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SYMPOSIUM REVIEW

Interdependent feedback regulation of breathing by the carotid bodies and the retrotrapezoid nucleus

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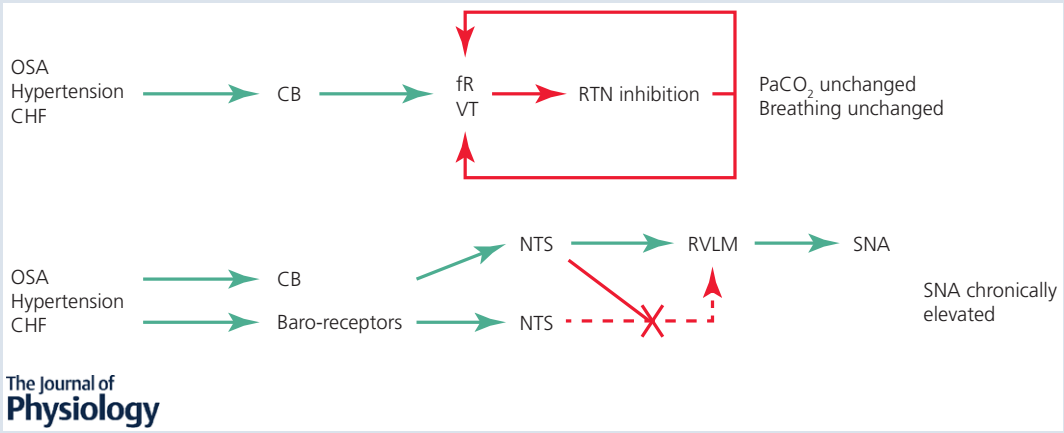

Abstract The retrotrapezoid nucleus (RTN) regulates breathing in a CO_2- and state-dependent manner. RTN neurons are glutamatergic and innervate principally the respiratory pattern generator; they regulate multiple aspects of breathing, including active expiration, and maintain breathing automaticity during non-REM sleep. RTN neurons encode arterial P_{CO_2}/pH via cell-autonomous and paracrine mechanisms, and via input from other CO_2-responsive neurons. In short, RTN neurons are a pivotal structure for breathing automaticity and arterial P_{CO_2} homeostasis. The carotid bodies stimulate the respiratory pattern generator directly and indirectly by activating RTN via a neuronal projection originating within the solitary tract nucleus. The indirect



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pathway operates under normo- or hypercapnic conditions; under respiratory alkalosis (e.g. hypoxia) RTN neurons are silent and the excitatory input from the carotid bodies is suppressed. Also, silencing RTN neurons optogenetically quickly triggers a compensatory increase in carotid body activity. Thus, in conscious mammals, breathing is subject to a dual and interdependent feedback regulation by chemoreceptors. Depending on the circumstance, the activity of the carotid bodies and that of RTN vary in the same or the opposite directions, producing additive or countervailing effects on breathing. These interactions are mediated either via changes in blood gases or by brainstem neuronal connections, but their ultimate effect is invariably to minimize arterial P_{CO_2} fluctuations. We discuss the potential relevance of this dual chemoreceptor feedback to cardiorespiratory abnormalities present in diseases in which the carotid bodies are hyperactive at rest, e.g. essential hypertension, obstructive sleep apnoea and heart failure.

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Abstract figure legend This schematic diagram is an attempt to explain why, in humans, obstructive sleep apnoea (OSA), essential hypertension or *mild* congestive heart failure (CHF) produce a chronic elevation of sympathetic tone (SNA) without concomitant change in breathing (frequency (f_R) or tidal volume (V_T)) and arterial P_{CO_2} (P_{aCO_2}). All three conditions are associated with increased carotid body activity which might be expected *a priori* to increase both breathing and SNA. At rest, breathing is unchanged, however, whereas SNA is elevated. We speculate that the reason why breathing is unchanged is the powerful countervailing influence of a reduction in central chemoreceptor activity (RTN) which restores P_{CO_2} homeostasis despite increased carotid body activity. By contrast, SNA might be chronically enhanced because its main buffering influence, the baroreflex, is actually reduced when the carotid bodies are activated. Because of the lack of breathing stimulation reported in humans, the present interpretation deliberately de-emphasizes the importance of increased cardiorespiratory coupling (the excitatory influence of the respiratory pattern generator on the circuits responsible for SNA generation) in causing the increase in SNA. Instead, a previously described direct excitatory pathway from second-order carotid body afferents (located within nucleus tractus solitarii (NTS)) to bulbospinal presympathetic neurons (RVLM) is invoked along with a reduced activity of the largely respiration-independent baroreflex feedback pathway between NTS and RVLM neurons. The present schematic diagram does not adequately describe the cardiorespiratory changes observed in animal models of OSA or CHF, especially in reduced preparations, or the cardiorespiratory status of humans with end-stage CHF, situations in which breathing is enhanced and/or irregular at rest. Under such conditions, increased cardiorespiratory coupling does contribute to the rise in SNA. Green arrows, pathway facilitated; red arrows, inhibitory pathway; red cross symbolizes the inhibitory effect of carotid body stimulation on the sympathetic baroreflex pathway.

Introduction

The retrotrapezoid nucleus (RTN) is a small group of medulla oblongata neurons that regulate breathing in a CO_2-dependent manner. Since 2004 a number of laboratories, including ours, have investigated the possibility that this nucleus might be the main hub of the central respiratory chemoreflex as well as the principal CNS site where CO_2 is sensed for the purpose of breathing regulation. The experimental evidence is briefly updated here (for prior reviews see Guyenet & Bayliss, 2015 and Guyenet *et al.* 2016).

For consistency with the main theme of the 2017 ISAC meeting, a large portion of our report focuses on how the carotid bodies, the body's main oxygen sensors (Prabhakar & Semenza, 2015), and central chemoreceptors cooperate in regulating arterial P_{CO_2}. For instance, the notion that, in conscious mammals, poikilocapnic hypoxia silences central respiratory chemoreceptors via alkalosis has long been accepted in spite of scant cellular evidence. We highlight here recent experimental data showing that RTN is silenced by hypoxia, pointing out that this result is consistent both with the postulated central respiratory chemoreceptor function of this nucleus and with the theory that hypoxia leads to a silencing of central respiratory chemoreceptors.

Finally, we discuss how recent findings concerning the interactions between carotid bodies and RTN may help explain the cardiorespiratory anomalies associated with various diseases in which the carotid bodies are hyperactive at rest, e.g. hypertension, heart failure, obstructive sleep apnoea.

The retrotrapezoid nucleus (RTN) – anatomical definition

The definition of RTN that we currently advocate relies on congruent developmental, biochemical and physiological evidence that was complemented more recently by

RNA-Seq and ISH data (Shi *et al.*, 2017). This cell group resides in the parafacial region, mainly below the facial motor nucleus and consists of around ~700 glutamatergic neurons in mice (~1900 in rats) that express homeobox transcription factor Phox2b and NK1 receptors, and lack mRNA for tyrosine-hydroxylase, choline acetyltransferase, glutamic acid decarboxylases, glycine transporter-2 and tryptophan hydroxylase (Guyenet & Bayliss, 2015; Guyenet *et al.* 2016). These neurons have a common developmental lineage (Egr-2, Phox2b, Atoh-1) (Ramanantsoa *et al.* 2011; Ruffault *et al.* 2015) and similar late embryonic (Thoby-Brisson *et al.* 2009), postnatal (Onimaru *et al.* 2014) and adult biochemical characteristics (Guyenet *et al.* 2016). The terms embryonic parafacial oscillator (Thoby-Brisson *et al.* 2009), Phox2b-positive parafacial neurons (Onimaru *et al.* 2014) and RTN neurons (Ramanantsoa *et al.* 2011; Ruffault *et al.* 2015; Guyenet *et al.* 2016) designate what are probably three developmental stages of the same neurons. In addition to expressing Phox2b, RTN neurons can be distinguished from surrounding neurons by the presence of neuromedin B mRNA and, typically (80–90%) by the presence of both GPR4 and TASK-2 (putative physiological proton detectors) (Fig. 1*A*–*C*).

RTN neurons, despite their small number, are heterogeneous; for example, prepro-galanin or prepro-enkephalin transcripts are detectable in only 82 and 67% of these neurons, respectively, and some neurons express only one of the two proton sensors (Shi *et al.*, 2017). We stress that our definition of RTN is based primarily on developmental and biochemical similarities (including pH sensitivity) between neurons. It does not imply that every RTN neuron has the same function, or that every RTN neuron responds equally to changes in P_{aCO_2}/pHa.

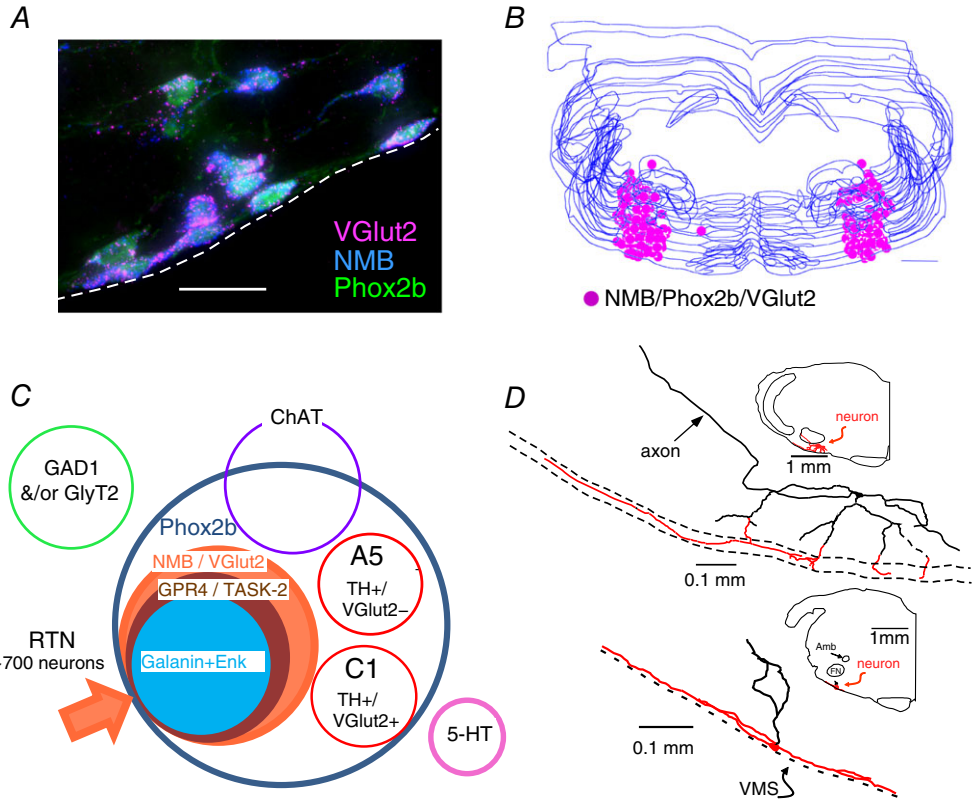

Figure 1. Anatomy of the retrotrapezoid nucleus
A, cluster of RTN neurons containing mRNA transcripts for VGlut2 and NMB. These neurons are immunoreactive for eGFP, the latter denoting the presence of Phox2b (transverse section, JX-99 – Phox2b-eGFP mouse; dashed line identifies the ventral medullary surface (VMS). Scale bar = 25 μm. Unpublished work of R. L. Stornetta. Every neuron is triple-labelled. *B*, computer-assisted mapping of RTN neurons (NMB+) in one mouse. Neurons identified in a one-in-three series of 30 μm-thick transverse sections between 6.65 and 5.63 mm behind bregma. Scale bar = 500 μm (from Shi *et al.* 2017, with modifications). *C*, Venn diagram representing the various cell populations located in the parafacial region of the mouse. RTN neurons are defined as positive for Phox2b, VGlut2 and NMB. A least 90% of these neurons contain putative proton sensor TASK-2 and ~82% contain GPR4 (Shi *et al.* 2017). *D*, location and dendritic structure of two RTN neurons from rat recorded and labelled juxtacellularly *in vivo*. Note presence of extensive dendrites in the marginal layer (ML) regardless of the position of the cell body (adapted from Mulkey *et al.* 2004).

In fact, as illustrated by the accompanying Venn diagram (Fig. 1*C*), 10–20% of the parafacial neurons that have the biochemical markers previously used to characterize RTN chemoreceptors do not express detectable levels of proton sensors in mice. These neurons do not express Fos after hypercapnia either (Shi *et al.*, 2017). In brief, the vast majority of RTN neurons do have biochemical and physiological properties consistent with a central chemoreceptor function but around 10–20% do not (Fig. 1*C*). Finally, a single marker, neuromedin B mRNA, identifies RTN chemoreceptors with 80–90% fidelity in mice (Shi *et al.*, 2017).

RTN innervates selectively four respiratory-related regions: the ventral respiratory column in its entirety, the Kölliker-Fuse region, select lateral parabrachial nuclei and the ventrolateral portion of the nucleus of the solitary tract (Fig. 2*A*) (Bochorishvili *et al.* 2012). In rodents, most RTN neurons have extensive dendrites just below

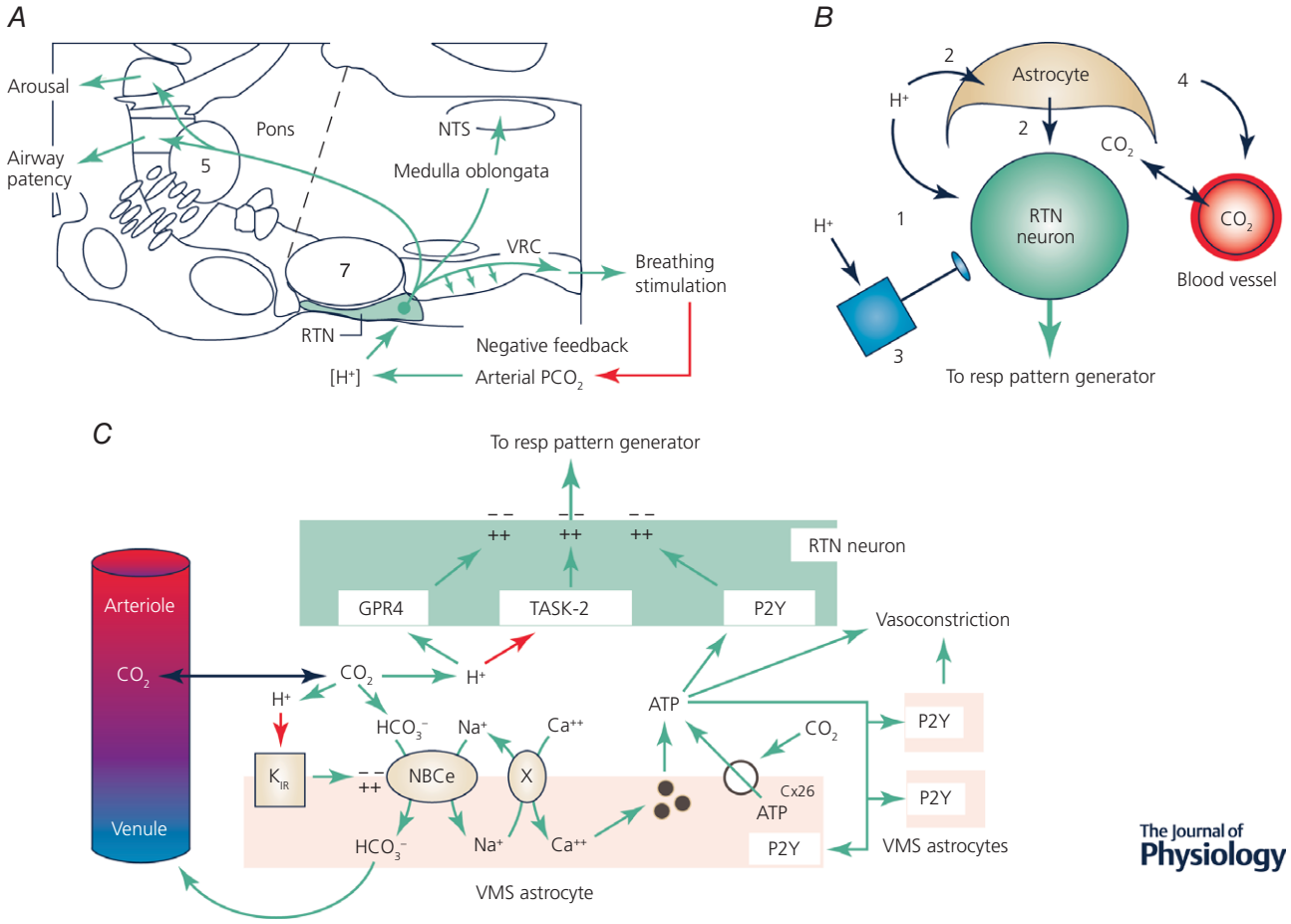

Figure 2. Retrotrapezoid nucleus: axonal projections and CO_2 sensing mechanisms
A, brain regions targeted by RTN neurons. Schematic summary of results from Bochorishvili *et al*. (2012). *B*, CO_2 detection by RTN neurons. Four mechanisms are thought to contribute to the exquisite sensitivity of RTN neurons to hypercapnia *in vivo*: (1) direct effect of proton on RTN neurons, (2) paracrine effect of protons mediated via acid-sensitive astrocytes, (3) excitatory inputs from other pH-sensitive neurons, (4) CO_2-induced vasoconstriction. The relative importance of each of the 4 mechanisms is unsettled. *C*, cell autonomous and paracrine mechanisms underlying the CO_2 sensitivity of RTN neurons. The cell-autonomous neuronal response to protons is mediated by TASK-2 (Kcnk5) and GPR4. RTN is surrounded by astrocytes that are depolarized by local acidification. The depolarization activates an electrogenic sodium–bicarbonate transporter (NBCe) which alkalizes the glial cytoplasm and simultaneously acidifies the extracellular space. This extracellular acidification likely potentiates the intrinsic response of RTN neurons to a given change in arterial pH/P_{CO_2}. Sodium entry via NBCe activation also stimulates sodium–calcium exchange. The rise in intracellular calcium causes exocytosis of gliotransmitters such as ATP. ATP may also be released through connexin26 hemichannels opened by carbamylation. ATP recruits neighbouring astrocytes and may depolarize RTN neurons. Acid-induced ATP release (from astrocytes or other unidentified cells) causes vasoconstriction, reduced wash-out of metabolically produced CO_2 and further acidification. Key supporting references: Mulkey *et al*. (2004); Erlichman & Leiter, (2010); Gestreau *et al*. (2010); Gourine *et al*. (2010); Huckstepp *et al*. (2010); Wenker *et al*. (2010); Wang *et al*. (2013); Kumar *et al*. (2015); Turovsky *et al*. (2016); Hawkins *et al*. (2017).

the ventral surface of the brain (Fig. 1*D*). This anatomical peculiarity suggests that these neurons could also respond to the chemical composition of the cerebrospinal fluid, CO_2 included.

Mechanisms responsible for the pH/CO_2 sensitivity of RTN neurons

Four mechanisms are thought to contribute to the CO_2-sensitivity of RTN neurons (Fig. 2*B*): (1) a cell-autonomous effect of protons on these neurons, (2) a paracrine effect mediated via surrounding CO_2-responsive astrocytes, (3) mono- or polysynaptic inputs from other CO_2-responsive neurons and (4) a vaso-constrictor effect of CO_2. Type-3 mechanisms include a polysynaptic input from the carotid bodies to RTN and direct inputs from CO_2 activated CNS serotonergic, noradrenergic or orexinergic neurons.

The intrinsic response of RTN neurons to $[H^+]$ is a depolarization mediated via proton sensors TASK-2 and GPR4 (Kumar *et al.* 2015; for review, see Guyenet *et al.* 2016). Surrounding astrocytes are also depolarized by acidification but via a different mechanism (K_{IR}4.1/5.1) (Gourine *et al.* 2010; Wenker *et al.* 2010). Astrocyte depolarization may further enhance the acidification of the RTN region relative to that of the plasma via the phenomenon called depolarization-induced alkalization, thereby increasing the sensitivity of RTN neurons to changes in arterial pH. Astrocytes may also activate RTN neurons by releasing ATP, prostaglandin E2 or D-alanine (Gourine *et al.* 2010; Beltran-Castillo *et al.* 2017; Forsberg *et al.* 2017; see legend to Fig. 2*C* for additional references and further details regarding these mechanisms). Finally, in the RTN region, hypercapnia may produce arteriolar constriction (Hawkins *et al.* 2017). This newly identified and unexpected vasoconstrictor effect of CO_2 may be unique to the ventrolateral medulla. *In vivo*, vasoconstriction likely causes further CO_2 retention, parenchymal acidification and ultimately activation of RTN neurons (Xie *et al.* 2006; Hawkins *et al.* 2017). The relative importance of the four mechanisms listed above is yet to be clearly determined and could vary depending on the characteristics of the hypercapnic stimulus (e.g. intensity, duration).

Evidence that RTN neurons mediate the central respiratory chemoreflex

The conclusion that RTN neurons mediate the central component of the ventilatory response to changes in arterial P_{CO_2} (Fig. 2*A*) relies on four congruent pieces of evidence. In anaesthetized rats, RTN neurons increase their firing rate on exposure to hypercapnia (0.5 Hz per 0.01 change in arterial pH (pH_a)), including when the carotid bodies or the nucleus of the solitary tract are disabled (Mulkey *et al.* 2004; Kumar *et al.* 2015; Wakai *et al.* 2015; Fig. 3*A*). Optogenetic activation of RTN produces very large increases in breathing and RTN inhibition elicits major breathing reductions (Abbott *et al.* 2011; Basting *et al.* 2015; Holloway *et al.* 2015; Fig. 3*B*). The breathing reduction caused by inhibiting RTN is linearly related to pH_a below a threshold of ~7.5; in quietly resting animals, these cells do not contribute to ventilation above this pH level (Fig. 3*C* and *D*). Thus, RTN neurons encode P_{aCO_2} (and pH_a) almost linearly *in vivo* with a pH_a recruitment threshold at around pH 7.5 and, most importantly, they appear to drive breathing in proportion to their discharge rate. A fourth and crucial piece of evidence is that RTN lesions performed by genetic means or via local injection of NK1R selective saporin-based toxins produce profound reductions of the hypercapnic ventilatory reflex (Nattie & Li, 2002; Dubreuil *et al.* 2008; Ramanantsoa *et al.* 2011; Takakura *et al.* 2014).

RTN neurons drive multiple aspects of breathing, including active expiration

Optogenetic stimulation of RTN neurons in quietly awake rodents increases breathing frequency and inspiratory amplitude; phasic activation of RTN also entrains the breathing cycle and elicits active expiration (Fig. 4*A*; Abbott *et al.* 2011; Burke *et al.* 2015; Holloway *et al.* 2015). These effects require the release of glutamate by RTN neurons (Holloway *et al.* 2015). Optogenetic activation of RTN also reduces expiratory flow immediately following inspiration (Fig. 4*A*). This phenomenon (expiratory brake) could be caused by enhanced diaphragmatic contraction and/or increased laryngeal resistance during the post-inspiratory phase of the breathing cycle.

In the arterially perfused rat preparation, non-selective inhibition of the RTN region or selective pharmacogenetic inhibition of RTN neurons eliminates the expiratory abdominal muscle outflow elicited by hypercapnia (Abdala *et al.* 2009; Marina *et al.* 2010). Thus, both gain (Burke *et al.* 2015) and loss of function experiments (Marina *et al.* 2010) show that RTN neurons stimulate multiple aspects of breathing, *including* active expiration. Whether RTN neurons and the proposed parafacial 'oscillator for active expiration' are one and the same, overlapping or entirely separate neuronal populations remains to be determined (Janczewski & Feldman, 2006; Pagliardini *et al.* 2011; Huckstepp *et al.* 2015, 2016; de Britto & Moraes, 2017).

The effect of RTN stimulation on breathing is state dependent

Optogenetic activation of RTN neurons produces substantially different effects on breathing depending on the state of vigilance (Fig. 4). The forced expiration

elicited during the E2 phase when rats are quietly awake disappears during non-REM sleep and the peak expiratory flow migrates to the early part of the expiratory phase (E1) denoting a simultaneous reduction of the expiratory brake (Burke *et al.* 2015; Fig. 4*A*). However, during this sleep phase, RTN stimulation still increases inspiratory rate and amplitude (Burke *et al.* 2015). In other words, the network responsible for active expiration and post-inspiratory brake is less responsive to excitatory inputs from RTN during non-REM sleep than during quiet

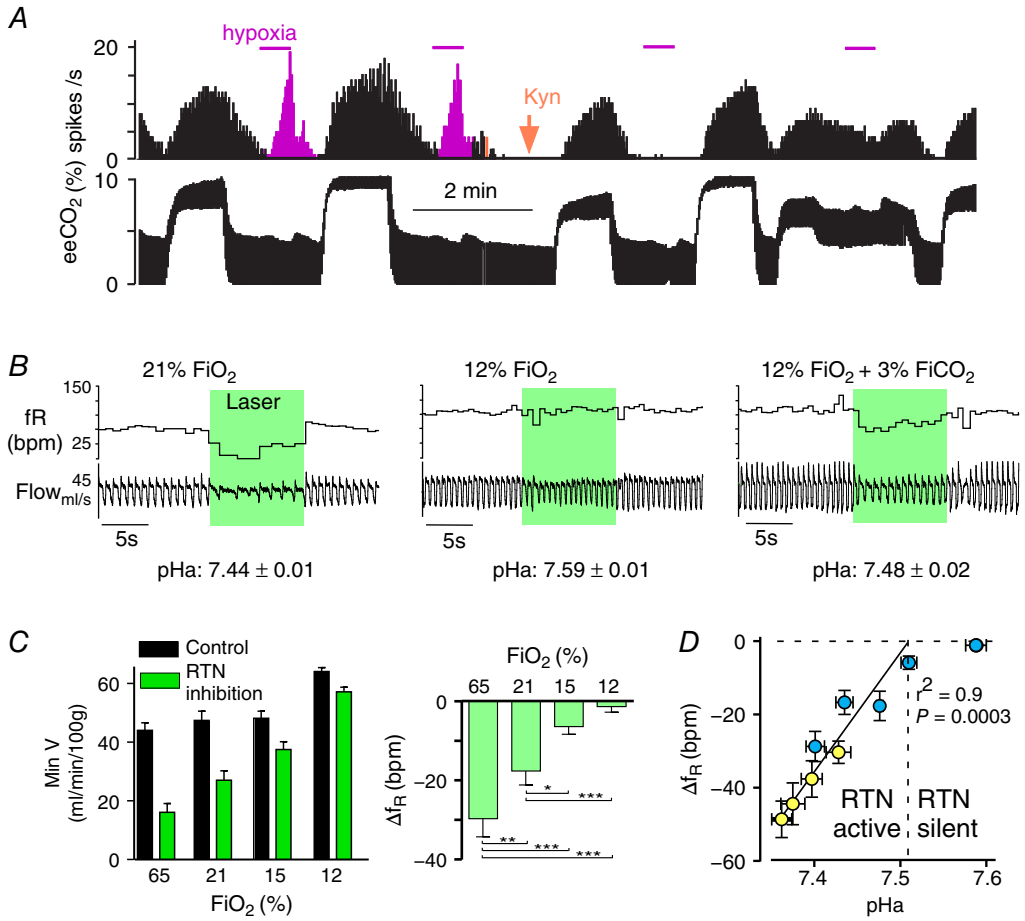

Figure 3. RTN-dependent and -independent activation of breathing by the carotid bodies
A, single-unit recording evidence that carotid body stimulation can activate RTN. This RTN neuron was activated by hypercapnia ($eeCO_2$: end-expiratory CO_2) and by brief hypoxia (magenta). Administration of kynurenate I.C.V. (orange arrow), a blocker of excitatory glutamatergic synapses, eliminated the effect of hypoxia selectively whereas the effect of hypercapnia, which is primarily mediated by cell-autonomous and paracrine effects of CO_2, persisted (redrawn from Mulkey *et al*. 2004). *B*, evidence that the carotid bodies activate breathing via a pathway that bypasses RTN. Bilateral opto-inhibition of RTN neurons (conscious rats) reduces breathing under normoxia (21% F_{I,O_2}) but has no effect under hypoxia (12% F_{I,O_2}). Breathing rate and amplitude increased under hypoxia despite RTN being silent, demonstrating that the CBs activated breathing via a pathway that bypasses RTN. The addition of a small amount of CO_2 restored the breathing reduction caused by RTN inhibition, suggesting that hypoxia-induced hyperventilation had inhibited RTN via the resultant respiratory alkalosis. Average plasma pH identified in 6 rats is shown below the representative examples (modified from Basting *et al*. 2015). *C*, breathing reduction elicited by bilateral optoinhibition of RTN is reduced under hypoxia. Left panel, minute volume before (black bars) and during RTN inhibition (green bars); right panel change in breathing frequency elicited by RTN inhibition. At 12% F_{I,O_2}, breathing is activated but RTN no longer contributes to breathing (right panel). At 15% F_{I,O_2} breathing is unchanged from room air but, as indicated in the right panel, a much smaller portion of the respiratory drive originates from RTN (modified from Basting *et al*. 2015). *D*, relationship between arterial pH and effect of RTN inhibition on breathing frequency (f_R) and plasma pH was manipulated with hypoxia. Respiratory alkalosis was compensated by adding CO_2 (blue symbols) or by inducing metabolic acidosis (acetazolamide, yellow symbols). Above pH_a 7.5, RTN inhibition has no effect on breathing consistent with single-unit evidence that the neurons are silent (e.g. panel *B*). Below pH 7.5, the breathing reduction elicited by RTN inhibition (change in breathing frequency is depicted) is a linear function of arterial pH, consistent with single-unit evidence (not shown) that RTN neurons are increasingly active (modified from Basting *et al*. 2015).

resting. Reduced release of wake-promoting transmitters (e.g. noradrenaline, serotonin, orexin) at strategic sites of the breathing network (abdominal motor and/or pre-motor neurons, expiratory 'oscillator') could underlie this reduced excitability.

The transition from non-REM to REM sleep brings about a different set of changes (Burke *et al.* 2015). As the rat enters REM sleep, RTN stimulation immediately ceases to elicit any effect on breathing frequency whereas inspiratory amplitude is still elevated (Burke *et al.* 2015). The converse is also true; selective optogenetic inhibition of RTN neurons reduces breathing rate and amplitude profoundly during non-REM sleep whereas, during REM sleep, inspiratory amplitude alone is decreased (Fig. 4*B*) (Burke *et al.* 2015). Finally, during quiet waking or non-REM sleep, the breathing cycle can be faithfully entrained by phasic stimulation of RTN at rates above the resting breathing frequency but no such entrainment can be produced during REM sleep (Burke *et al.* 2015). This change probably denotes a fundamental difference

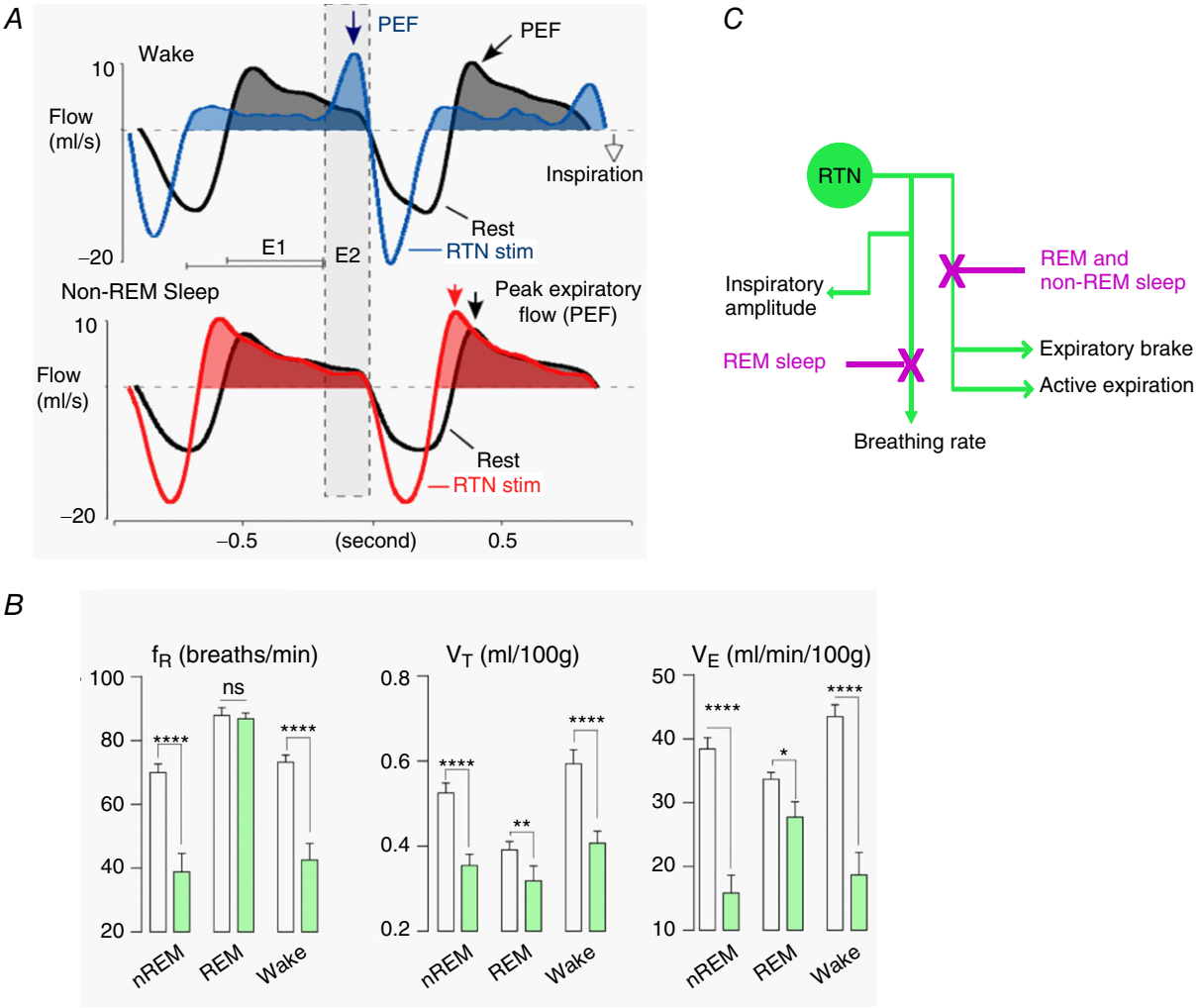

Figure 4. RTN activates multiple breathing parameters in a state-dependent fashion
A, unilateral phasic optogenetic activation of RTN (channelrhodopsin-2; trains of 3 light pulses; trains delivered at a rate slightly above resting breathing frequency) during quiet waking entrains the respiratory pattern generator, increases inspiratory amplitude, produces active expiration (note the distinctive peak-expiratory flow, PEF, during late expiratory phase, E2) and activates the expiratory brake (note reduced expiratory flow during early expiration, E1). During non-REM sleep (same rat), RTN activation still entrained the pattern generator and increased inspiratory amplitude but active expiration and the expiratory brake were no longer elicited (from Burke *et al*. 2015, with slight modifications). *B*, bilateral optogenetic inhibition of RTN (archaerhodopsin, 10 s light pulses; green bars) reduces respiratory frequency (f_R) and tidal volume (V_T) during quiet resting and non-REM sleep. During REM sleep RTN inhibition has no effect on f_R but still reduces V_T, albeit to a lesser degree. This evidence indicates that RTN controls breathing frequency only when the pattern generator is auto-rhythmic (slow wave sleep or quiet awake state) but not when it is externally driven, as in REM sleep. Adapted from Basting *et al*. 2015). *C*, schematic representation of the four known effects of RTN on breathing and their differential regulation by the state of vigilance.

in the way inspiration is generated in different arousal states. During anaesthesia, quiet waking and non-REM sleep, inspiration is probably generated autonomously by the relatively regular discharges of the preBötzinger complex and its surround (Kam *et al.* 2013; Rybak *et al.* 2014). During REM sleep, as during voluntary control, the expiratory phase is likely controlled by inputs originating outside this circuitry (Richter & Smith, 2014). The optogenetic evidence obtained in rats indicates that RTN neurons regulate breathing frequency only when the pontomedullary respiratory circuit is in auto-rhythmic mode (anaesthesia, quiet rest and non-REM as opposed to active behaviour or REM sleep) (Fig. 4*B*; Abbott *et al.* 2011; Burke *et al.* 2015). Thus, besides driving inspiratory amplitude and active expiration, RTN is a key regulator of breathing auto-rhythmicity. Frequency regulation, like inspiratory amplitude control is mediated primarily by glutamate and possibly by direct projections of RTN to the rhythmogenic neurons of the preBötzinger complex (Bochorishvili *et al.* 2012; Holloway *et al.* 2015). The simplest, though yet untested, mechanism could be that RTN increases the breathing rate by accelerating the inter-burst depolarization rate of preBötzinger complex rhythmogenic neurons.

The mechanism by which RTN controls breathing frequency is speculative at this point. However, the loss of frequency control by RTN during REM sleep is consistent with breathing frequency being virtually insensitive to hypercapnia during this stage of sleep (reviewed by Coote, 1982; additional citations in Horner *et al.* 2002; Burke *et al.* 2015). The effect of hypoxia (HVR) and that of pulmonary afferents (Hering-Breuer reflex) on breathing frequency is also greatly diminished during this sleep stage.

In short, in rodents, RTN neurons and CNS hypercarbia elicit similar effects on a broad range of respiratory motor outflows (Abbott *et al.* 2011). This is consistent with the notion that RTN controls lung ventilation by modulating inspiratory, expiratory and post-inspiratory (glottis, diaphragm) muscle activity in an orderly manner according to the intensity of the chemoreceptor stimulus. Like those of CO_2, the effects of RTN on breathing are profoundly state dependent, the most dramatic aspect being the loss of effect of RTN on breathing frequency during REM sleep (Fig. 4*C* for summary).

The carotid bodies stimulate breathing via and independently of RTN

Except during REM sleep, carotid bodies and central chemoreceptors provide a substantial tonic drive to breathe, even at rest under normoxic and normocapnic conditions (Smith *et al.* 1995; Pan *et al.* 1998; Dahan *et al.* 2007; Burke *et al.* 2015). Hypoxia and hypercapnia have supra-additive effects on breathing, which are at least partly caused by the robust potentiative effect of acid and low P_{O_2} on the activity of carotid body afferents (Daristotle & Bisgard, 1989; Kumar & Prabhakar, 2012). Centrally, carotid body afferent activity and central chemoreceptors have additive or supra-additive effects on breathing in intact and unanaesthetized mammals whereas hypo-additive effects are observed in an arterially perfused rat preparation (Duffin & Mateika, 2013; Teppema & Smith, 2013; Wilson & Day, 2013; Smith *et al.* 2015).

An excitatory polysynaptic pathway between carotid body afferents and RTN neurons has been identified by single unit recordings in anaesthetized rats (Fig. 5; pathway 1); under normocapnia, RTN neurons are powerfully excited by brief hypoxia or by intravenous administration of cyanide (Fig. 3*A*; Mulkey *et al.* 2004; Takakura *et al.* 2006). This activation requires the integrity of the carotid sinus nerves and the nucleus of the solitary tract (NTS); it may be mediated by a direct excitatory connection between NTS second-order neurons and RTN (Fig. 5, pathway 1; Takakura *et al.* 2006). The existence of this connection suggests that the carotid bodies activate breathing in part by stimulating RTN.

The carotid bodies also undoubtedly stimulate breathing via pathways that bypass RTN (pathway 2, Fig. 5). Experiments conducted in an arterially perfused preparation indicated that the carotid bodies can activate breathing when brain perfusate P_{CO_2} is reduced to levels that should be well below central chemoreceptor threshold (Fiamma *et al.* 2013). Evidence that the carotid bodies can stimulate breathing via pathways that bypass RTN was also obtained in unanaesthetized rats. RTN inhibition (optogenetic) reduces breathing rate and amplitude in rats breathing room air or CO_2-enriched air but produces no effect in rats exposed to 12% inspired O_2 fraction (F_{I,O_2}) implying that the RTN had been silenced during hypoxia; the addition of a minimal amount of CO_2 (F_{I,CO_2}

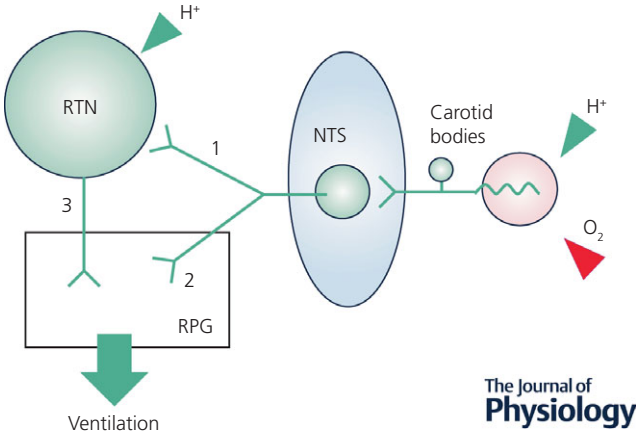

Figure 5. RTN-dependent (1) and RTN-independent pathways (2) from the carotid bodies to the respiratory pattern generator (RPG)

3%, in 12% F_{I,O_2}) restored opto-inhibition of breathing, indicating that RTN neurons were reactivated (Fig. 3*B* and *C*; Basting *et al.* 2015). Accordingly, in an intact rat, the respiratory alkalosis elicited by hypoxic hyperventilation appears to reduce the excitability of RTN neurons to such a degree that they no longer respond to the excitatory input from the carotid bodies. Yet, despite RTN being silenced, breathing is maintained or even elevated if F_{I,O_2} is low enough (~12% or less in Sprague-Dawley rats). This can only be explained by the existence of strong inputs from the carotid bodies to the respiratory pattern generator that bypass RTN.

In short, in unanaesthetized mammals, humans included, the interaction between carotid bodies and central chemoreceptors is at least additive and often potentiative. The carotid bodies activate the respiratory pattern generator via RTN and independently of this nucleus. The indirect pathway through RTN could perhaps explain why the interaction between carotid body afferents and central chemoreceptors can be supra-additive.

Circumstances under which the activity of RTN and carotid bodies vary in the opposite direction

Under most physiological conditions, the activity of RTN and carotid bodies changes in parallel (e.g. during hypoventilation, when both increase). However, there are occasions when their activity is regulated in opposite directions. One such case, discussed in the previous paragraph in another context, is poikilocapnic hypoxia; increasing degrees of carotid body activation and the ensuing respiratory alkalosis lead to correspondingly larger inhibition of RTN neurons until these cells become silent, somewhere around 12% F_{I,O_2} in conscious rats (Fig. 3*B* and *C*).

A second example is when RTN is selectively inhibited using optogenetics in conscious rats breathing room air; breathing returns quickly towards control and a breathing overshoot is observed after the optical inhibition is terminated (Fig. 6*A*). Breathing recovery and overshoot are primarily mediated by an increase in respiratory frequency (f_R) and are absent when the carotid bodies are either silenced by hyperoxia (65% F_{I,O_2}) or surgically denervated (Fig. 6*A* and *B*). Thus, *within this specific time frame*, both recovery and overshoot of breathing following RTN inhibition result from carotid body activation rather than from reactivation of RTN or other central chemoreceptors (interpretation in Fig. 6*C*; Basting *et al.* 2016).

Parallel or opposite changes in RTN *vs.* carotid body activity enhance arterial P_{CO_2} stability; implication for diseases in which the carotid bodies are hyperactive

The activity of peripheral and central chemoreceptors usually varies in the same direction because the most common trigger is hypo- or hyperventilation, perturbations that are characterized by opposite changes in arterial P_{O_2} and P_{CO_2}. In such situations the feedback exerted by carotid bodies and RTN is additive or even potentiative (Smith *et al.* 2015); its obvious function is to minimize arterial P_{CO_2} fluctuations.

During hypoxia, the activity of carotid bodies and RTN varies in opposite directions. By reducing the magnitude of the hyperventilation, RTN inhibition minimizes the respiratory alkalosis elicited by the increase in carotid body activity (Basting *et al.* 2015). Thus, in this situation, RTN inhibition also contributes to arterial P_{CO_2} stability, at least until F_{I,O_2} reaches a level low enough that oxygen delivery takes precedence over CO_2 homeostasis and ventilation increases substantially. In conscious rats, RTN inhibition elicits a rapid compensatory increase in carotid body activity that also largely and rapidly restores breathing (Basting *et al.* 2016). Thus, again under this circumstance, the activity of the carotid bodies and that of RTN change in opposite directions and the ultimate result of the increase in carotid body activity is to buffer the change in arterial P_{CO_2} (hypercapnia in this case) caused by a sudden decrease in central chemoreceptor activity that was not caused by reduced arterial P_{CO_2}. In other words, the network is built to favour P_{CO_2} homeostasis at the expense of P_{O_2} stability until hypoxaemia becomes severe and oxygen delivery becomes the overriding priority.

Carotid body hyperactivity consisting of hypersensitivity to hypoxia and aberrant 'tonicity' occurs in animal models of essential hypertension, heart failure and obstructive sleep apnoea (OSA) (Kumar & Prabhakar, 2012; Del Rio *et al.* 2013; Pijacka *et al.* 2016*a*,*b*). This hyperactivity is not driven by a change in arterial blood gases and contributes to the pathogenic increase in sympathetic nerve activity present in these human diseases and their animal models (Pijacka *et al.* 2016*a*,*b*).

In human essential hypertension, OSA, mild to moderate congestive heart failure, sympathetic tone and blood pressure are elevated but, unlike in some animal models of these diseases, *resting* ventilation under normoxia is unchanged (Narkiewicz *et al.* 1999*a*,*b*; Guardiola *et al.* 2004; Sinski *et al.* 2012; Del Rio *et al.* 2016). Based on our experiments on rodents, we suggest that in the waking state, a mild and sustained rise in carotid body activity may have little effect on breathing because a simultaneous reduction in central chemoreceptor (RTN) activity restores P_{aCO_2} and breathing to control (Basting *et al.* 2015). By contrast sympathetic stimulation could be sustained because its main countervailing influence, the baroreflex, is reduced when the carotid bodies are activated (McBryde *et al.* 2013).

A change in central cardiorespiratory coupling has been repeatedly invoked to explain the increased SNA associated with OSA, hypertension and CHF (Zoccal *et al.* 2008; Molkov *et al.* 2011; Menuet *et al.* 2017).

The evidence largely derives from semi-*in vitro* preparations in which the intensity of the respiratory drive cannot be precisely compared between animals; without such quantification, it is near impossible to distinguish between normal coupling (increased phasic SNA caused by increased central respiratory drive in a particular preparation) and abnormal coupling (increased phasic SNA despite normal activity of the respiratory network). Since breathing is unchanged in OSA or hypertensive patients at rest, only an abnormally large coupling, evidence for which is very limited, could make a difference (Fatouleh & Macefield, 2011). Finally, a change in breathing or cardiorespiratory coupling is not the only way in which carotid body afferent activity could enhance sympathetic tone: carotid body stimulation increases the discharge of ventrolateral medullary presympathetic neurons via a direct excitatory pathway from the NTS (Koshiya & Guyenet, 1996; Dempsey *et al.* 2017).

The ability of central chemoreceptors to compensate for an increase in carotid body activity is evidently limited by the amount of tonic excitatory drive that RTN neurons, and possibly additional central chemoreceptors, contribute to the respiratory pattern generator at rest. Under more severe hypoxia in rodents, ventilation does rise. Based on experiments in rats breathing 12% F_{I,O_2}, this rise occurs despite RTN being silenced. Under pathological conditions such as severe heart failure in humans, carotid body hyperactivity at rest may be high enough to overcome the loss of central chemoreceptor drive and hyperventilation is the result (Marcus *et al.* 2014). Chronic intermittent hypoxia (CIH), an animal model of OSA (Fletcher, 2001) sometimes produces a

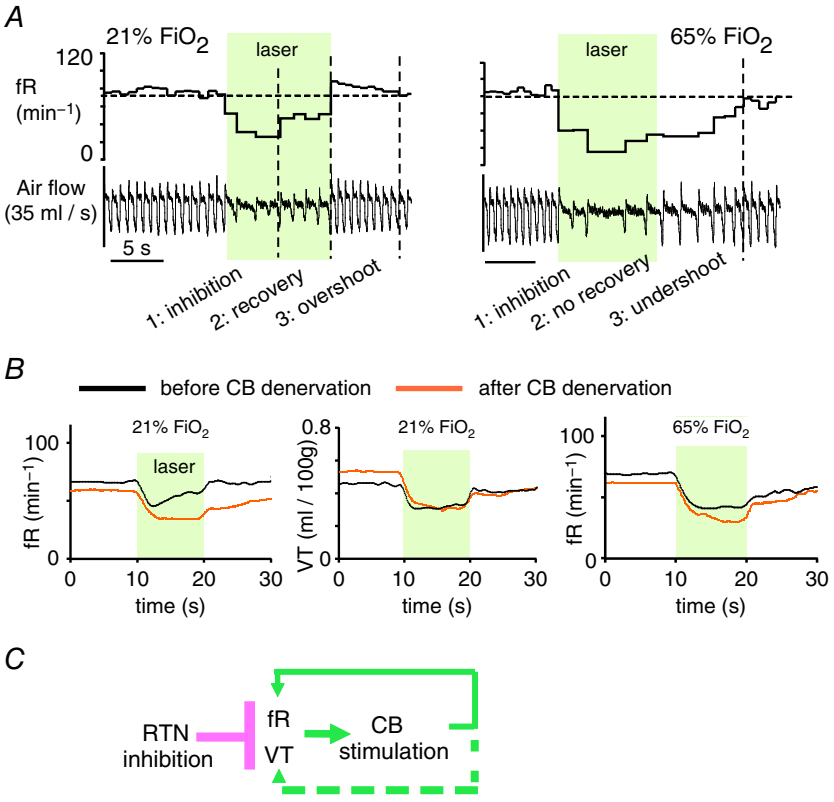

Figure 6. Carotid body activation rapidly restores breathing during RTN inhibition
A, representative breathing response to instant bilateral optogenetic inhibition of RTN in a conscious rat. Under normoxia, the nadir occurs within 5 s and is followed by a gradual recovery. A brief overshoot occurs when the laser is switched off. Under hyperoxia, to silence the carotid bodies, the recovery phase is not observed and breathing inhibition persists for some time after the light is switched off. Recovery and overshoot of f_R are therefore attributable to carotid body stimulation. Adapted from Basting *et al.* (2016). *B*, mean breathing response to bilateral opto-inhibition of RTN in conscious rats ($N = 6$). Left panel: frequency response before and 7 days after bilateral CB denervation (same 6 rats). Middle panel: tidal volume (V_T) response before and after CB denervation. Note that, contrary to f_R, V_T inhibition is unaffected by CB denervation. Right panel: under hyperoxia, the breathing response to RTN opto-inhibition is no longer affected by CB denervation. From Basting *et al.* (2016). Confidence intervals removed for clarity. *C*, sequence of events (schematic): RTN inhibition reduces both f_R and V_T causing a decrease in alveolar ventilation and subsequent carotid body activation via the ensuing changes in blood gases. In rats, the carotid bodies stimulate breathing by increasing breathing frequency primarily; this effect quickly mitigates the hypoventilation elicited by RTN inhibition.

persistent increase in breathing rate and amplitude in rats (Reeves & Gozal, 2006) and may even elicit a permanent state of active expiration (Zoccal *et al.* 2008). CIH also modifies the discharge characteristics and hypoxic sensitivity of the preBötzinger complex in slices, denoting some persistent changes in cellular properties or a circuit configuration (Garcia *et al.* 2017). Animal models in which resting ventilation is elevated or disrupted at rest (CIH, heart failure) may present with a level of carotid body afferent activity considerably higher than in the human disease they are designed to mimic. These models may also experience brainstem abnormalities caused by direct effects of repeated cycles of hypoxia/re-oxygenation on brainstem circuits.

Summary and concluding remarks

The retrotrapezoid nucleus is a small cluster of glutamatergic neurons that have a well-defined developmental lineage (Egr-2, Phox2b, Atoh-1) and biochemical markers (e.g. Phox2b, NMB, VGlut2). These neurons selectively innervate the pontomedullary regions that harbour the respiratory pattern generator.

Most RTN neurons are CO_2 activated and respond with high sensitivity to changes in arterial pH *in vivo* (0.5 Hz per 0.01 pH). Collectively, RTN neurons drive breathing in direct proportion to arterial [H+] with a pH recruitment threshold close to 7.5 in conscious rats. The pH sensitivity of RTN neurons is at least partly intrinsic and relies on proton receptors GPR4 or TASK-2. This pH sensitivity is boosted by paracrine mechanisms mediated by pH-sensitive astrocytes and by a regionally specific vasoconstrictive effect of CO_2. RTN neurons also receive excitatory inputs from the carotid bodies. Finally, input from serotonergic and other classes of CNS neurons likely potentiate the response of RTN neurons response to CO_2. An upcoming challenge will be to assess the relative contribution of these various mechanisms to the CO_2 sensitivity of RTN neurons under different circumstances *in vivo*.

RTN neurons regulate alveolar ventilation and pulmonary CO_2 excretion by facilitating multiple aspects of breathing (inspiratory amplitude, breathing rate, active expiration and, possibly, airway patency). The effect of RTN on these parameters is differentially gated or modulated according to the state of vigilance. Specifically, during REM sleep, the breathing rate is affected neither by hypercapnia not by RTN stimulation and the active expiration triggered by RTN stimulation during quiet waking disappears during slow-wave sleep. Each of these breathing parameters could conceivably be regulated by a dedicated subset of RTN neurons. Whether RTN and the parafacial oscillator for active expiration (Huckstepp *et al.* 2016) are distinct or overlapping sets of neurons is unsettled.

At this time, there is no compelling reason to assume that the sole function of RTN is central respiratory chemoreception. RTN could also mediate the hyperpnoea of exercise as suggested by a Fos expression study (Barna *et al.* 2014); this possibility is consistent with the fact that these neurons stimulate active expiration (Marina *et al.* 2010; Abbott *et al.* 2011). Whether RTN also contributes to the emotional control of breathing and to diet- or obesity-associated changes in ventilation has not been investigated.

The peripheral and the central respiratory chemoreflexes are highly interdependent. The breathing network is especially responsive to hypercapnic hypoxia, the consequence of hypoventilation, because RTN and the carotid bodies have cumulative excitatory effects on the respiratory pattern generator and RTN neurons receive an excitatory polysynaptic input from the carotid body afferents. The network is therefore poised to respond with extreme sensitivity to the slightest degree of hypo- or hyperventilation by triggering a breathing change of the opposite sign that restores arterial P_{CO_2} to normal.

Under other circumstances, a primary change in the activity of one type of chemoreceptor triggers a change of opposite sign in the other (e.g. RTN inhibition during hypoxia, or carotid body activation following a sudden reduction in RTN activity). These interactions are probably driven by changes in blood gases rather than by cross-talk between brainstem pathways implicated in peripheral *vs.* central chemoreception but this point is not definitively established.

Chronically elevated carotid body activity underlies the increased sympathetic tone and hypertension associated with OSA and essential hypertension. In conscious humans, the rise in SNA is not accompanied by a change in resting breathing. As a tentative explanation we propose that when carotid body afferent activity is only moderately increased, breathing homeostasis is restored by a countervailing reduction in the activity of central chemoreceptors, notably RTN. As suggested previously (Pijacka *et al.* 2016*a*), SNA may remain chronically elevated because of a reduction in the baroreflex. Also, because resting breathing is unchanged in OSA and hypertension, we also suggest that the emphasis on cardiorespiratory coupling as the underlying cause of the increased SNA may be exaggerated and that the role of pathways that directly link carotid body afferents to the vasomotor presympathetic neurons of the RVLM should be further evaluated.

Finally, the theory that poikilocapnic hypoxia silences central respiratory chemoreceptors via alkalosis in conscious mammals has been axiomatic for decades but its cellular basis has long remained obscure. RTN is indeed switched off by poikilocapnic hypoxia despite the persistent or increased activation of the respiratory pattern generator (Basting *et al.* 2015). This evidence is consistent

with the original theory regarding the effects of alkalosis on central chemoreceptors while, at the same time, providing additional evidence that RTN may indeed be the hub of the central respiratory chemoreflex.

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Additional information

Competing interests

None declared.

Author contributions

All authors are current or very recent members of our laboratories (P.G.G. and D.A.B.). They have read, edited and contributed to and approved this review. P.G.G., D.A.B. and R.L.S. wrote the manuscript. P.G.G., D.A.B., R.L.S., R.K., Y.S., B.B.H., G.M.P.R.S., T.M.B., S.B.G.A. and I.C.W. designed and performed many of the experiments discussed in this review. All persons listed as authors qualify for authorship and all those who qualify are listed.

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