

# The essential role of peripheral respiratory chemoreceptor inputs in maintaining breathing revealed when CO<sub>2</sub> stimulation of central chemoreceptors is diminished

Marie-Noëlle Fiamma<sup>1,2</sup>, Edward T. O'Connor<sup>1</sup>, Arijit Roy<sup>1</sup>, Ines Zuna<sup>1</sup> and Richard J. A. Wilson<sup>1</sup>

<sup>1</sup>Hotchkiss Brain Institute, Department of Physiology and Pharmacology, University of Calgary, Calgary, Alberta, Canada

<sup>2</sup>Université Pierre et Marie Curie-Paris 06, ER 10 UPMC, Paris, F-75013 France

## Key points

- Central sleep apnoea is a condition characterized by oscillations between apnoea and hyperpnoea during sleep, which can have many serious health implications.
- Each ventilatory overshoot following an apnoea attenuates peripheral chemoreceptor input which, in turn, has the potential to cause a further apnoea.
- In a decerebrate, vagotomized, *in situ* rat preparation, we show that central apnoeas can be overcome both physiologically (with high peripheral CO<sub>2</sub>) and pharmacologically (with peripheral pituitary adenylate cyclase-activating peptide).
- We also show that the central apnoeic threshold, i.e. the CO<sub>2</sub> level at which the animal stops breathing, can be lowered by increasing peripheral chemoreceptor stimulation.
- These data suggest that stimulation of peripheral chemoreceptors may prevent central apnoeas, re-affirming the peripheral chemoreceptors as possible therapeutic targets for some sleep apnea phenotypes.

**Abstract** Central sleep apnoea is a condition characterized by oscillations between apnoea and hyperpnoea during sleep. Studies in sleeping dogs suggest that withdrawal of peripheral chemoreceptor (carotid body) activation following transient ventilatory overshoots plays an essential role in causing apnoea, raising the possibility that sustaining carotid body activity during ventilatory overshoots may prevent apnoea. To test whether sustained peripheral chemoreceptor activation is sufficient to drive breathing, even in the absence of central chemoreceptor stimulation and vagal feedback, we used a vagotomized, decerebrate dual-perfused *in situ* rat preparation in which the central and peripheral chemoreceptors are independently and artificially perfused with gas-equilibrated medium. At varying levels of carotid body stimulation (CB  $P_{O_2}/P_{CO_2}$ : 40/60, 100/40, 200/15, 500/15 Torr), we decreased the brainstem perfusate  $P_{CO_2}$  in 5 Torr steps while recording phrenic nerve activity to determine the central apnoeic thresholds. The central apnoeic thresholds decreased with increased carotid body stimulation. When the carotid bodies were strongly stimulated (CB 40/60), the apnoeic threshold was  $3.6 \pm 1.4$  Torr  $P_{CO_2}$  (mean  $\pm$  SEM,  $n = 7$ ). Stimulating carotid body afferent activity with either hypercapnia (60 Torr  $P_{CO_2}$ ) or the neuropeptide pituitary adenylate cyclase-activating peptide restored phrenic activity during central apnoea. We conclude that peripheral stimulation shifts the central apnoeic threshold to very hypocapnic levels that would likely increase the CO<sub>2</sub> reserve and have a protective effect on

M.-N.F. and E.T.O.C. are joint first authors.

breathing. These data demonstrate that peripheral respiratory chemoreceptors are sufficient to stave off central apnoeas when the brainstem is perfused with low to no CO<sub>2</sub>.

(Resubmitted 24 October 2012; accepted after revision 24 January 2013; first published online 28 January 2013)

**Corresponding author** R. J. A. Wilson: Department of Physiology and Pharmacology, Hotchkiss Brain Institute, Faculty of Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, Canada T2N 4N1. Email: wilsonr@ucalgary.ca

**Abbreviations** CSN, carotid sinus nerve; DPP, dual-perfused preparation; OSA, obstructive sleep apnoea; PACAP, pituitary adenylate cyclase-activating peptide; REM, rapid eye movement; RTN, retrotrapezoid nucleus.

## Introduction

Inputs from central (brainstem) and peripheral (carotid bodies) respiratory chemoreceptors are integrated within the respiratory control system to protect blood gases against potentially deleterious fluctuations (O'Regan & Majcherczyk, 1982). Respiratory chemoreceptors are important during wakefulness and essential during sleep (Spengler *et al.* 2001; Dempsey *et al.* 2004), determining the efficacy of phasic vagal feedback from stretch receptors in the lung, regulating upper and lower airway calibre, and modulating the respiratory rhythm generator and/or respiratory motor neurons to sustain breathing (Kinkead *et al.* 1994; Spyer & Gourine, 2009; Nattie, 2011). Consequently, abnormalities in brainstem and/or carotid body chemoreflex function might be a primary cause of breathing instability during sleep and a possible target for pharmacotherapeutics for some sleep apnoea phenotypes (Kiwull-Schöne *et al.* 2008). Here, we use a rodent model to test whether tonic carotid body stimulation is capable of sustaining breathing when the brainstem CO<sub>2</sub> chemoreflex is silenced.

Many brainstem areas appear to be endowed with endogenous respiratory CO<sub>2</sub> chemosensitivity (ability to detect local changes in  $P_{\text{CO}_2}$  and initiate ventilatory responses), including the nucleus tractus solitarius, raphe, retrotrapezoid nucleus (RTN), the locus coeruleus and the cerebellar fastigial nucleus (Martino *et al.* 2007; Guyenet, 2008; Corcoran *et al.* 2009; Gargaglioni *et al.* 2010; Nattie, 2011). However, as the central chemoreflex responds slowly to changes in  $P_{\text{aCO}_2}$  relative to the fluctuations that occur in sleep apnoea, the role of central chemoreceptors in periodic breathing has been questioned (Smith *et al.* 2003). In contrast to the central chemoreflex, the response of the carotid body-mediated chemoreflex to changes in blood gases occurs within 3–5 s, an order of magnitude faster than that of the brainstem chemoreflex (Carroll *et al.* 1991).

Since their discovery, the carotid bodies have been considered as the primary oxygen sensors, responding vigorously to precipitous falls in  $P_{\text{aO}_2}$  that normally accompany asphyxia. In sleep-disordered breathing, such a response may be critical after an apnoea or hypopnea to trigger arousal, open the airways and/or

kick-start breathing motor patterns. However, carotid bodies also play an important role in sustaining eupnoeic ventilation under normoxic conditions (Forster *et al.* 2008; Mouradian *et al.* 2012) and are reported to respond vigorously to changes in  $P_{\text{CO}_2}$  (Cunningham, 1987). Thus, elevated CO<sub>2</sub> causes increased  $[\text{Ca}^{2+}]_i$  in isolated glomus cells and carotid sinus nerve (CSN) activity in a range of preparations, from the isolated perfused carotid body preparations to *in vivo* preparations (Iturriaga, 1993; Linton *et al.* 1995; Cummings & Wilson, 2005). But perhaps most telling, with few exceptions (Lugliani *et al.* 1971; da Silva *et al.* 2011), carotid body denervation in awake humans, lambs, goats, ponies, piglets and rats causes a pronounced hypoventilation, with an accompanying increase in  $P_{\text{aCO}_2}$  of 5–15 Torr (Wade *et al.* 1970; Bisgard *et al.* 1976; Olson *et al.* 1988; Praud *et al.* 1992; Côté *et al.* 1996; Pan *et al.* 1998; Lowry *et al.* 1999; Timmers *et al.* 2003; Mouradian *et al.* 2012). Despite the increase in  $P_{\text{aCO}_2}$  being of sufficient magnitude to cause cerebrospinal fluid acidosis, the central chemoreceptors are unable to compensate.

These observations raise the possibility that, in addition to the carotid bodies' classic role in terminating apnoeas by triggering vigorous sympathetic and respiratory responses, output from the carotid bodies may also be necessary for preventing apnoeas during eupnoea. Daristotle *et al.* (1990) addressed this issue directly in goats by selectively making the carotid body hypocapnic using an extracorporeal circuit (carotid bodies isolated from systemic circulation). In awake animals, reducing the carotid body  $P_{\text{CO}_2}$  by 10.9 Torr decreased ventilation by 24% and increased  $P_{\text{aCO}_2}$  by 5.6 Torr. In 4 of 14 animals the hypoventilation was accompanied by ventilatory instability, including apnoea (Daristotle *et al.* 1990). Smith and Dempsey used a similar approach and carotid body denervation to investigate the importance of the carotid body in sustaining breathing during non-rapid eye movement (REM) sleep in conscious dogs. In line with Daristotle's observations, they found that arterial hypocapnia caused by a transient ventilatory overshoot was much more likely to cause apnoea if the hypocapnia was sensed by the carotid body (Nakayama *et al.* 2003; Smith *et al.* 2003).

If exposure to hypocapnia causes breathing instability and apnoea by reducing the activity of the carotid body, then sustaining activity of the carotid body should be a rational strategy for preventing apnoeas. This argument is supported by remarkable data obtained by Berkenbosch *et al.* using an anaesthetized cat preparation that separates perfusion of the pons and brainstem from systemic circulation, thereby allowing blood gases at the carotid bodies and brainstem to be controlled independently (Berkenbosch *et al.* 1984). Providing the carotid bodies were stimulated, the breathing pattern remained stable even when the  $P_{\text{CO}_2}$  of the blood supplied to the brainstem was reduced to 3.8 Torr. However, the authors considered it likely that their result was in part caused by the vasoconstriction that accompanies extreme hypocapnia, changing cerebral blood flow and allowing systemic circulation into the medulla. A further caveat was revealed recently with discovery of a population of hypothalamic orexin-containing neurons with exquisite sensitivity to  $\text{CO}_2$ : it is possible that not all central respiratory chemoreceptors are located in the brainstem (Williams *et al.* 2007).

As pharmacological stimulation of the carotid body might yet provide a therapeutic avenue to treat sleep apnoea in carefully phenotyped patients, the aim of our study was to test whether peripheral respiratory chemoreceptor activation was sufficient to sustain respiratory rhythm generation in the absence of functional central chemoreceptors, vagal feedback and descending projections from higher centres, in a non-anaesthetized preparation. For these experiments, we used a decerebrate, vagotomized dual-perfused rat preparation (illustrated in Fig. 1A) based on the *in situ* working heart brainstem preparation in which carotid bodies and brainstem are independently perfused with artificial saline (Paton, 1996; Day & Wilson, 2005, 2007, 2009). In this preparation, the circle of Willis is cut preventing the possibility of cross-contamination between the perfusate to the brainstem and carotid bodies. We found that with asphyxia-like (hypoxic-hypercapnia) stimulation of the carotid bodies, breathing motor output persisted even when brainstem perfusion  $P_{\text{CO}_2}$  approached 0 Torr, demonstrating that peripheral respiratory chemoreceptors are sufficient to stave off central apnoeas caused by severe brainstem hypocapnia. We also show that hypercapnic or pharmacological stimulation of the carotid bodies can overcome central apnoea.

## Methods

Experimental procedures were approved by the University of Calgary Animal Care Committee, and were in accordance with Canadian law. Experiments were conducted with juvenile male Sprague–Dawley albino

rats (weight 100–150 g;  $\approx$  4–6 weeks old; Charles River, Quebec, Canada).

### Dual-perfused preparation (DPP)

Rats were weighed, lightly anaesthetized in a bell jar with halothane till recumbent and then prepared for experimentation. In the initial experiments, animals were given an intra-peritoneal injection of sodium pentobarbital (30–50 mg kg<sup>-1</sup>) or intubated and ventilated with 2% halothane. The efficacy of anaesthesia was assessed by testing for absence of response to noxious tail pinch and corneal reflex. In rapid succession, rats were cut in half just below the diaphragm and placed in ice-cold perfusate containing (in mM): NaCl, 115; NaHCO<sub>3</sub>, 24; KCl, 4; CaCl<sub>2</sub>, 2; MgSO<sub>4</sub>, 1; NaH<sub>2</sub>PO<sub>4</sub>, 1.25; glucose, 10; sucrose, 12; equilibrated with 95% O<sub>2</sub>–5% CO<sub>2</sub>. Next, the skin from the top of the head was removed. Animals were then placed in the experimental chamber in a supine position and fixed with ear bars. The descending aorta was cannulated with a double-lumen catheter. One lumen of the catheter was connected to a peristaltic pump (Gilson Minipuls 3) and used to perfuse the descending aorta retrogradely with warm (33°C) perfusate, equilibrated with 40 Torr  $P_{\text{CO}_2}$  in O<sub>2</sub> (central perfusion). The other lumen was attached to a pressure transducer to monitor perfusion pressure. Once cannulated, the speed of the peristaltic pump was increased so as to ramp perfusion pressure to 60 mmHg over the first few minutes using a custom-built computer-controlled feedback system. Next, the preparation was decerebrated at the mid-collicular level (approximate level of lambda). All tissue rostral to the decerebration was removed, as was the remaining cortex dorsal to the colliculi rendering the preparation insentient. The face and any remaining skin were also removed. The common carotid arteries were exposed, the jugular veins and vagus nerves sectioned, and the arteries tied off above the clavicles and cannulated. More recent experiments used a slightly different approach to preparing the DPP because we found it significantly increased the yield of viable preparations. In this revised approach, anaesthesia was maintained via a facemask supplied with 2–4% halothane. The efficacy of anaesthesia was assessed by testing for the absence of response to noxious tail pinch and corneal reflex. The animal was then placed in ice-cold perfusate and the skin removed rostrally from the abdomen to the cranium. While still breathing, the preparation was decerebrated, as above, at the mid-collicular level. All tissue rostral to the decerebration was removed, as was the remaining cortex dorsal to the colliculi. The animal was then rapidly sectioned just below the diaphragm, placed in the experimental chamber and fixed in a supine position with ear bars. The descending aorta was cannulated, as above, and the perfusion pressure

gradually increased to 60 mmHg. Both vagi were sectioned at the cervical level, followed by cannulation of the carotid arteries.

A separate peristaltic pump (Gilson Minipuls 3) was used to perfuse both common carotid arteries (peripheral perfusion). Following cannulation, the flow through each common carotid artery was ramped up to 15 ml min<sup>-1</sup>.

Up to this stage in the dissection, central and peripheral perfusions were from the same tonometer (300 ml). Next, carotid and brainstem compartments were supplied with perfusate from separate tonometers with different gas compositions (carotid body: 100 Torr  $P_{O_2}$ , 35 Torr  $P_{CO_2}$  in N<sub>2</sub>; brainstem: 35 Torr  $P_{CO_2}$  in O<sub>2</sub>). Perfusate exiting the preparation was collected in the base of the recording chamber and returned to the tonometers for re-equilibration and recirculation. The central perfusion pressure was ramped to 90 mmHg.

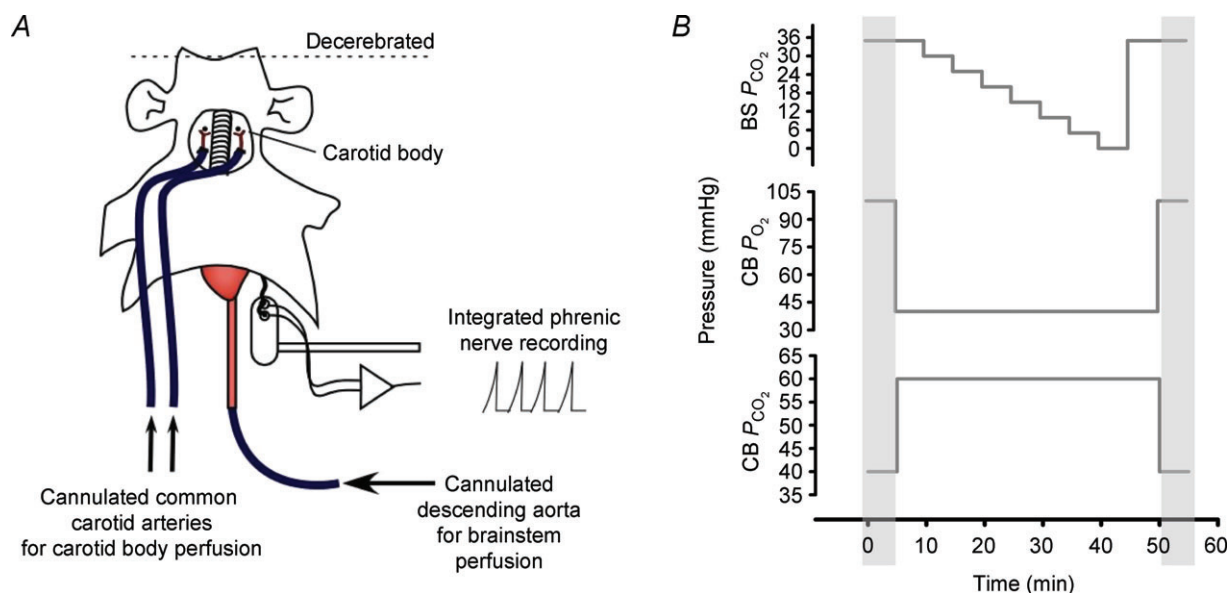
### Electrophysiology

The left phrenic nerve was located and dissected free of surrounding tissues, leaving a small portion of diaphragm attached at the distal end. The nerve was placed in a custom holder that prevented the nerve from drying out and facilitated long-term extracellular recordings. The phrenic neurogram was amplified (A-M Systems Differential AC Amplifier Model 1700), filtered (low cut-off, 300 Hz; high cut-off, 5 kHz), rectified and integrated (Amplitude

Demodulator; Saga Tech), computer archived (Axon Instruments Digidata 1322A and Axoscope 9.0) at a sampling rate of 50 Hz, and analysed off-line.

### Experimental protocols

**Protocol 1.** A graphic description of the protocol can be found in Fig. 1B: 35–40 min following dissection and pressure equilibration, the baseline condition was established: brainstem perfusate 35 Torr  $P_{CO_2}$  in O<sub>2</sub> and carotid bodies perfusate, 40 Torr  $P_{CO_2}$  and 100 Torr  $P_{O_2}$  in N<sub>2</sub>. Then, the carotid bodies were stimulated with 60 Torr  $P_{CO_2}$  and 40 Torr  $P_{O_2}$  in N<sub>2</sub> for 5 min without any change in the perfusate to the brainstem (stimulated conditions). Next, with the carotid bodies stimulated, brainstem perfusate  $P_{CO_2}$  was decreased by 5 Torr (balance with O<sub>2</sub>) every 5 min to find the central apnoeic threshold, defined as the brainstem perfusate  $P_{CO_2}$  at or below which phrenic bursts were abolished (Dempsey & Skatrud, 1986; Boden *et al.* 1998). In some cases, phrenic bursts continued for 5 min even when brainstem perfusate  $P_{CO_2}$  was 0 Torr. Once the apnoeic threshold was obtained, or brainstem perfusate  $P_{CO_2}$  was 0 Torr but phrenic bursts persisted, the  $P_{CO_2}$  of the brainstem perfusate was returned to baseline conditions for 5 min (35 Torr  $P_{CO_2}$  in O<sub>2</sub>), while maintaining stimulation to the carotid bodies. Finally, the baseline conditions used at the start of the protocol were re-imposed: brainstem perfusate 35 Torr  $P_{CO_2}$  in O<sub>2</sub> and



**Figure 1. Schematic of the DPP and Protocol 1**

*A*, the key feature of the DPP used for this study is independent artificial perfusion of the carotid bodies (via ligation, cannulation and perfusion of the common carotid arteries) and brainstem (via cannulation and retrograde perfusion of the descending aorta). The DPP is vagotomized and decerebrated (non-anaesthetized). We use phrenic activity to measure neuronal ventilation. *B*, illustration of protocol.  $P_{CO_2}$  and/or  $P_{O_2}$  in the central (brainstem; BS) and peripheral (carotid body; CB) perfusate for the CB 40/60 group. The  $P_{CO_2}$  in the brainstem perfusate was balanced with O<sub>2</sub>. The  $P_{CO_2}$  and  $P_{O_2}$  in carotid body perfusate were balanced with N<sub>2</sub>.

carotid bodies perfusate, 40 Torr  $P_{\text{CO}_2}$  and 100 Torr  $P_{\text{O}_2}$  in  $\text{N}_2$ . This protocol was repeated in separate preparations where the carotid body perfusate was maintained at 100 Torr  $P_{\text{O}_2}$  with 40 Torr  $P_{\text{CO}_2}$ , as well as both 200 and 500 Torr  $P_{\text{O}_2}$  with 15 Torr  $P_{\text{CO}_2}$  ( $n = 6/\text{group}$ ; see Figs 3 and 4).

Only preparations in which phrenic discharges returned when brainstem perfusate  $P_{\text{CO}_2}$  was increased (following determination of the apnoeic threshold) were included in the analysis. Every phrenic burst over the protocol period was analysed.

**Protocol 2.** Following dissection, baseline conditions were established as in Protocol 1. Next, the gas equilibrating the tonometer supplying the brainstem was made severely hypocapnic (10 Torr  $P_{\text{CO}_2}$ , in  $\text{O}_2$ ) without changing the gas equilibrating the carotid bodies (100 Torr  $P_{\text{O}_2}$ , 40 Torr  $P_{\text{CO}_2}$  in  $\text{N}_2$ ). After apnoea had developed, a 1 min bolus of pituitary adenylate cyclase-activating peptide (PACAP)-38 (final concentration: 100 nM) was added to the peripheral perfusate before it reached the preparation. Six preparations were used for this protocol, three with CSN intact and the remainder with CSN denervated.

**Protocol 3.** Baseline conditions were established as in Protocol 1, and the gas equilibrating the tonometer supplying the brainstem was made severely hypocapnic (10 Torr  $P_{\text{CO}_2}$ , in  $\text{O}_2$ ) without changing the gas equilibrating the carotid bodies (100 Torr  $P_{\text{O}_2}$ , 40 Torr  $P_{\text{CO}_2}$  in  $\text{N}_2$ ). After apnoea had developed, the gas equilibrating the carotid bodies was made hypercapnic (60 Torr  $P_{\text{CO}_2}$ ). Both brainstem and carotid body perfusate were returned to baseline gas values 5 min after breathing had resumed.

### Phrenic neurogram analysis

The following respiratory variables were quantified from the integrated phrenic neurogram using custom-written software (RJAW): period ( $T_{\text{TOT}}$ ), respiratory rate ( $f_{\text{R}}$ , 60 times the inverse of the period), time to peak ( $T_{\text{p}}$ ), inspiratory duration ( $T_{\text{I}}$ ), expiratory duration ( $T_{\text{E}}$ ), neural tidal volume ( $nV_{\text{T}}$ , the peak phrenic amplitude), neural minute ventilation ( $n\dot{V}_{\text{E}}$ , the product of  $f_{\text{R}}$  and  $nV_{\text{T}}$ ) and eupnoeic index (EI,  $T_{\text{p}}$  divided by  $T_{\text{I}}$ ). A schematic of this quantification has been previously published (Day & Wilson, 2005). Respiratory variables were averaged in 60 s bins.  $nV_{\text{T}}$  and  $n\dot{V}_{\text{E}}$  were normalized to the last minute of the initial baseline section (baseline 1: minutes 4–5) for graphical representation.

### Statistical analysis

For statistical tests of each respiratory variable, the last time bin (between minutes 4 and 5) of each experimental condition was analysed. The data were not normally distributed owing to the occurrence of apnoea, so we used non-parametric statistics. We used the Friedman's two-way ANOVA by ranks test to determine the merit of the null hypothesis that respiratory variables were the same between conditions. To determine at what condition apnoea was statistically present, we used a series of one-sample Wilcoxon signed rank tests to test the null hypothesis that respiratory rate during each condition had a value of zero. Grouped data are given as mean  $\pm$  SEM.

## Results

### Central apnoeic threshold at varying levels of carotid body activity

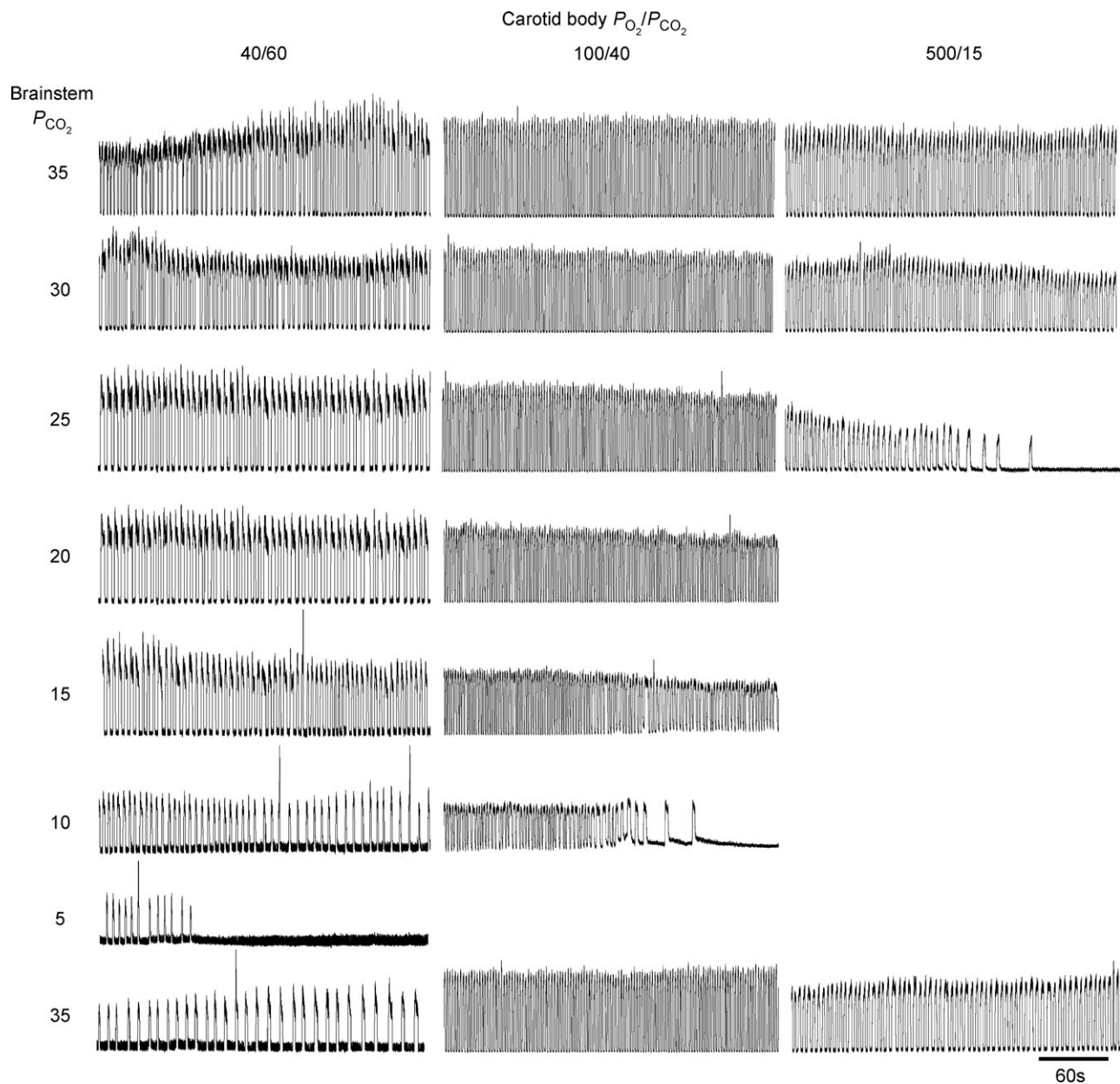
Examples showing the effects of lowering brainstem perfusate  $P_{\text{CO}_2}$  on phrenic nerve activity when the carotid bodies are stimulated with perfusate containing  $P_{\text{O}_2}/P_{\text{CO}_2}$  of 40/60, 100/40 and 500/15 Torr are illustrated in Fig. 2. As can be seen, rate and neural tidal volume decrease with brainstem perfusate  $P_{\text{CO}_2}$  but, in the case of the hypoxic-hypercapnic carotid body, apnoea is not apparent until the brainstem perfusate is severely hypocapnic (nominally 0 Torr  $P_{\text{CO}_2}$ ; see Wilson *et al.* 2001). A similar degree of resistance to central apnoea was apparent across all preparations in which the carotid bodies were stimulated with hypoxic-hypercapnia (Fig. 3). Thus, in two of nine preparations, rhythmic phrenic bursts continued even when brainstem perfusate  $P_{\text{CO}_2}$  was zero. In the remaining seven preparations, progressive reduction in brainstem perfusate  $P_{\text{CO}_2}$  eventually caused cessation of phrenic activity, but the  $P_{\text{CO}_2}$  reached when cessation occurred (approximating the central apnoeic threshold) was  $3.57 \pm 1.43$  Torr. In three of these seven preparations, phrenic activity only stopped when brainstem perfusate  $P_{\text{CO}_2}$  was nominally 0 Torr. The highest central apnoeic threshold in the seven preparations was between 10 and 15 Torr  $P_{\text{CO}_2}$ . To statistically evaluate the level of brainstem perfusate  $P_{\text{CO}_2}$  required to obtain apnoea across all nine preparations, we used a series of one-sample Wilcoxon signed rank tests (null hypothesis: breaths per minute equals zero). Accordingly, from a statistical perspective, when the carotid body was stimulated by 40 Torr  $P_{\text{O}_2}$  and 60 Torr  $P_{\text{CO}_2}$ , a brainstem perfusate  $P_{\text{CO}_2}$  of 10 Torr or more was sufficient to prevent apnoea. When output from the carotid body was attenuated by increasing carotid body  $P_{\text{O}_2}$  and decreasing  $P_{\text{CO}_2}$ , the mean central apnoeic thresholds increased. Thus, with CB  $P_{\text{O}_2}/P_{\text{CO}_2}$  of 100/40, 200/15 and 500/15, apnoeic

thresholds were  $7.0 \pm 0.5$ ,  $11.7 \pm 0.4$  and  $16.7 \pm 0.9$  Torr  $P_{\text{CO}_2}$ , respectively. From a statistical perspective, the brainstem perfusate  $P_{\text{O}_2}$  required to prevent apnoea for each of these levels of carotid body stimulation was 15, 20 and 30 Torr, respectively ( $n = 6/\text{group}$ ).

### Effect on rate versus neuronal tidal volume

Although changing brainstem perfusate  $P_{\text{CO}_2}$  affected all respiratory variables (Friedman's two-way ANOVA by rank,  $P < 0.001$  for all variables), respiratory variables were not equally affected. With the carotid bodies

stimulated with hypoxic-hypercapnia, both  $nV_{\text{T}}$  and  $n\dot{V}_{\text{E}}$  were approximately proportional to brainstem perfusate  $P_{\text{CO}_2}$ , with both timing and volume variables affected (Fig. 3). Whereas the latter was influenced progressively by a decrease in brainstem perfusate  $P_{\text{CO}_2}$ , timing variables were relatively intransient compared with  $nV_{\text{T}}$  until severe levels of hypocapnia were reached. At these severe levels, both  $T_{\text{I}}$  and  $T_{\text{E}}$  were affected. Specifically, as brainstem perfusate  $P_{\text{CO}_2}$  decreased,  $T_{\text{I}}$  first increased above baseline levels (from  $0.75 \pm 0.03$  s to  $1.07 \pm 0.07$  s at 15 Torr brainstem perfusate  $P_{\text{CO}_2}$ ) before falling to zero (Fig. 4). This trend was apparent in seven of the nine preparations with

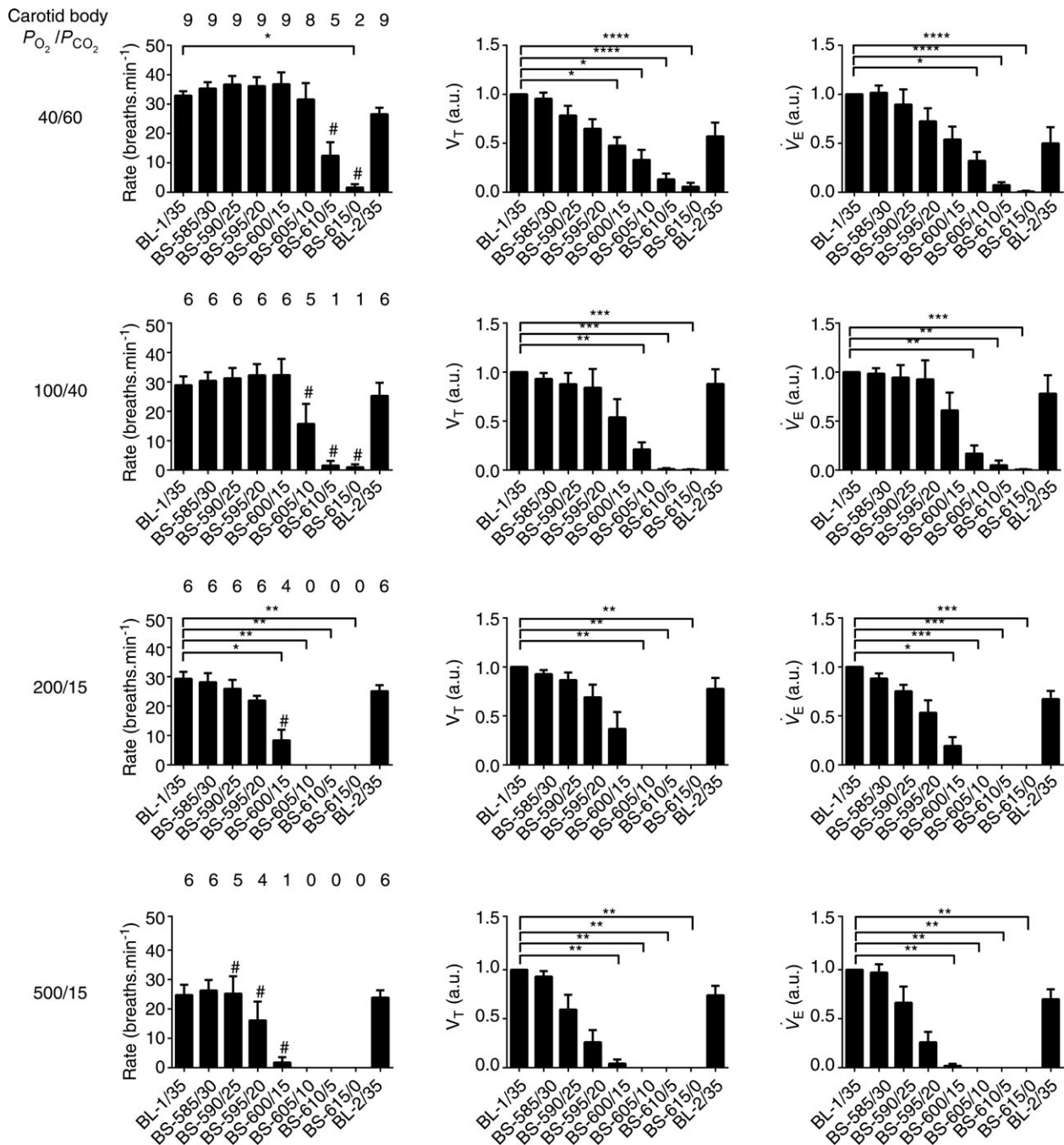


**Figure 2. Stimulating the carotid body staves off apnoeas**

Rectified integrated phrenic neurograms of representative preparations subjected to Protocol 1. With removal of carotid body stimulation, the apnoeic threshold is raised in a linear fashion.

the carotid bodies stimulated with hypoxic-hypercapnia, as well as in all preparations with carotid body  $P_{O_2}/P_{CO_2}$  levels of 100/40 and 200/15 Torr, but was absent in the 500/15 Torr group.

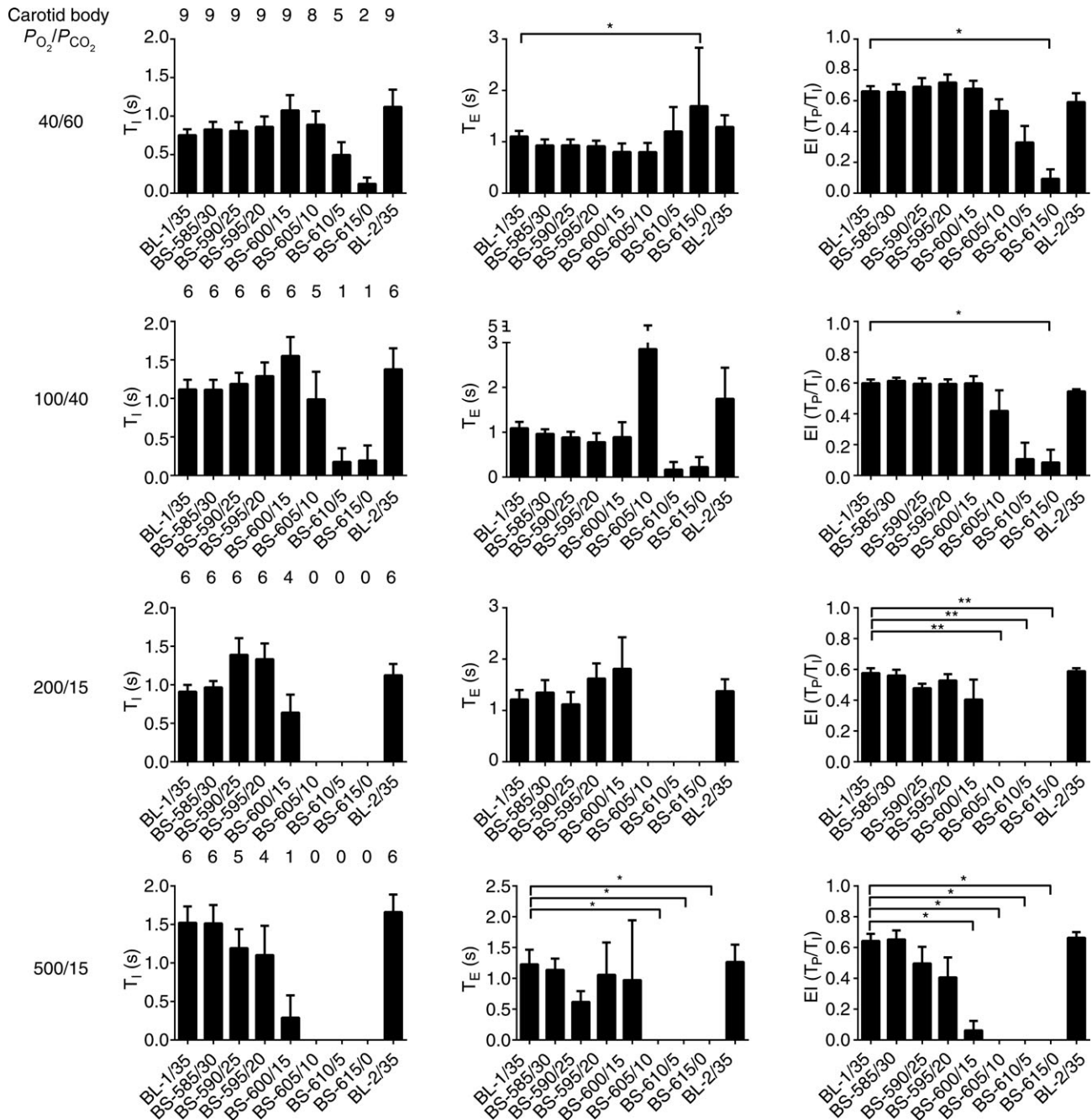
To determine the effects of brainstem hypocapnia on burst shape, which in turn may indicate oxygenation status of brainstem tissue, we quantified a eupnoeic index (EI; see Materials and methods for derivation). An EI of 0



**Figure 3. Respiratory rate,  $nV_T$  and  $nV_E$  at different levels of brainstem  $P_{CO_2}$**   
 Bins represent average values of respiratory variables over 60 s (between minutes 4 and 5, i.e. the last 60 s bin) of each experimental condition. The numbers above rate data represent the number of animals in which phrenic bursts persisted at each condition.  $nV_T$ : amplitude of phrenic in arbitrary units (a.u.);  $nV_E$ :  $nV_T$  multiplied by breaths per minute in arbitrary units (a.u.). Error bars represent SEM. Friedman test with Dunn's multiple comparisons test, \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ; \*\*\*\* $P \leq 0.0001$ . Wilcoxon signed rank test (hypothetical mean of 0), # $P > 0.05$ , thus not significantly different to 0 (apnoea). BL, baseline; BS-585/30, brainstem perfusate gas pressure of 585 Torr  $P_{O_2}$  and 30 Torr  $P_{CO_2}$ .

indicates a rapid-onset decrementing burst, whereas an EI of 1 indicates a ramping burst with a sudden offset. We consider bursts with EI below 0.5 to be gasp-like, and those above 0.5 to be eupnoeic. Changing brainstem perfusate  $P_{CO_2}$  had a significant effect on the EI (Friedman's two-way ANOVA by rank:  $P < 0.01$ ; Fig. 4).

On average, with the carotid bodies stimulated, bursts remained eupnoeic even with brainstem perfusate  $P_{CO_2}$  as low as 5 Torr (EI was  $0.59 \pm 0.06$ ; with only one preparation having an EI below 0.5). Nonetheless, between 15 and 0 Torr, EI diminished such that at 0 Torr the EI of the two preparations that continued to generate bursts was



**Figure 4.**  $T_1$ ,  $T_E$  and EI at different levels of brainstem  $P_{CO_2}$ . Bins represent average values of respiratory variables over 60 s (between minutes 4 and 5, i.e. the last 60 s bin) of each experimental condition.  $T_1$ : duration of phrenic burst;  $T_E$ : interburst interval;  $T_P$ : time from onset of phrenic burst to burst peak; EI: eupnoeic index ( $T_P/T_1$ ) where, at the extremes, a value of 0 represents a rapid-onset decrementing burst and a value of 1 is an augmenting burst with rapid offset. Error bars represent SEM. Friedman test with Dunn's multiple comparisons test, \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ .



0.42 ± 0.05. All other groups followed a broadly similar pattern, with EI diminishing at higher brainstem perfusate  $P_{\text{CO}_2}$ , in line with the relative apnoeic threshold/decreasing carotid body stimulation, i.e. the lower the level of carotid body activity, the higher the level of central  $\text{CO}_2$  at which EI diminished, whilst exhibiting a similar pattern.

### Pharmacological stimulation of the carotid body during central apnoea

To determine whether pharmacological stimulation of the carotid body is sufficient to overcome central apnoea, we perfused the common carotid artery with the stress peptide PACAP (Fig. 5). We used six preparations for these experiments, three with the CSN intact and three with it denervated. In all preparations, with the carotid body perfusate having a  $P_{\text{O}_2}$  and  $P_{\text{CO}_2}$  of 100 and 40 Torr, lowering the brainstem perfusate  $P_{\text{CO}_2}$  to 10 Torr caused a long-duration apnoea. In CSN intact preparations, adding a 1 min bolus of PACAP to the peripheral perfusate, giving a final concentration of 100 nM, promptly terminated the apnoea by causing a pronounced and long-lived increase in phrenic nerve activity. In preparations with the CSN denervated prior to PACAP application, application of PACAP had no immediate effect: apnoea persisted during the 3<sup>rd</sup> min of PACAP exposure. Thus, the stimulatory effects of PACAP over the first 3 min were largely dependent on an intact CSN (+CSN/–CSN:  $P < 0.001$ ). These data suggest that pharmacological stimulation of the carotid body is a viable method of overcoming central apnoeas.

Interestingly, while PACAP failed to restore phrenic activity within the first 3 min in all denervated preparations tested, a transient recovery from apnoea (lasting 3–4 min) occurred in all denervated preparations sometime thereafter (4–6 min; Fig. 5B). This suggests that a second component of the respiratory controller is also stimulated by PACAP. The considerable delay in response to PACAP with CSN denervated suggests this second component may be located within central respiratory or autonomic nuclei (Farnham *et al.* 2008; Peña, 2010; Inglott *et al.* 2011).

### Hypercapnic stimulation of the carotid body during central apnoea

To determine whether hypercapnic stimulation of the carotid body is sufficient to overcome central apnoea, we perfused the common carotid artery with normoxic-hypercapnic solution. In all preparations, with the carotid body perfusate having a  $P_{\text{O}_2}$  and  $P_{\text{CO}_2}$  of 100 and 40 Torr, lowering the brainstem perfusate  $P_{\text{CO}_2}$  to between 5 and 10 Torr caused a long-duration apnoea.

Upon stimulation of the carotid body with perfusate containing  $P_{\text{O}_2}$  and  $P_{\text{CO}_2}$  of 100 and 60 Torr, phrenic activity promptly resumed in all preparations (Fig. 6).

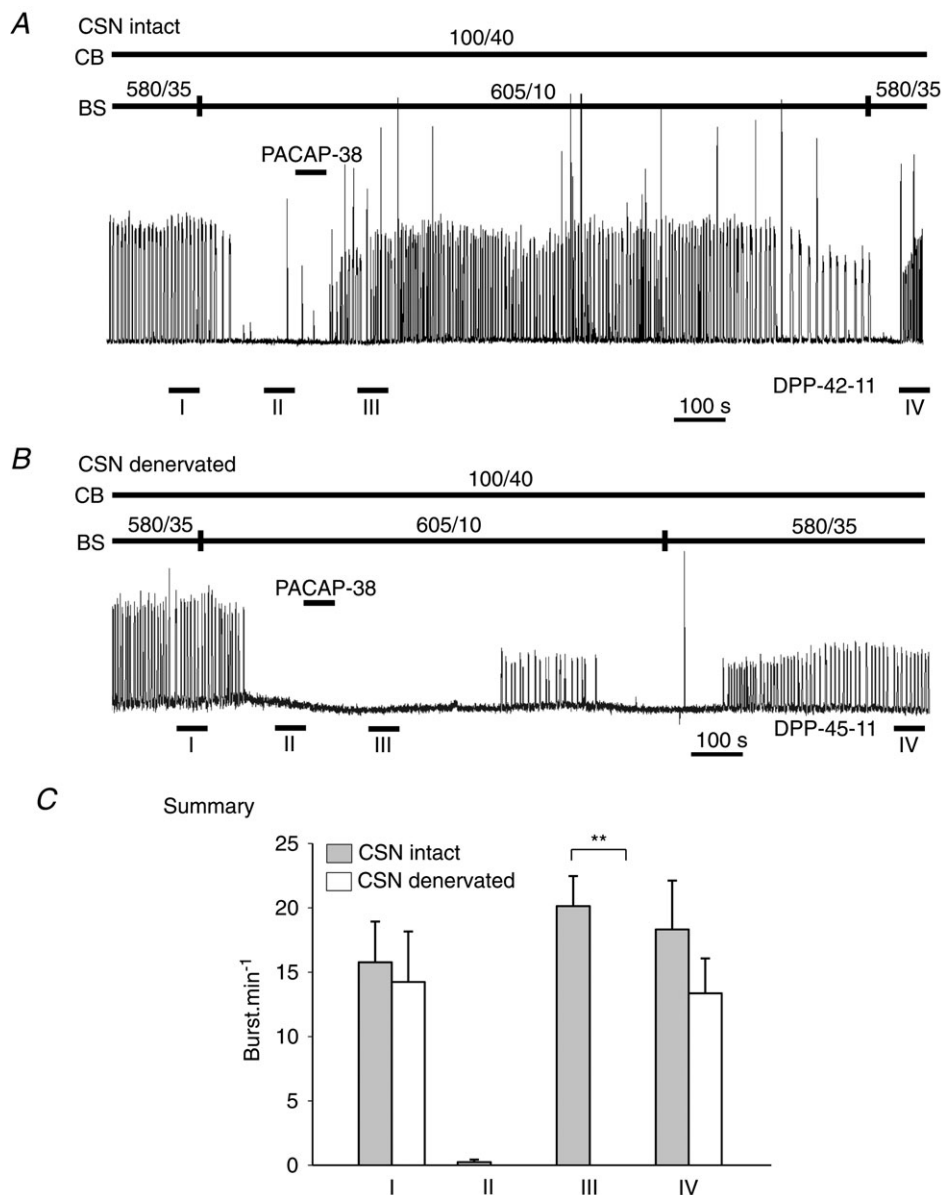
## Discussion

Our data demonstrate that providing the peripheral chemoreceptors are stimulated, respiratory rhythm generation is highly resilient to brainstem perfusion with low  $P_{\text{CO}_2}$  even in the absence of descending input from the cerebrum, vagal feedback and anaesthetics. When the carotid bodies are stimulated, the main effect of brainstem  $P_{\text{CO}_2}$  on phrenic activity is a reduction in burst amplitude. Only at extreme levels of brainstem perfusate hypocapnia (less than 10 Torr  $P_{\text{CO}_2}$ ) is frequency and shape affected: as  $P_{\text{CO}_2}$  approaches zero, frequency slows and bursts transition from incrementing to gasp-like. These data suggest that: (a) the peripheral chemoreceptors are sufficient to drive breathing through an excitatory pathway onto the respiratory rhythm generator that does not require central chemosensitivity; and (b) stimulating the carotid body may provide a therapeutic avenue to stave off apnoea caused by central hypocapnia. In addition, we demonstrate that specific carotid body hypercapnia is sufficient to overcome central apnoea, complicating interpretation of several recent studies suggesting that rodent carotid bodies do not contribute to  $\text{CO}_2$  chemosensitivity (Mouradian *et al.* 2012).

Our data are corroborated by data from an anaesthetized cat model in which the brainstem was perfused independently of systemic circulation, albeit with intact vagal input from the lungs and the cerebrum present (Berkenbosch *et al.* 1984). Those data also suggest the resilience of the respiratory rhythm generator to severe central hypocapnia when peripheral chemoreceptors are stimulated. In the cat preparation, extreme hypocapnia may have caused changes in cerebral circulation resulting in compromised independence of brainstem and systemic perfusate; the authors considered it likely that brainstem chemoreceptors had been exposed to the hypercapnic and hypoxic systemic perfusate used to stimulate the peripheral chemoreceptors. In addition, artificial perfusion in the cat model was limited to the brainstem and did not include recently discovered hypothalamic neurons that exhibit an exquisite  $\text{CO}_2$  sensitivity on par with that of RTN neurons (Williams *et al.* 2007). The *in situ* DPP escapes both these confounders as the circle of Willis is sectioned, preventing cross-contamination between peripheral and central perfusate. Given the gross differences between the cat preparation and the DPP (cat *versus* rat, normothermia *versus* mild hypothermia, baroreflex and heart intact *versus* artificial pump and pressure feedback system, blood *versus* artificial perfusate, vagus intact *versus* vagotomized, and anaesthetized *versus*

decerebrate), the similar observations give credence to the notion that central chemosensitivity is not absolutely necessary for breathing *per se*, while peripheral chemoreceptors alone are sufficient to maintain activity of the respiratory rhythm generator, at least under normoxic

conditions. This suggestion is further endorsed by the recent findings of Ramanantsoa *et al.* (2011). They showed that normal, rhythmic breathing can persist in transgenic mice deficient in RTN neurons and effectively lacking respiratory CO<sub>2</sub> sensitivity. This rhythm is, however,



**Figure 5. Excitation of carotid body with stress peptide pituitary adenylate cyclase-activating peptide (PACAP) overcomes central apnoeas**

In all preparations, central apnoea was produced by reducing brainstem  $P_{\text{CO}_2}$  from 35 to 10 Torr. *A*, rectified integrated phrenic neurogram of a representative preparation exposed to PACAP during apnoea with carotid sinus nerve (CSN) intact. Note the rapid, sustained reversal of central apnoea on application of PACAP. *B*, rectified integrated phrenic neurogram of a representative preparation exposed to PACAP during apnoea with CSN denervated. Note the lack of immediate response to PACAP; the response to PACAP only occurs after several minutes and is not sustained. *C*, summary data. I: brainstem normocapnic; II: brainstem hypocapnic; III: third minute after perfusing the carotid bodies with 100 nM PACAP; IV: period of washout used for quantification. The carotid body was maintained normocapnic and normoxic throughout the protocol. Error bars represent SEM. \*\*Indicates significant difference ( $P < 0.001$ ) between carotid body intact *versus* carotid body denervated preparations. BS, brainstem; CB, carotid body; DPP, dual-perfused preparation.

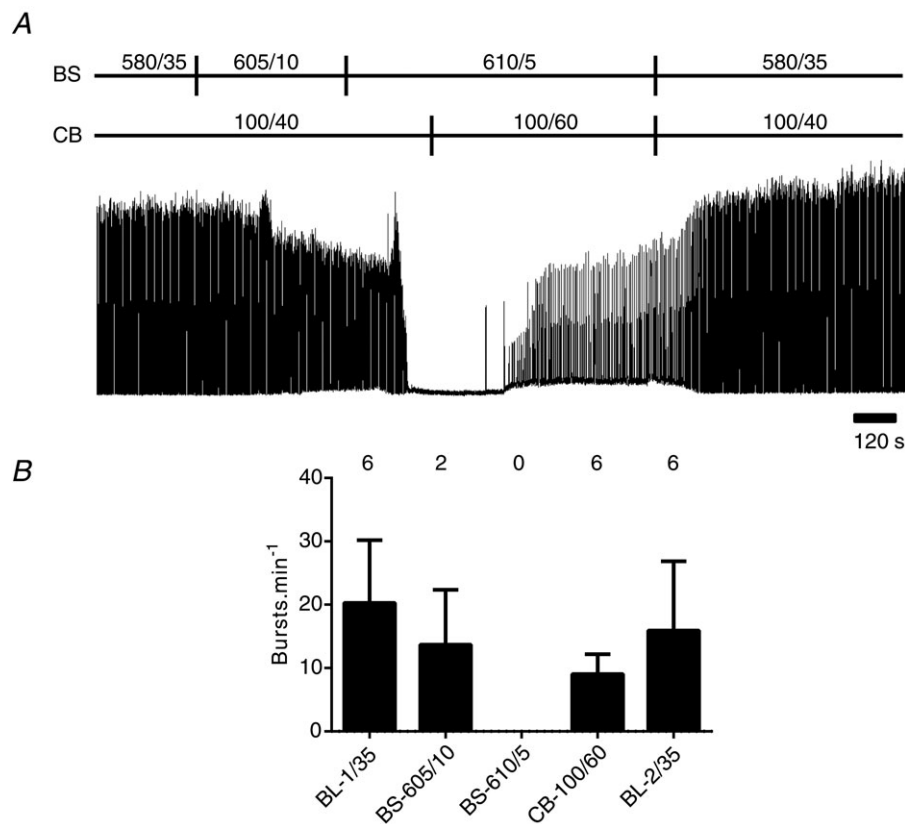
severely disrupted by 'physiological denervation' of the carotid body, whereby hyperoxia essentially silences the peripheral chemoreceptors, resulting in periodic breathing (deep breathing interrupted by prolonged apnoeas). This suggests that although the peripheral chemoreceptors are capable of supporting a normal, rhythmic breathing pattern in the absence of central chemoreceptors, central chemoreceptors are required to support breathing under certain conditions.

### Caveats and comparison of results to conscious animals

*In situ* perfused brainstem preparations are growing in popularity as models for investigating respiratory control because they produce a eupnoeic-like motor pattern similar to that seen *in vivo*, have open autonomic and respiratory feedback loops, lack confounding influences from higher brain centres and anaesthetics, and allow precise experimental control of arterial gases and pressure (Dutschmann *et al.* 2000; Wilson *et al.* 2001). As with any reduced preparation, these advantages have to be carefully

weighed against a number of caveats (for an extensive discussion of the merits of the DPP see Day & Wilson (2005, 2007)). In the context of the current study, two key issues are: one, whether brainstem tissue is adequately oxygenated when severely hypocapnic; and two, whether a decerebrate preparation is more or less relevant than data from other preparations.

Although the artificial perfusate supplied to the brainstem of the *in situ* preparation has a high  $P_{O_2}$  ( $P_{O_2} > 500$  Torr), it has a much lower oxygen-carrying capacity than blood. During normocapnia, tissue  $P_{O_2}$  measurements suggest that the brainstem is hyperoxic, but with the cerebral vasoconstriction associated with severe hypocapnia, the possibility exists that the tissue becomes hypoxic (Wilson *et al.* 2001). However, we observed a stable eupnoeic index until the  $P_{CO_2}$  in the brainstem perfusate dropped below 10 Torr (see Fig. 4), suggesting that respiratory centres were oxygenated sufficiently so as not to cause rapid-onset, decrementing ('gasp-like') phrenic bursts that usually go hand-in-hand with brainstem hypoxia (Fewell, 2005). Some preparations produced gasp-like bursts as perfusate  $P_{CO_2}$  approached zero. Thus, when the carotid bodies are stimulated, the



**Figure 6. Excitation of carotid body with hypercapnia overcomes central apnoeas**

In all preparations, central apnoea was produced by reducing brainstem  $P_{CO_2}$  from 35 to between 5 and 10 Torr. *A*, rectified integrated phrenic neurogram of a representative preparation exposed to 60 Torr  $P_{CO_2}$  during apnoea. *B*, summary data. Note that breathing resumed in all cases with hypercapnic stimulation of the carotid body. Error bars represent SEM. BL, baseline; BS, brainstem; CB, carotid body.

demise of respiratory bursts at low  $P_{\text{CO}_2}$  may be as much a consequence of indirect effects of low  $\text{CO}_2$  on cerebrovascular resistance resulting in hypoxia, as directly caused by loss of  $\text{CO}_2$  stimulation of brainstem respiratory chemoreceptors.

A main advantage of the DPP is that it reduces the respiratory chemoreflex control system to its most fundamental components, chiefly brainstem and carotid body, and therefore one might expect insights very different from data derived solely from conscious animals that are influenced by higher command centres that coordinate diverse behaviours. As we have reviewed previously,  $\text{CO}_2$  chemosensitivity is shifted in the hypocapnic direction in all decerebrate preparations, including the dog, cat and rat (Day & Wilson, 2005, 2007). Currently, the factor(s) that causes this shift is unknown, but we assume it relates to 'descending wakefulness drive' and possibly involves orexin neurons that project to the brainstem from the hypothalamus (Williams *et al.* 2007; Gestreau *et al.* 2008; Lazarenko *et al.* 2011; Nattie, 2011). Loss of such an input with decerebration may make the brainstem circuit abnormally sensitive to peripheral chemoreceptor input, explaining the current results. However, the fact that denervation of the carotid body causes pronounced hypoventilation in conscious animals (see Introduction) suggests that the brainstem respiratory control is always heavily dependent on carotid body input even in conscious animals when the carotid bodies and chemosensitivity in general are considered to serve a lesser role. In this respect, we note that at altitude, where the carotid body is stimulated by hypoxia (and  $\text{CO}_2$  is 'blown off'), ventilation continues despite often intense hypocapnia. While other factors may be at play in this example, including hypothalamic, descending and adrenal-dependent influences, the most parsimonious explanation is that, in some cases at least, the peripheral chemoreflex helps sustain ventilation during times of diminished central chemoreflex (Duffin, 2010).

### Effect of low brainstem $P_{\text{CO}_2}$ on respiratory burst frequency and amplitude

With carotid body stimulation, timing variables reflecting the output of the respiratory rhythm generation (i.e.  $T_{\text{T}}$ ,  $T_{\text{E}}$ ,  $f_{\text{R}}$ ) were constant over a large range of brainstem perfusate  $P_{\text{CO}_2}$ : only when  $P_{\text{CO}_2}$  approached the apnoeic threshold were such variables affected. In contrast,  $nV_{\text{T}}$  and  $n\dot{V}_{\text{E}}$  decreased linearly with hypocapnia. Thus, in the vagotomized *in situ* preparation, when the carotid bodies are stimulated, the carotid bodies are the dominant influence on the respiratory rhythm generator, and the role of brainstem  $P_{\text{CO}_2}$  is largely restricted to the modulation of downstream mechanisms that determine the amplitude of motor neuron output. If central chemosensitivity is

largely mediated by one population of neurons, such as the RTN as some have speculated (Guyenet, 2008; Guyenet *et al.* 2010), then the central chemoreceptors would not appear to contribute substantially to respiratory rhythm generation. On the other hand, if central chemosensitivity is shared by multiple sites (for example, the RTN, raphe, nucleus tractus solitarius and/or locus coeruleus; Nattie & Li, 2009), then it is possible that endowing the brainstem with excitation from the carotid body saturates the activity of populations that modulate rhythm generation (e.g. RTN), but has only mild effects on the dynamic range of the populations that control burst pattern (amplitude).

### Stimulating the carotid body as a therapeutic strategy for central sleep apnoea?

During non-REM sleep, animals and humans are critically dependent upon chemoreceptor feedback to protect blood gases (Spengler *et al.* 2001; Dubreuil *et al.* 2008). Control theory and experimental data suggest that increases in peripheral and central chemoreflex gain may be primary factors in causing some forms of sleep-disordered breathing, destabilizing breathing by contributing to ventilatory overshoots in response to blood gas perturbations (Longobardo *et al.* 1982; Cherniack, 1984; Khoo, 2000; Kiwull-Schöne *et al.* 2008).

Smith and Dempsey have proposed that ventilatory sensitivity to  $P_{\text{CO}_2}$  below eupnoeic levels may be a key determinant of ventilatory instability during sleep (Dempsey *et al.* 2004). According to the Smith and Dempsey doctrine, the high gain chemoreflexes associated with sleep apnoea lead to oscillations in  $P_{\text{aCO}_2}$ .  $P_{\text{aCO}_2}$  perturbations below eupnoeic levels diminish carotid body activity, decreasing  $n\dot{V}_{\text{E}}$ , and if  $P_{\text{aCO}_2}$  is reduced far enough, apnoea results (i.e. Dempsey *et al.* 2004; Dempsey, 2005). In support of their doctrine, Smith and Dempsey used an extracorporally perfused carotid body conscious dog model to demonstrate that the withdrawal of capnic stimuli from the carotid body during hyperpnoea likely plays a critical role in causing apnoeas, leading to further respiratory oscillations (Nakayama *et al.* 2003; Smith *et al.* 2003). If the sensitivity of the carotid bodies is too high, such that the withdrawal of capnic stimuli shuts off carotid body excitation too readily causing apnoea, we suggest mild tonic, pharmaceutical stimulation of the carotid body may be all that is required to stave off apnoeas and thereby improve ventilatory stability. To illustrate this, we showed that a bolus of the stress neuropeptide PACAP, when applied through the common carotid artery, is capable of overcoming central apnoeas in the DPP. Consistent with previous studies demonstrating that arterially perfused PACAP strongly excites the carotid body (Ishizuka *et al.* 1992; Runcie *et al.* 1995), we demonstrate that the effect of PACAP on overcoming central apnoeas was dependent on

intact carotid body afferents; that some activity persisted after transecting the CSN suggests that PACAP excites peripheral targets outside the carotid sinus, the location of which is currently under investigation.

Normally, tonic stimulation of the carotid bodies results in hyperventilation; the respiratory system blows off CO<sub>2</sub> leading to arterial hypocapnia (Kumar, 2009). If carotid body stimulation has no effect on the central apnoeic threshold, then one would expect that apnoeas would be more likely, not less. However, our results suggest carotid body stimulation reduces the central apnoeic threshold substantially, making apnoeas less likely. Specifically, in the current study using the DPP, with the peripheral chemoreceptors stimulated with hypoxic-hypercapnia, two of nine preparations had no central apnoeic threshold and, in the remainder, the central apnoeic threshold was  $3.6 \pm 1.4$  Torr  $P_{\text{CO}_2}$ . We also showed that this could be raised, in a linear fashion, up to  $16.7 \pm 0.9$  Torr  $P_{\text{CO}_2}$  by attenuating carotid body output (by increasing  $P_{\text{O}_2}$  and decreasing  $P_{\text{CO}_2}$ ), confirming previous work from our lab (Day & Wilson, 2005). Thus, stimulating the carotid bodies caused a potentially protective hypocapnic shift in the apnoeic threshold of  $\sim 13$  Torr  $P_{\text{CO}_2}$ .

In humans with central sleep apnoea, drugs that stimulate respiration tend to stabilize breathing, but this is not the case in patients with obstructive sleep apnoea (OSA). While the carbonic anhydrase inhibitor acetazolamide has a substantial effect on the apnoea/hypopnoea index, most likely through acidification of the blood by action at the kidney (White *et al.* 1982; Javaheri, 2006) or reductions in the sensitivity of the ventilatory control system (Edwards *et al.* 2012), drugs that target neuronal components of respiration control have only mild effects on breathing stability in patients with OSA (Sabosky *et al.* 2009; Kohler & Stradling, 2011). For example, while theophylline stabilizes breathing in central sleep apnoea, in two of three randomized control studies, theophylline is reported to cause only a mild reduction in the apnoea/hypopnoea index in OSA (reviewed in Veasey *et al.* 2006). Similarly, the effects of naloxone, doxapram and almatrine, all of which are reported to stimulate the carotid body to some degree, have underwhelming effects on the apnoea-hypopnea index in OSA (Krieger *et al.* 1982; Guillemainault & Hayes, 1983; Atkinson *et al.* 1985; Suratt *et al.* 1986; Hackett *et al.* 1987; Olievier *et al.* 1987; Yost, 2006). These extensive observations underline the fact that carotid body stimulants are not suitable for the treatment of all types of sleep apnoea, especially when collapse of the airway may be exacerbated by increased inspiratory effort. Therefore, to be effective, pharmaceuticals that target the carotid body will need to be tailored to specific disease phenotypes (White, 2005).

In conclusion, the current data from the decerebrate, vagotomized *in situ* rat preparation suggest that the peri-

pheral respiratory chemoreceptors are demigod chemoreceptors capable of sustaining the frequency of the respiratory rhythm generator in the absence of vagal feedback or substantive central respiratory chemoreceptor activity. With the respiratory rhythm sustained by input from the peripheral chemoreceptors, the role of the central chemoreceptor is one of determining tidal volume. Given the Smith–Dempsey doctrine that predicts removal of capnic stimuli from the carotid body is the primary cause of apnoea, and assuming the demonstration herein that carotid body stimulation is capable of shifting the apnoeic threshold to very low levels of  $P_{\text{CO}_2}$  is reflective of the role of the carotid body in intact sleeping humans, we surmise that pharmacological treatments that selectively stimulate the carotid bodies may be an effective treatment for some forms of sleep apnoea.

## References

- Atkinson RL, Suratt PM, Wilhoit SC & Recant L (1985). Naloxone improves sleep apnea in obese humans. *Int J Obes* **9**, 233–239.
- Berkenbosch A, van Beek JH, Olievier CN, De Goede J & Quanjer PH (1984). Central respiratory CO<sub>2</sub> sensitivity at extreme hypocapnia. *Respir Physiol* **55**, 95–102.
- Bisgard GE, Forster HV, Orr JA, Buss DD, Rawlings CA & Rasmussen B (1976). Hypoventilation in ponies after carotid body denervation. *J Appl Physiol* **40**, 184–190.
- Boden AG, Harris MC & Parkes MJ (1998). Apneic threshold for CO<sub>2</sub> in the anesthetized rat: fundamental properties under steady-state conditions. *J Appl Physiol* **85**, 898–907.
- Carroll JL, Canet E & Bureau MA (1991). Dynamic ventilatory responses to CO<sub>2</sub> in the awake lamb: role of the carotid chemoreceptors. *J Appl Physiol*, **71**, 2198–2205.
- Cherniack NS (1984). Sleep apnea and its causes. *J Clin Invest* **73**, 1501–1506.
- Corcoran AE, Hodges MR, Wu Y, Wang W, Wylie CJ, Deneris ES & Richerson GB (2009). Medullary serotonin neurons and central CO<sub>2</sub> chemoreception. *Resp Physiol Neurobiol* **168**, 49–58.
- Côté A, Porras H & Meehan B (1996). Age-dependent vulnerability to carotid chemodenervation in piglets. *J Appl Physiol* **80**, 323–331.
- Cummings KJ & Wilson RJA (2005). Time-dependent modulation of carotid body afferent activity during and after intermittent hypoxia. *Am J Physiol Regul Integr Comp Physiol* **288**, R1571–R1580.
- Cunningham DJ (1987). Studies on arterial chemoreceptors in man. *J Physiol* **384**, 1–26.
- da Silva GSF, Giusti H, Benedetti M, Dias MB, Gargaglioni LH, Branco LGS & Glass ML (2011). Serotonergic neurons in the nucleus raphe obscurus contribute to interaction between central and peripheral ventilatory responses to hypercapnia. *Pflugers Arch* **462**, 407–418.
- Daristotle L, Berssenbrugge AD, Engwall MJ & Bisgard GE (1990). The effects of carotid body hypocapnia on ventilation in goats. *Respir Physiol* **79**, 123–135.

- Day TA & Wilson RJA (2005). Specific carotid body chemostimulation is sufficient to elicit phrenic poststimulus frequency decline in a novel *in situ* dual-perfused rat preparation. *Am J Physiol Regul Integr Comp Physiol* **289**, R532–R544.
- Day TA & Wilson RJA (2007). Brainstem PCO<sub>2</sub> modulates phrenic responses to specific carotid body hypoxia in an *in situ* dual perfused rat preparation. *J Physiol* **578**(Pt 3), 843–857.
- Day TA & Wilson RJA (2009). A negative interaction between brainstem and peripheral respiratory chemoreceptors modulates peripheral chemoreflex magnitude. *J Physiol* **587**(Pt 4), 883–896.
- Dempsey JA (2005). Crossing the apnoeic threshold: causes and consequences. *Exp Physiol* **90**, 13–24.
- Dempsey JA, Smith CA, Przybylowski T, Chenuel B, Xie A, Nakayama H & Skatrud JB (2004). The ventilatory responsiveness to CO<sub>2</sub> below eupnoea as a determinant of ventilatory stability in sleep. *J Physiol* **560**(Pt 1), 1–11.
- Dempsey J & Skatrud J (1986). A sleep-induced apneic threshold and its consequences. *Am Rev Respir Dis* **133**, 1163–1170.
- Dubreuil V, Ramanantsoa N, Trochet D, Vaubourg V, Amiel J, Gallego J, Brunet JF & Goridis C (2008). A human mutation in Phox2b causes lack of CO<sub>2</sub> chemosensitivity, fatal central apnea, and specific loss of parafacial neurons. *Proc Natl Acad Sci U S A* **105**, 1067–1072.
- Duffin J (2010). The role of the central chemoreceptors: a modeling perspective. *Resp Physiol Neurobiol* **173**, 230–243.
- Dutschmann M, Wilson RJ & Paton JF (2000). Respiratory activity in neonatal rats. *Auton Neurosci: Basic* **84**, 19–29.
- Edwards BA, Sands SA, Eckert DJ, White DP, Butler JP, Owens RL, Malhotra A & Wellman, A (2012). Acetazolamide improves loop gain but not the other physiological traits causing obstructive sleep apnoea. *J Physiol* **590**(Pt 5), 1199–211.
- Farnham MMJ, Li Q, Goodchild, AK & Pilowsky PM (2008). PACAP is expressed in sympathoexcitatory bulbospinal C1 neurons of the brain stem and increases sympathetic nerve activity in vivo. *Am J Physiol Regul Integr Comp Physiol* **294**, R1304–R1311.
- Fewell JE (2005). Protective responses of the newborn to hypoxia. *Resp Physiol Neurobiol* **149**, 243–255.
- Forster HV, Martino P, Hodges M, Krause K, Bonis J, Davis S & Pan L (2008). The carotid chemoreceptors are a major determinant of ventilatory CO<sub>2</sub> sensitivity and of PaCO<sub>2</sub> during eupneic breathing. *Adv Exp Med Biol* **605**, 322–326.
- Gargaglioni LH, Hartzler LK & Putnam RW (2010). The locus coeruleus and central chemosensitivity. *Resp Physiol Neurobiol* **173**, 264–273.
- Gestreau C, Bévengut M & Dutschmann M (2008). The dual role of the orexin/hypocretin system in modulating wakefulness and respiratory drive. *Curr Opin Pulm Med* **14**, 512–518.
- Guilleminault C & Hayes B (1983). Naloxone, theophylline, bromocriptine, and obstructive sleep apnea. Negative results. *Bull Eur Physiopathol Respir* **19**, 632–634.
- Guyenet PG (2008). The 2008 Carl Ludwig Lecture: retrotrapezoid nucleus, CO<sub>2</sub> homeostasis, and breathing automaticity. *J Appl Physiol* **105**, 404–416.
- Guyenet PG, Stornetta RL & Bayliss DA (2010). Central respiratory chemoreception. *J Comp Neurol* **518**, 3883–3906.
- Hackett PH, Roach RC, Harrison GL, Schoene RB & Mills WJ Jr (1987). Respiratory stimulants and sleep periodic breathing at high altitude. Almitrine *versus* acetazolamide. *Am Rev Respir Dis* **135**, 896–898.
- Inglott MA, Farnham MMJ & Pilowsky PM (2011). Intrathecal PACAP-38 causes prolonged widespread sympathoexcitation via a spinally mediated mechanism and increases in basal metabolic rate in anesthetized rat. *Am J Physiol Heart Circ Physiol* **300**, H2300–H2307.
- Ishizuka Y, Kashimoto K, Mochizuki T, Sato K, Ohshima K & Yanaihara N (1992). Cardiovascular and respiratory actions of pituitary adenylate cyclase-activating polypeptides. *Regul Pept* **40**, 29–39.
- Iturriaga R (1993). Carotid body chemoreception: the importance of CO<sub>2</sub>-HCO<sub>3</sub><sup>-</sup> and carbonic anhydrase (review). *Biol Res* **26**, 319–329.
- Javaheri S (2006). Acetazolamide improves central sleep apnea in heart failure: a double-blind, prospective study. *Am J Respir Crit Care Med* **173**, 234–237.
- Khoo MC (2000). Determinants of ventilatory instability and variability. *Respir Physiol* **122**, 167–182.
- Kinkead R, Filmyer WG, Mitchell GS & Milsom WK (1994). Vagal input enhances responsiveness of respiratory discharge to central changes in pH/CO<sub>2</sub> in bullfrogs. *J Appl Physiol* **77**, 2048–2051.
- Kiwull-Schöne H, Teppema L, Wiemann M, Kalhoff H & Kiwull P (2008). Pharmacological impact on loop gain properties to prevent irregular breathing. *J Physiol Pharmacol* **59**, 37–45.
- Kohler M & Stradling JR (2011). Pitfalls of clinical trials on pharmacological treatment for obstructive sleep apnoea: future directions. *Expert Opin Invest Drugs* **20**, 1033–1037.
- Krieger J, Mangin P & Kurtz D (1982). Almitrine and sleep apnoea. *Lancet* **320**, 210.
- Kumar P (2009). Systemic effects resulting from carotid body stimulation-invited article. *Adv Exp Med Biol* **648**, 223–233.
- Lazarenko RM, Stornetta RL, Bayliss DA & Guyenet PG (2011). Orexin A activates retrotrapezoid neurons in mice. *Resp Physiol Neurobiol* **175**, 283–287.
- Linton RA, Band DM & McLoughlin P (1995). Adaptation of carotid chemoreceptors to step increases in PaCO<sub>2</sub> in anesthetized cats. *J Neurophysiol* **74**, 2707–2712.
- Longobardo GS, Gothe B, Goldman MD & Cherniack NS (1982). Sleep apnea considered as a control system instability. *Respir Physiol* **50**, 311–333.
- Lowry TF, Forster HV, Pan LG, Serra A, Wenninger J, Nash R, Sheridan D & Franciosi RA (1999). Effects on breathing of carotid body denervation in neonatal piglets. *J Appl Physiol* **87**, 2128–2135.
- Lugliani R, Whipp BJ, Seard C & Wasserman K (1971). Effect of bilateral carotid-body resection on ventilatory control at rest and during exercise in man. *N Engl J Med* **285**, 1105–1111.
- Martino PF, Davis S, Opansky C, Krause K, Bonis JM, Pan LG, Qian B & Forster HV (2007). The cerebellar fastigial nucleus contributes to CO<sub>2</sub>-H<sup>+</sup> ventilatory sensitivity in awake goats. *Resp Physiol Neurobiol* **157**, 242–251.

- Mouradian GC, Forster HV & Hodges MR (2012). Acute and chronic effects of carotid body denervation (CBD) on ventilation and chemoreflexes in three rat strains. *J Physiol* **590**, 3335–3347.
- Nakayama H, Smith CA, Rodman JR, Skatrud JB & Dempsey JA (2003). Carotid body denervation eliminates apnea in response to transient hypocapnia. *J Appl Physiol* **94**, 155–164.
- Nattie E (2011). Julius H. Comroe, Jr, Distinguished Lecture: central chemoreception: then . . . and now. *J Appl Physiol* **110**, 1–8.
- Nattie E & Li A (2009). Central chemoreception is a complex system function that involves multiple brain stem sites. *J Appl Physiol* **106**, 1464–1466.
- O'Regan RG & Majcherczyk S (1982). Role of peripheral chemoreceptors and central chemosensitivity in the regulation of respiration and circulation. *J Exp Biol* **100**, 23–40.
- Olivier CN, Berkenbosch A, Degoede J & Kruyt EW (1987). Almitrine bismesylate and the central and peripheral ventilatory response to CO<sub>2</sub>. *J Appl Physiol* **63**, 66–74.
- Olson EB Jr, Vidruk EH & Dempsey JA (1988). Carotid body excision significantly changes ventilatory control in awake rats. *J Appl Physiol* **64**, 666–671.
- Pan LG, Forster HV, Martino P, Strecker PJ, Beales J, Serra A, Lowry TF, Forster MM & Forster AL (1998). Important role of carotid afferents in control of breathing. *J Appl Physiol* **85**, 1299–1306.
- Paton JF (1996). A working heart-brainstem preparation of the mouse. *J Neurosci Meth* **65**, 63–68.
- Peña F (2010). PACAP modulates the respiratory rhythm generated in the brainstem slice preparation. *Adv Exp Med Biol* **669**, 119–122.
- Praud JP, Canet E & Bureau MA (1992). Chemoreceptor and vagal influences on thyroarytenoid muscle activity in awake lambs during hypoxia. *J Appl Physiol* **72**, 962–969.
- Ramanantsoa N, Hirsch MR, Thoby-Brisson M, Dubreuil V, Bouvier J, Ruffault PL, Matrot B, Fortin G, Brunet JF, Gallego J & Golidis C (2011). Breathing without CO<sub>2</sub> chemosensitivity in conditional Phox2b mutants. *J Neurosci* **31**, 12880–12888.
- Runcie MJ, Ulman LG & Potter EK (1995). Effects of pituitary adenylate cyclase-activating polypeptide on cardiovascular and respiratory responses in anaesthetised dogs. *Regul Pept* **60**, 193–200.
- Saboisky JP, Chamberlin NL & Malhotra A (2009). Potential therapeutic targets in obstructive sleep apnoea. *Expert Opin Ther Targets* **13**, 795–809.
- Smith CA, Nakayama H & Dempsey JA (2003). The essential role of carotid body chemoreceptors in sleep apnea. *Can J Physiol Pharmacol* **81**, 774–779.
- Spengler CM, Gozal D & Shea SA (2001). Chemoreceptive mechanisms elucidated by studies of congenital central hypoventilation syndrome. *Respir Physiol* **129**, 247–255.
- Spyer KM & Gourine AV (2009). Chemosensory pathways in the brainstem controlling cardiorespiratory activity. *Phil Trans R Soc B* **364**, 2603–2610.
- Suratt PM, Wilhoit SC, Brown ED & Findley LJ (1986). Effect of doxapram on obstructive sleep apnea. *Bull Eur Physiopathol Respir* **22**, 127–131.
- Timmers HJLM, Wieling W, Karemaker JM & Lenders JWM (2003). Denervation of carotid baro- and chemoreceptors in humans. *J Physiol* **553**(Pt 1), 3–11.
- Veasey SC, Guilleminault C, Strohl KP, Sanders MH, Ballard RD & Magalang, UJ (2006). Medical therapy for obstructive sleep apnea: a review by the Medical Therapy for Obstructive Sleep Apnea Task Force of the Standards of Practice Committee of the American Academy of Sleep Medicine. *Sleep* **29**, 1036–1044.
- Wade JG, Larson CP Jr, Hickey RF, Ehrenfeld WK & Severinghaus JW (1970). Effect of carotid endarterectomy on carotid chemoreceptor and baroreceptor function in man. *N Engl J Med* **282**, 823–829.
- White DP (2005). Pathogenesis of obstructive and central sleep apnea. *Am J Respir Crit Care Med* **172**, 1363–1370.
- White DP, Zwillich CW, Pickett CK, Douglas NJ, Findley LJ & Weil JV (1982). Central sleep apnea. Improvement with acetazolamide therapy. *Arch Intern Med* **142**, 1816–1819.
- Williams RH, Jensen LT, Verkhatsky A, Fugger L & Burdakov D (2007). Control of hypothalamic orexin neurons by acid and CO<sub>2</sub>. *Proc Natl Acad Sci U S A* **104**, 10685–10690.
- Wilson RJ, Remmers JE & Paton JF (2001). Brain stem PO<sub>2</sub> and pH of the working heart-brain stem preparation during vascular perfusion with aqueous medium. *Am J Physiol Regul Integr Comp Physiol* **281**, R528–R538.
- Yost CS (2006). A new look at the respiratory stimulant doxapram. *CNS Drug Rev* **12**, 236–249.

### Author contributions

This study was performed at the University of Calgary. All authors contributed to this study: M.-N.F., I.Z. and R.J.A.W. developed the concept, performed initial experiments, analysed data and wrote the first draft of the manuscript; E.T.O.C., A.R. and R.J.A.W. designed substantive additional experiments; these additional experiments were performed and analysed by E.T.O.C. and A.R.; E.T.O.C. and R.J.A.W. wrote the final draft of the manuscript. All authors approved the final version to be published.

### Acknowledgements

This study was funded by the Canadian Institutes of Health Research. Salary support for R.J.A.W. was provided by Alberta Innovates-Health Solutions (AIHS). M.-N.F. was supported in part by a grant 'Legs Poix' of the Chancellerie des Universités de Paris, France, and by a grant from the Bourse de voyage de la Société de Pneumologie de Langue Française (Boehringer-Ingelheim). I.Z. was funded by the AIHS (both HYRS program and summer studentship). The authors thank Sherry Moore and Dr Trevor Day for technical assistance.