypercapnic ventilatory responses in conscious rats

Under resting conditions [normocapnia (<0.3% CO_2)/hyperoxia (>60% O_2; to reduce the drive from the peripheral chemoreceptors)], blockade of vesicular release mechanisms in preBötC astrocytes by virally driven expression of TeLC (preBötC-TeLC/RTN-DREADD$_{Gi}$ group) was associated with lower respiratory frequency f_R (by ∼13%; $p = 0.002$, unpaired *t* test) and reduced minute ventilation V_E (by 19%; $p = 0.032$, unpaired *t* test; Fig. 4*A*), consistent with the previously reported data (Sheikhbahaei, Turovsky et al., 2018).

The ventilatory response to hypercapnia was reduced in rats expressing TeLC in preBötC astrocytes. The peak f_R response to 6% inspired CO_2 was reduced by 18% [168 (18) min^{-1} *vs.* 204 (20) min^{-1} in control animals; $p = 0.021$, unpaired *t* test; Fig. 4*A*], and peak V_E response was reduced by 21% ($p = 0.018$, unpaired *t* test; Fig. 4*A*). TeLC expression in the preBötC had no effect on V_T under any conditions (Fig. 4*A*).

Inhibition of RTN neurons after CNO administration (2 mg kg^{-1}, I.P.) had no effect on resting ventilation (in conditions of 60% inspired oxygen). Peak increases in ventilation in response to 6% CO_2 were significantly reduced when RTN neurons were inhibited (Fig. 4*B*). After CNO administration, CO_2-induced increases in V_T and V_E were smaller in the RTN-DREADD$_{Gi}$ group; V_T was reduced by 18% ($p = 0.006$, unpaired *t* test), and V_E was similarly reduced by 18% ($p = 0.010$, unpaired *t* test). Inhibition of RTN neurons had no effect on the respiratory frequency under any conditions (Fig. 4*B*).

Combined blockade of exocytotic release mechanisms in preBötC astrocytes by virally driven expression of TeLC, and inhibition of RTN neurons expressing DREADD$_{Gi}$ following systemic administration of CNO (in the preBötC-TeLC/RTN-DREADD$_{Gi}$ group), led to a 65% reduction in the ventilatory response to CO_2 ($p < 0.001$, unpaired *t* test; Fig. 4*B*). These data suggest that in conscious animals in conditions when the drive

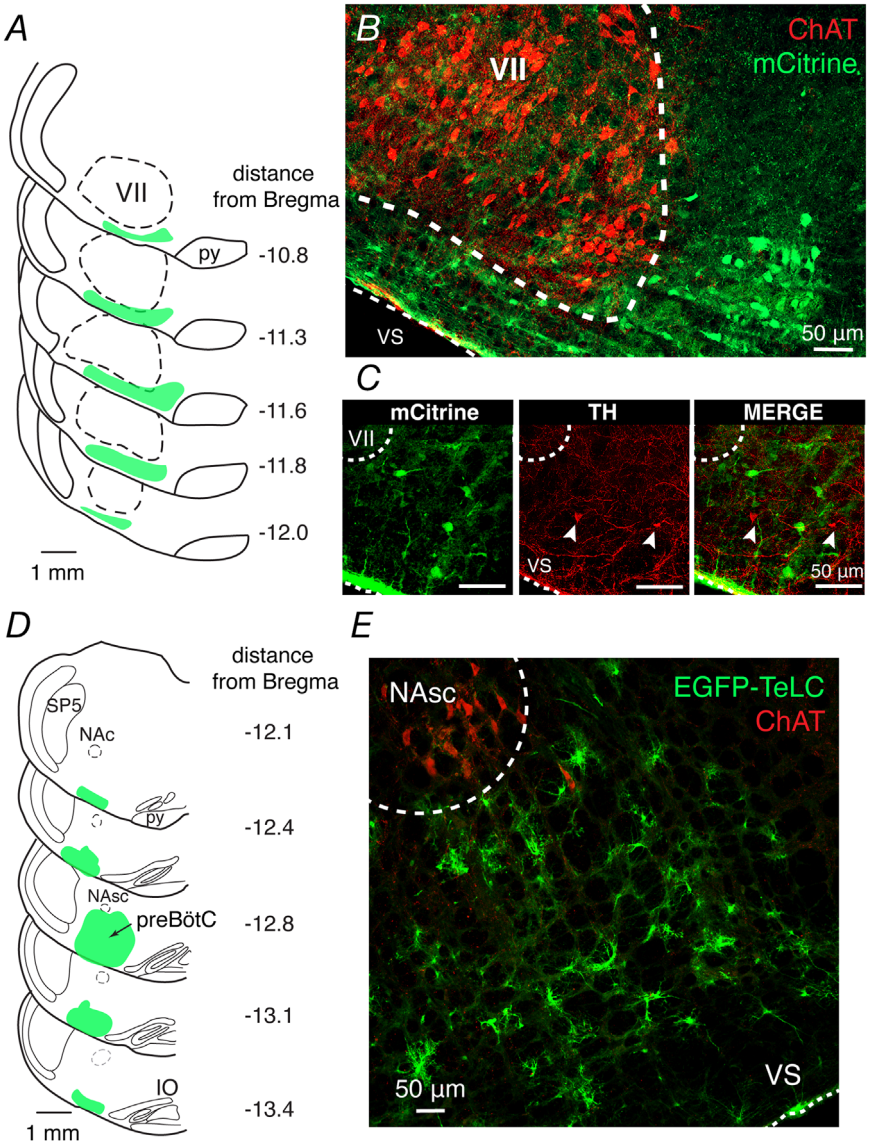

Figure 3. Targeting RTN (retrotrapezoid nucleus) neurons to express DREADD$_{Gi}$ and preBötC astrocytes to express tetanus toxin light chain (TeLC)
A, Schematic illustration of the extent of DREADD$_{Gi}$ expression after GFP immunostaining (within coloured regions shown schematically) in the ventrolateral regions of the medulla oblongata in representative reconstructed serial coronal brainstem sections of adult rats. Adeno-associated viral vectors designed to drive the expression of DREADD$_{Gi}$-mCitrine were used to target RTN neurons bilaterally. The peak density of transduced neurons was within the RTN (extending rostrocaudally from bregma −12.1 and −10.8 mm), with limited expression in the immediately adjacent rostral and caudal areas of the ventrolateral medullary reticular formation, including the lateral parafacial area. *B*, Representative confocal images illustrating GFP immunostaining corresponding to the brainstem section −11.6 mm (from bregma). The expression of DREADD$_{Gi}$-mCitrine in RTN neurons was amplified with anti-GFP immunostaining. *C*, Higher-magnification images showing that neurons transduced to express DREADD$_{Gi}$ (green, left panel) do not express TH (tyrosine hydroxylase, white arrowheads; red, middle and merged images). *D*, Schematic illustration from a representative adult rat brainstem showing the extent of TeLC expression (revealed by GFP immunostaining, green-coloured regions) in the ventrolateral medulla oblongata in astrocytes of the preBötC. Adenoviral vector designed to drive the expression of EGFP-TeLC was used to target preBötC regions bilaterally. *E*, A representative, high-magnification confocal image illustrating GFP immunolabeling (and therefore TeLC expression) at the preBötC region of maximum expression (−12.8 mm relative to bregma). VS, ventral surface. Abbreviations: py, pyramids; NAsc, semi-compact division of the nucleus ambiguous; IO, inferior olive; SP5, spinal trigeminal nucleus; VII, facial nucleus; VS, ventral surface.

from the peripheral chemoreceptors is reduced by hyperoxia, RTN neurons mediate the CO_2-induced increases in tidal volume, whereas preBötC mechanisms contribute to the increases in respiratory frequency.

The contribution of carotid body chemoreceptors, RTN neurons and preBötC astrocytes to hypercapnic respiratory responses in anaesthetized rats

Blockade of exocytotic release mechanisms in preBötC astrocytes or the expression of $DREADD_{Gi}$ in the RTN did not affect the homeostatic control of blood gases (Table 1). The magnitude of the initial (control) response to CO_2 (5 min), expressed as a percentage increase from the baseline PNA amplitude (% ΔPNA), was not significantly different between the groups of experimental animals [controls: increase 114 (54)%; RTN-$DREADD_{Gi}$: increase by 103 (45)%; preBötC-TeLC/RTN-$DREADD_{Gi}$: increase by 71 (21)%; $p = 0.27$, one-way ANOVA (F (2, 12) = 1.434); Figs 5 and 6*A*]. CBD decreased PNA response to CO_2 by ∼28% in the control and RTN-$DREADD_{Gi}$ groups [first response: increase by 109 (47)%; after CBD: increase by 81 (21)%; $p = 0.028$; $n = 10$ per group, paired t test; Figs 2, 5, 6*B*]. Analysis of the pooled data obtained in the control, RTN-$DREADD_{Gi}$ and preBötC-TeLC/RTN-$DREADD_{Gi}$ experimental groups revealed that CBD decreased the hypercapnic respiratory response across all three experimental groups by 25% [first response: increase by 96 (44)%; response after CBD, increase by 71 (23)%, $p = 0.007$, $n = 15$ per group, paired t test; Fig. 6*C*].

To confirm that we could effectively inhibit the RTN neuronal population, we evaluated the effects of CNO administration on CO_2-induced increases in the EMG_{ABD}

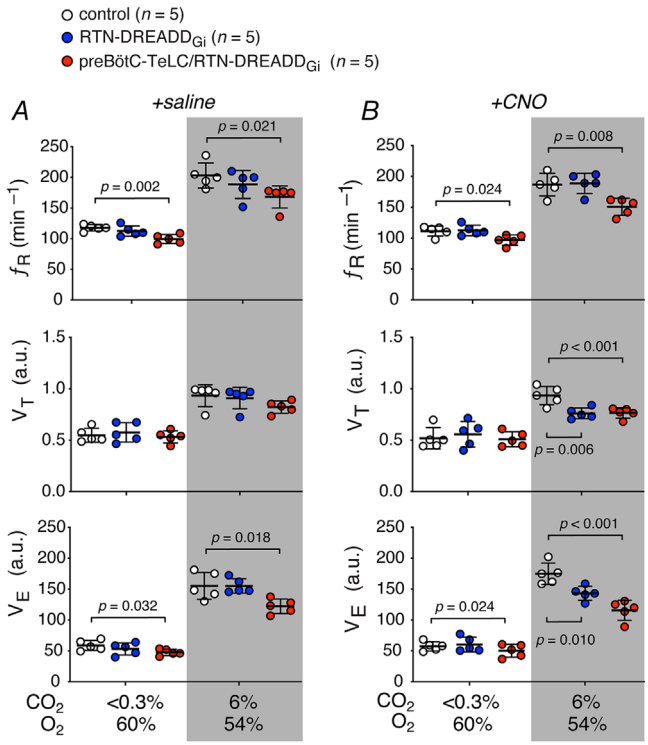

Figure 4. Contributions of RTN (retrotrapezoid nucleus) neurons and preBötC astrocytes to the development of the ventilatory response to CO_2
A, Group data illustrating the effect of $DREADD_{Gi}$ expression in RTN neurons (without CNO) and TeLC (tetanus toxin light chain) expression in preBötC astrocytes on CO_2-induced increases in f_R, V_T and V_E in conscious rats under conditions of peripheral chemoreceptor inhibition by hyperoxia. *B*, Group data illustrating the effect of inhibition of $DREADD_{Gi}$-expressing RTN neurons (administration of CNO, 2 mg kg^{-1}; I.P.) and TeLC expression in preBötC astrocytes on CO_2-induced increases in f_R, V_T and V_E in conscious rats. Each data point represents the measurement obtained in one animal. p-Values – unpaired t test.

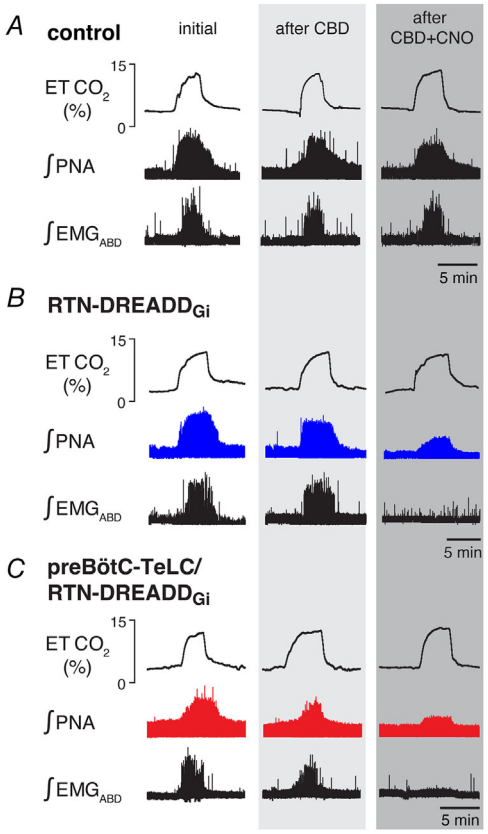

Figure 5. Recruitment of expiratory activity by CO_2 is blocked after acute inhibition of $DREADD_{Gi}$-expressing RTN (retrotrapezoid nucleus) neurons in anaesthetized rats
Time-condensed records illustrating changes in the amplitude of integrated phrenic nerve activity (∫PNA) and abdominal EMG ($\int EMG_{ABD}$) in response to repeated hypercapnic challenges (10%–12% CO_2, 5 min) in control (preBötC-CatCh-EGFP/RTN-ChR2-EGFP) (*A*), RTN-$DREADD_{Gi}$ (*B*) and preBötC-TeLC/RTN-$DREADD_{Gi}$ (*C*) rats. CNO abolished the $\int EMG_{ABD}$ expiratory activity in animals expressing $DREADD_{Gi}$ in the RTN.

activity. CNO administration after CBD effectively abolished recruitment of late-expiratory EMG_{ABD} activity evoked by CO_2 in both RTN-$DREADD_{Gi}$ and preBötC-TeLC/RTN-$DREADD_{Gi}$ experimental animals (Figs 2 and 5), consistent with the previously reported data (Marina et al., 2010).

To determine the contribution of RTN neurons to the inspiratory component of the hypercapnic response, we analysed the evoked responses using two complementary methods. First, we compared the magnitude of CO_2-induced PNA increases in RTN-$DREADD_{Gi}$ and preBötC-TeLC/RTN-$DREADD_{Gi}$ to that recorded in the control group. A between-subjects analysis showed that CO_2-evoked PNA responses were reduced after CNO administration by ∼47% in RTN-$DREADD_{Gi}$ rats ($p < 0.001$, unpaired t test; Fig. 7*A*) and by ∼70% in preBötC-TeLC/RTN-$DREADD_{Gi}$ animals ($p < 0.001$, unpaired t test; Fig. 7*A*).

Second, we performed a within-subject analysis of the CO_2-induced PNA responses obtained before and after CBD and CNO administration where the responses were expressed as percentages of the first (control) and second hypercapnic responses (after CBD). In two groups of experimental animals transduced to express $DREADD_{Gi}$ in the RTN (RTN-$DREADD_{Gi}$ and preBötC-TeLC/RTN-$DREADD_{Gi}$), CNO administration led to a significant reduction in the hypercapnic respiratory response (reduction by ∼54% when compared to the initial response; $p = 0.001$, paired t test; Fig. 7*B*; and by ∼37% when compared to the CO_2 response recorded after CBD; $p = 0.002$, paired t test; Fig. 7*B*). These data suggest that in anaesthetized rats in the absence of the

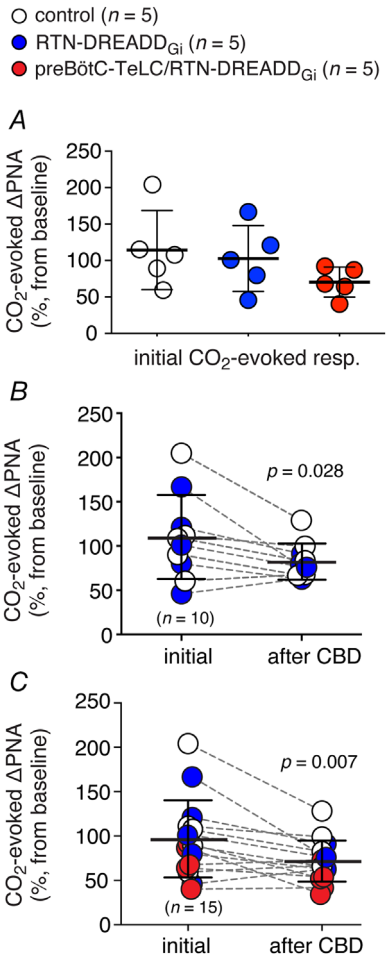

Figure 6. Contribution of the carotid body chemoreceptors to the hypercapnic respiratory response in anaesthetized rats
A, Initial CO_2-evoked (10%–12% CO_2, 5 min) changes in the amplitude of phrenic nerve activity relative to baseline activity. *B* and *C*, Effects of carotid body denervation (CBD) on CO_2-evoked increases in the amplitude of phrenic nerve activity in control and RTN-$DREADD_{Gi}$ animals (*B*) and combined in all animals from three experimental groups (*C*). Each data point represents the measurement obtained in one animal. p – unpaired t test (*A*) and paired t test (*B* and *C*).

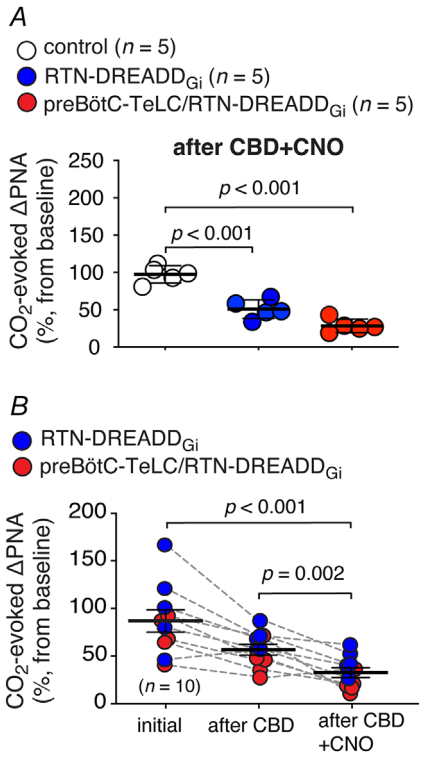

Figure 7. Contribution of the RTN (retrotrapezoid nucleus) neurons to the hypercapnic respiratory response in anaesthetized rats
A, Summary data illustrating the effect of CNO (2 mg kg^{-1}, I.V.) on CO_2-induced (10%–12% CO_2, 5 min) increases in phrenic nerve activity in control, RTN-$DREADD_{Gi}$ and preBötC-TeLC/RTN-$DREADD_{Gi}$ experimental groups. *B*, Effects of CNO on CO_2-evoked increases in the amplitude of phrenic nerve discharge compared to the initial responses (top) and responses evoked in conditions of CBD (carotid body denervation, bottom). Each data point represents the measurement obtained in one animal. p – unpaired t test (*A*) and paired t test (*B*).

carotid body input, the RTN mechanisms contribute approximately one-third to the overall respiratory response to CO_2.

To evaluate the contribution of preBötC astrocytes to the hypercapnic respiratory response, we next compared the magnitude of the CO_2-induced increases in PNA between the RTN-DREADD$_{Gi}$ and preBötC-TeLC/RTN-DREADD$_{Gi}$ experimental animals. The first response to CO_2 in the preBötC-TeLC/RTN-DREADD$_{Gi}$ group was not significantly different from that recorded in the RTN-DREADD$_{Gi}$ group [increase by 71 (21)% *vs.* 103 (45)%, $n = 5$ per group; $p = 0.18$, unpaired t test; Fig. 8; see also Fig. 5*A*]. Blood gas analysis showed no differences between preBötC-TeLC/RTN-DREADD$_{Gi}$ and the other two experimental groups (Table 1). After CBD, the PNA increases induced by CO_2 were reduced by ∼31% in the preBötC-TeLC/RTN-DREADD$_{Gi}$ group compared to that in the RTN-DREADD$_{Gi}$ group ($p = 0.024$; unpaired t test). These data suggest that in anaesthetized rats, in the absence of the carotid body input, preBötC astrocytes contribute approximately one-third to the overall respiratory response to CO_2. Subsequent RTN inhibition by application of CNO reduced the amplitude of CO_2-induced increase in PNA by a further 42% ($p = 0.012$, unpaired t test; Fig. 8).

Discussion

In this study, we further investigated the role of the preBötC in the mechanisms of central respiratory CO_2 sensitivity. The data obtained show that under general anaesthesia, CO_2 sensitivity of the preBötC is mediated by astrocytes that contribute 31% to the overall respiratory response to hypercapnia. This contribution is similar to that of the peripheral chemoreceptors of the carotid body (28%) and the RTN mechanisms (37%). Experiments conducted in conscious animals showed that preBötC mechanisms mediate CO_2-induced increases in the respiratory frequency, whereas RTN neurons are responsible for CO_2-induced increases in tidal volume.

Removal of the carotid body input decreases the respiratory frequency at rest and increases the variability of breathing, and has a major impact on the ventilatory responses to hypoxia and hypercapnia (Andronikou et al., 1988; Angelova et al., 2015; Dahan et al., 2007; Forster et al., 2008; Nakayama et al., 2003; Sheikhbahaei et al., 2017; Smith et al., 2003; Timmers et al., 2003). The data obtained in the present study are in agreement with the results of previous studies, demonstrating that the chemosensory drive from the carotid bodies is responsible for ∼30% of the respiratory sensitivity to CO_2 (Andronikou et al., 1988; Dahan et al., 2007; Gautier & Bonora, 1979; Izumizaki et al., 2004; Olson et al., 1988).

Viral targeting of the RTN neurons to express inhibitory receptors was established and validated in our earlier studies (Huckstepp et al., 2015; Korsak et al., 2018; Marina et al., 2010). In this study, histological analysis of every sixth 50 μm section identified ∼60 transduced neurons per side, which translates to ∼3600 neurons expressing DREADD$_{Gi}$. Although we used immunohistochemical detection of mCitrine, it is possible that we may have underestimated the number of transduced neurons. However, the application of CNO completely abolished active expirations, a result similar to that obtained in studies that used selective chemogenetic inhibition of Phox2b-expressing RTN neurons (Marina et al., 2010). This suggests that a critical number of RTN neurons were effectively inhibited in our experiments, consistent with the proposed key role of the RTN neurons in controlling the active expirations (Marina et al., 2010). The percentage reduction in CO_2-evoked inspiratory (phrenic nerve activity) response after inhibition of DREADD$_{Gi}$-expressing RTN neurons observed in this study was similar to that reported previously (Bhandare et al., 2020; Korsak et al., 2018; Marina et al., 2010).

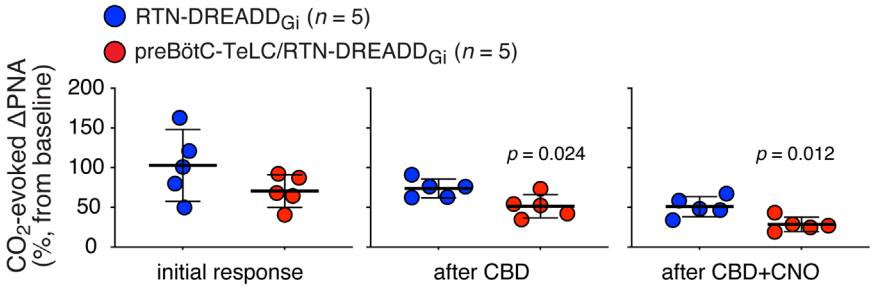

Figure 8. Contribution of preBötC (preBötzinger complex) astrocytes to the hypercapnic respiratory response in anaesthetized rats
Group data illustrating the contribution of preBötC astrocytes to CO_2-induced (10%–12% CO_2, 5 min) increases in phrenic nerve activity in control conditions, after CBD (carotid body denervation) and then after the administration of CNO (2 mg kg^{-1}, I.V.) in RTN-DREADD$_{Gi}$ and preBötC-TeLC/RTN-DREADD$_{Gi}$ experimental groups. Each data point represents the measurement obtained in one animal. p – unpaired t test.

In conscious rats when the inputs from the peripheral chemoreceptors were reduced by hyperoxia, the inhibition of the RTN neurons had no effect on resting respiratory activity as previously observed in normoxic conditions (Marina et al., 2010). This observation differs from the data showing reduction in breathing frequency and tidal volume observed with optogenetic inhibition of RTN neurons under similar conditions (Basting et al., 2015; Burke et al., 2015), suggesting that RTN inhibition may have been more effective in these experiments. However, the CO_2-evoked increases in tidal volume and minute ventilation were reduced by ∼20% in our experiments. In anaesthetized and artificially ventilated rats in conditions of bilateral CBD, RTN inhibition decreased the respiratory response to CO_2 by 37%, similar to that reported previously (Huckstepp et al., 2015; Marina et al., 2010). These results are consistent with the recently reported data, suggesting that in awake rodents RTN neurons may be less responsive to CO_2 than under general anaesthesia (Bhandare et al., 2020).

A growing body of evidence suggests that in addition to chemosensory responses evoked by CO_2 in RTN neurons, central respiratory chemosensitivity relies on astroglial signalling mechanisms (Gourine & Dale 2022; Gourine et al., 2010; Sobrinho et al., 2017; Turk et al. 2022). This role for astrocytes was suggested within the brainstem regions of the caudal parapyramidal area (van de Wiel et al., 2020), preBötC (Beltrán-Castillo et al., 2017; Sheikhbahaei, Turovsky et al., 2018) and RTN (Gourine et al., 2010). Indeed, brainstem astrocytes are sensitive to physiological changes in extracellular pH, responding with robust elevations in intracellular Ca^{2+} (Gourine et al., 2010; Turovsky et al., 2016), which triggers the exocytotic release of gliotransmitters, such as ATP/adenosine and/or D-serine (Erlichman et al., 2010; Kasymov et al., 2013). Accumulating evidence also suggests that CO_2 has an independent direct effect on breathing by a mechanism involving connexin-26-expressing glial cells that reside on the ventral surface of the medulla (Huckstepp et al., 2010; Meigh et al., 2013; van de Wiel et al., 2020). The actions of gliotransmitters released in response to hypercapnia are believed to cause propagation of Ca^{2+}-excitation between astrocytes, and activation of the brainstem RTN and preBötC neurons (Beltrán-Castillo et al., 2017; Gourine et al., 2010; Kasymov et al., 2013; Marina et al., 2018; Sheikhbahaei, Turovsky et al., 2018).

The preBötC has chemosensory properties (Solomon, 2003a, 2003b; Solomon et al., 1999, 2000), and some preBötC neurons respond to acidification *in vitro* (Koizumi et al., 2010). PreBötC astrocytes can modulate the respiratory response to hypercapnia via the release of D-serine and/or ATP (Beltrán-Castillo et al., 2017; Sheikhbahaei, Turovsky et al., 2018; Turk et al., 2022). The data obtained in this study further support the earlier observations in conscious animals that astrocytes within preBötC rhythm-generating circuits contribute to CO_2-induced respiratory responses (Sheikhbahaei, Turovsky et al., 2018). In conscious animals, interfering with exocytotic release mechanisms in preBötC astrocytes decreased baseline f_R and concomitantly V_E but had no effect on V_T.

These data support the conclusions reached by Grey et al. (2001) and Nattie (2000), who suggested that the contribution of preBötC mechanism(s) to the overall respiratory response to CO_2 is ∼20%–25%. Indeed, in conscious animals, lung ventilation during resting breathing (eucapnia) and at the peak of the CO_2 response was reduced by ∼20% when vesicular release mechanisms in preBötC astrocytes were blocked. Thus, astroglial signalling in the preBötC plays an important role in orchestrating the ventilatory response to CO_2. Whether this reflects direct sensitivity of preBötC astrocytes to CO_2 or astroglial modulation of preBötC network excitability to chemosensory inputs remains to be established. We have not demonstrated that preBötC astrocytes are sensitive to changes in pH/CO_2, but such sensitivity has been shown for other brainstem astrocytes, including astrocytes that reside in the RTN (Gourine & Dale 2022; Kasymov et al., 2013).

Simultaneous inhibition of RTN neuronal population and blockade of exocytotic release mechanisms in the preBötC astrocytes decreased minute ventilation by ∼35% in conscious rats, suggesting that the RTN and preBötC mechanisms may control distinct components of the ventilatory response (V_T and f_R, components, respectively). In experiments conducted in anaesthetized rats, the effect of inhibiting RTN neurons and preBötC astrocytes on phrenic nerve response was found to be additive, resulting in ∼70% reduction of the CO_2 response. Although we cannot rule out the decreased excitability of the respiratory network due to the removal of the carotid body input, our results are consistent with previously reported data, suggesting that urethane anaesthesia may reduce the drive from some chemosensitive regions (Massey & Richerson, 2017). In our experimental conditions, carotid body denervation led to a 28% reduction in the response, whereas combined blockade of astroglial signalling in the preBötC, bilateral RTN inhibition and carotid body denervation reduced the CO_2-induced response by about 70%. These data support the hypothesis of distributed CO_2 chemosensitivity, which proposes that the CO_2-sensitive drive to breathe is mediated by interacting peripheral and central mechanisms, including the interactions between chemosensitive brainstem neurons and glial cells.

Experimental limitations

In this study, to determine the relative contribution of the RTN neuronal population to the hypercapnic respiratory

response, we used a viral vector with a pan-neuronal promoter. We did not target the specific subpopulation of RTN neurons that express the specific markers of the chemosensitive neurons, and inhibition of other neurons within the region could potentially have changed the respiratory response to CO_2 (Cleary et al., 2021). The physiological data showing complete blockade of active expirations and significant (by ∼40%) reduction in the inspiratory response to CO_2 were found to be very similar to that reported in the preceding studies (Marina et al. 2010; Nattie & Li, 2002, 2006; Takakura et al., 2014), suggesting that a critical number of RTN neurons were successfully inhibited in these experiments. In addition, interpretation of these results is complicated because the role of the carotid body input in modulating the CO_2 sensitivity of both sites remains unknown.

Perspectives

The data obtained in this study support the hypothesis that respiratory sensitivity to CO_2 is mediated by the carotid bodies and several brainstem sites (including preBötC and RTN), with each site contributing a fraction of the hypercapnic ventilatory response (Nattie, 1999, 2000, 2001; Nattie & Li, 2009; Spyer & Thomas, 2000).

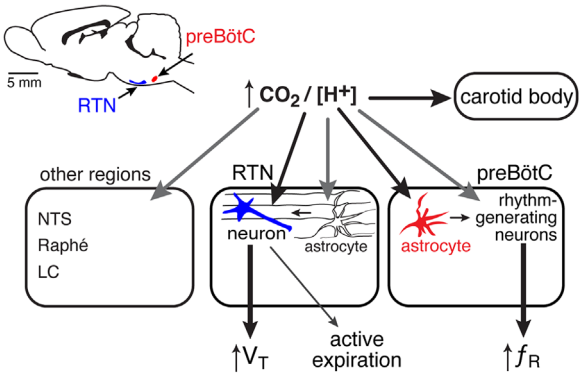

Figure 9. Respiratory CO_2 chemosensitivity
Increases in P_{CO2}/[H^+] activate chemosensors of the carotid body and several central sites of the medulla oblongata and pons (Nattie, 1999, 2000, 2001; Spyer & Thomas, 2000; Nattie & Li, 2009). This study showed that the contributions of the carotid bodies, preBötC astrocytes, and RTN mechanisms to the CO_2-evoked respiratory response are quantitatively similar. PreBötC astrocytes mediate CO_2-induced increases in f_R, whereas RTN mechanisms mediate increases in V_T. Direct excitatory effects of P_{CO2}/[H^+] on RTN and preBötC neurons are also indicated. A separate component (∼30%) of the CO_2 drive to breathe is hypothesized to originate from the chemosensitive sites located in other areas of the brainstem, including the nucleus tractus solitarius (NTS), raphé nuclei and locus coeruleus (Teppema et al., 1997; Nattie, 1999; Richerson, 2004; Nattie & Li, 2009; Erlichman et al., 2009; Marina et al., 2010; Cui et al., 2011). Abbreviations: LC, locus coeruleus; NTS, nucleus tractus solitarius; preBötC, preBötzinger complex; RTN, retrotrapezoid nucleus. Grey arrows depict data from other studies.

Our model (Fig. 9) proposes that increases in the arterial and brainstem tissue P_{CO2}/[H^+] are detected by the carotid body, RTN, preBötC and other regions (Cui et al., 2011; Erlichman et al., 2009; Fu et al., 2019; Huckstepp et al., 2010; Marina et al., 2010; Nattie & Li, 2009; Nattie, 1999; Richerson, 2004; Teppema et al., 1997; van de Wiel et al., 2020; Wu et al., 2019). PreBötC mechanisms involve astroglial signalling, primarily controlling the frequency component of the ventilatory response to CO_2.

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Additional information

Data availability statement

The data generated in the figures are provided in the source files at https://figshare.com/ and are also available from the corresponding author upon reasonable request.

Competing interests

The authors declare no competing financial interests.

Author contributions

S.S., J.C.S. and A.V.G. designed the experiments. S.S., N.M. and V.R. performed the experiments. S.S., N.M., J.C.S. and A.V.G. analysed and interpreted the data. S.S., N.M., J.C.S. and A.V.G. wrote the manuscript. S.K. and G.D.F. revised the article critically for important intellectual content. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Supporting information

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

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