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Central respiratory chemoreception

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Abstract

Brain PCO_2 is sensed primarily via changes in $[H^+]$. Small pH changes are detected in the medulla oblongata and trigger breathing adjustments that help maintain arterial PCO_2 constant. Larger perturbations of brain CO_2/H^+, possibly also sensed elsewhere in the CNS, elicit arousal, dyspnea and stress, and cause additional breathing modifications. The retrotrapezoid nucleus (RTN), a rostral medullary cluster of glutamatergic neurons identified by co-expression of *Phoxb* and *Nmb* transcripts, is the lynchpin of the central respiratory chemoreflex. RTN regulates breathing frequency, inspiratory amplitude, and active expiration. It is exquisitely responsive to acidosis in vivo and maintains breathing auto-rhythmicity during quiet waking, slow-wave sleep, and anesthesia. The RTN response to $[H^+]$ is partly an intrinsic neuronal property mediated by proton sensors TASK-2 and GPR4 and partly a paracrine effect mediated by astrocytes and the vasculature. The RTN also receives myriad excitatory or inhibitory synaptic inputs including from $[H^+]$-responsive neurons (e.g., serotonergic). RTN is silenced by moderate hypoxia. RTN inactivity (periodic or sustained) contributes to periodic breathing and, likely, to central sleep apnea. RTN development relies on transcription factors Egr2, Phox2b, Lbx1 and Atoh1. *PHOX2B* mutations cause congenital central hypoventilation syndrome; they impair RTN development and consequently the central respiratory chemoreflex.

Keywords

Central respiratory chemoreception; chemoreflex; chemoreceptors; hypercapnia; hypoxia; breathing; sleep apnea; brainstem; periodic breathing

Central respiratory chemoreception (CRC) is a type of interoception. As defined by Nattie (2012), CRC "refers to the detection of changes in CO_2/H^+ within the brain and the associated effects on breathing". The principal stumbling block of CRC research has always been the coordinating conjunction "and". Essentially all proteins (channels, receptors, enzymes, etc.) are titratable at some level of pH and therefore all cells are sensitive to $[H^+]$ variations; the challenge of CRC research is to determine causality between a molecular and cellular effect of pH and breathing stimulation.

In other areas of sensory neurobiology, the term "receptor" refers to neurons for which, increasingly, the relevant molecular detector is identified, i.e., thermoreceptors are neurons

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that contain the temperature-sensitive channels TRPV1 or TRPM8, baroreceptors are cells that contain the stretch-activated channels Piezo1/2, photoreceptors are specialized neurons that contain light-sensitive pigments. By analogy, central respiratory chemoreceptors can be defined as neurons that encode local changes in brain PCO_2, probably via changes in [H+], and regulate breathing (Figure 1A) (Loeschcke, 1982). A molecular detector for [H+] (co-expression of GPR4 and TASK-2) has been proposed in the case of the retrotrapezoid nucleus (RTN) (Gestreau et al., 2010; Kumar et al., 2015) but the notion that a central respiratory chemoreceptor is a multicellular sensor comprised of neurons whose sensitivity to CO_2 may be both an intrinsic property and the result of local paracrine mechanisms is rapidly gaining momentum (Figure 1B). A neuron whose response to CO_2 is exclusively the result of synaptic inputs from CO_2-responsive neurons located elsewhere does not qualify as a respiratory chemoreceptor although bona fide chemoreceptor neurons may derive a portion of their CO_2 sensitivity from synaptic inputs (Figure 1B).

The multicellular chemoreceptor concept compounds the difficulty of demonstrating that a particular cell type (neuron, astrocyte, pericyte, blood vessel) is a true respiratory chemoreceptor. The principal difficulty is to show unequivocally that the cell's response to CO_2/[H+] is an intrinsic property and that the cell can activate breathing as a result of this property. Five pieces of evidence are required to establish that a cell of any type is a bona fide respiratory chemoreceptor (or a CO_2-sensing component of a multicellular chemosensor): 1) Cell-specific activation and inhibition have opposite effects on breathing; 2) Cell-specific inhibition blunts effects of CO_2 on breathing; 3) Cell activity is CO_2/H^+-modulated in vivo; 4) Cell modulation by CO_2/H^+ is at least partly a direct effect; 5) Disrupting the mechanism for direct CO_2/H^+ sensing selectively in the presumptive chemoreceptor cell interferes with CO_2-stimulated breathing. Criteria 1-2 are necessary but not sufficient because the observed effects could result from modulation of the output that is independent of the PCO_2 sensing mechanism. Criteria 3 is also necessary but not sufficient because the observed effect could result from paracrine or synaptic mechanisms and the activation of the cell by CO_2 could be a network effect rather than a direct effect of a local change in PCO_2. Criteria 4 and 5 are clearly the most stringent and criteria 5 the only one that can be viewed as both necessary and sufficient. For these five criteria to be fulfilled, it is critical to have unambiguous methods to identify, record and selectively manipulate both the specific cell group and the direct CO_2/H^+ sensing mechanisms. To date, no candidate central respiratory chemoreceptor has met the full evidentiary requirement, as we will discuss, but substantial support has accrued for certain candidates that we will highlight.

The breathing stimulation elicited by a rise in brain PCO_2, defined as the hypercapnic ventilatory reflex (HCVR), was identified long ago (Loeschcke, 1982; Pappenheimer et al., 1965; Haldane and Priestley, 1905). Despite considerable progress (for recent reviews see: (Nattie and Li, 2012; Guyenet et al., 2019) the cells involved in CO_2 detection (neurons, astrocytes, blood vessels), their CNS location, and the molecular identity of the CO_2/H^+ detectors are still incompletely identified. The prevailing notion is that CO_2 is detected via the proxy of [H^+] but other results support an independent role of molecular CO_2 and/or bicarbonate (Nattie and Li, 2012; Guyenet and Bayliss, 2015; Meigh et al., 2013; Goncalves and Mulkey, 2018). As to the location of the respiratory chemoreceptor neurons, two opposing views have been championed. Originally, CRC was attributed to effects of

[H^+] that are sensed at one or a few spots on the ventral surface of the medulla oblongata (Loeschcke, 1982). A more recent view, the distributed chemosensitivity theory, posits that CRC is an emergent property of the CNS i.e., the aggregate effects of acidification that is sensed throughout the pontomedullary respiratory pattern generator and myriad brain regions that control it (Nattie and Li, 2012). Both viewpoints offer valid arguments that will be addressed.

The RTN is a major focus of this review. This nucleus resides at the ventral surface of the medulla oblongata, in a region suspected since the 1960s of harboring respiratory chemoreceptor neurons because breathing increases when it is topically acidified (Guyenet and Bayliss, 2015; Loeschcke, 1982; Mitchell et al., 1963). These neurons respond vigorously to hypercapnia in vivo, to CO_2/H^+ in vitro (Mulkey et al., 2004) and play a pivotal role in the regulation of breathing by CO_2 (Basting et al., 2015; Souza et al., 2018). Their phenotype and early developmental lineage are known in detail. Also, RTN has clear disease relevance (sleep apnea, congenital hypoventilation)(Goridis and Brunet, 2010). Finally, the cellular and molecular mechanisms implicated in their CO_2 sensitivity have been studied more thoroughly than that of any other candidate central respiratory chemoreceptor neurons.

Several important aspects of CRC (e.g., history, alpha-stat theory, technical details on microdialysis, advantages and limitations of plethysmography) are not or minimally addressed here. They have been clearly and exhaustively reviewed previously (Nattie and Li, 2012). Instead, this review emphasizes recent scientific findings related to the molecular, cellular, integrative, and developmental aspects of CRC.

We will air and briefly discuss all major hypotheses. The proposed interpretations reflect our perception of the strengths and weaknesses of the available evidence. The following prior reviews provide other perspectives on selected aspects of CRC (Nattie and Li, 2012; Teran et al., 2014; Nattie, 2011; Gourine and Kasparov, 2011; Duffin, 2010; Duffin, 2005; Huckstepp and Dale, 2011; Putnam, 2010).

Homeostatic and other functions of central respiratory chemoreception

A discussion of CRC should probably start by recognizing that a sudden rise in brain CO_2 affects breathing in two general ways depending on the intensity of the stimulus and the state of vigilance (Figure 2). The first is a simple feedback regulation of alveolar ventilation by arterial PCO_2; the other is the complex consequence of CO_2-induced arousal, stress and resulting behavior. The feedback component has the lowest threshold (< 0.01pH) and regulates breathing in a manner that is graded with the intensity of the stimulus ($\Delta PaCO_2$); this mechanism, including pH sensors and neural circuit, is probably confined to the lower brainstem (Figure 2, left side). In intact unanesthetized mammals, humans included, the feedback mechanism operates without causing any aversive sensation (dyspnea), stress or arousal; it is presumably responsible for most, perhaps all the breathing adjustments (increase or decrease) elicited by small perturbations of $PaCO_2$. The feedback component of CRC operates by activating the autorhythmic lower brainstem circuitry that maintains breathing at rest during wake and during non-rapid eye movement (NREM) sleep or

anesthesia. This network is described in detail elsewhere in this volume (chapter 1). Here, the emphasis will be placed on how this network is regulated by PCO_2 in the central nervous system (CNS).

Higher levels of $PaCO_2$ trigger additional effects on breathing that we will call the behavioral component of the HCVR (Figure 2, right side). A high CO_2 level signals the presence of a serious environmental or breathing abnormality, for example airway obstruction. Its effects on breathing depend on whether dyspnea, stress, arousal, and probably, increased metabolism resulting from increased muscle tone are elicited (Forster et al., 2012). Like any behavioral response, its specific manifestations engage vast regions of the brain and are probably species-specific. Unlike the feedback component, the behavioral component of CRC is not simply proportional to the stimulus intensity. For example, CO_2-induced arousal from sleep, is an all-or-none event triggered when the $PaCO_2$ reaches a given threshold. As soon as the animal wakes up, a step increase in breathing is observed which cannot be explained by an abrupt increase in brain PCO_2 or its proxy $[H^+]$ and denotes a sudden network response. The neural mechanisms responsible for CO_2 arousal are covered comprehensively in Chapter 7 of this volume. Also, in humans, dyspnea is not an all-or-none phenomenon; its intensity does increase with the degree of hypercapnia but the aversive sensation and, presumably the resulting cardiorespiratory responses, also have a $PaCO_2$ threshold well above that of the feedback component of the HCVR if breathing is not restricted (Buchanan and Richerson, 2009; Banzett et al., 2008). Dyspnea is reviewed in detail in chapter 11 of this volume.

The question can be raised, at least rhetorically, whether the real “purpose” of CRC is CO_2 homeostasis or to sound the alarm in case of severe hypoventilation. Arousal from sleep is a crucial survival mechanism and, during exercise, “central command” is clearly more important to PCO_2 stability than CRC (Forster et al., 2012; Eldridge et al., 1981). However, CRC fine-tunes PCO_2 homeostasis under all circumstances and its role is perhaps most critical during slow-wave sleep (SWS) or anesthesia because it prevents central apnea and the deleterious effects of brain hypoxia.

The relative importance of the feedback and behavioral components of CRC to the HCVR is hard to disambiguate, especially in animal experiments. The magnitude of the reflex varies considerably across rat strains for example and a burrowing species like the mole rat has almost no HCVR (Mouradian et al., 2012; Clayson et al., 2020). Such differences could reflect strain- or species-related differences in either the feedback or the behavioral component of CRC. The HCVR is most often studied in rodents during the day when they tend to be asleep. The hypercapnia typically used ($FiCO_2$ from 5 to 10%) inevitably triggers the behavioral component of the reflex but the EEG is rarely recorded to assess the degree of vigilance of the animal. Furthermore, the aversive quality of the hypercapnic stimulus, which rodents are also able to smell, hence the potential stress, is untested. In short, what is typically being measured is a mixed HCVR with lower brainstem and behavioral components whose relative contribution likely varies depending on the intensity of the stimulus, the species or strain. Extrapolation to humans of results obtained with small laboratory animals, especially mice, is also fraught because the neural mechanisms underlying the sensation of dyspnea and its behavioral consequences

are unknown. In this review, we argue that many brain neurons currently considered as respiratory chemoreceptors (e.g., the orexinergic neurons and the locus coeruleus) could be activated by hypercapnia principally as a result of CO_2-induced arousal, stress or a behavioral change rather than as a direct effect of CO_2/pH on these neurons.

1. The retrotrapezoid nucleus (RTN)

The RTN is probably the main nodal point through which changes in brain PCO_2 regulate breathing (Guyenet et al., 2019; Guyenet et al., 2016). This small bilaterally symmetric structure (~700 total neurons in mice) has also gained prominence as a prototypical respiratory chemoreceptor because the biochemical and multi-cellular basis of its pH sensitivity are understood in much greater details than that of other candidates. Finally, the suggestion that RTN fails to develop in patients with the congenital central hypoventilation syndrome (CCHS) has also boosted scientific interest in this structure (Stornetta et al., 2006; Amiel et al., 2003; Dubreuil et al., 2008).

1.1. Definition of the RTN—

The RTN is part of the reticular formation. As such it is not an architectonically recognizable nucleus but a collection of neurons that have a common developmental lineage and closely related transcriptome. These characteristics must be precisely defined to avoid misunderstandings concerning the function of the heterogeneous brain region where these neurons reside. A brief retrospective is thus required. The name RTN originally referred to neurons of unknown phenotype and function located below the facial motor nucleus caudal to the trapezoid body, which were identified by their axonal projections to or through the rostral ventral respiratory group (rVRG); the name has subsequently been used to describe the brain region that contains these neurons (Smith et al., 1989; Nattie and Li, 1995). Here we use the term RTN to describe a specific group of excitatory neurons located within this previously defined anatomical region, which express a combinatorial set of histochemical markers (described below). The importance of defining these cells based on a specific neurochemical phenotype is underscored by two recent observations. First, according to a single cell RNA-sequencing study of the RTN "region" ~70% of the non-cholinergic neurons (i.e., reticular formation neurons as opposed to facial motor neurons) are inhibitory and only ~11% contain markers that define the bona fide excitatory RTN neurons (Cleary et al., 2021). Secondly, the RTN "region" contains at least two types of neurons with projections to the ventral respiratory column one of which lacks chemoreceptor properties and the biochemical markers of the RTN proper (Magalhaes et al., 2021).

Bona fide RTN neurons express vesicular transporter-2 (VGlut2, *Slc17a6*), the homeodomain transcription factor Phox2b, the substance P receptor (NK1R), and lack the defining markers of catecholaminergic, serotonergic, cholinergic, GABAergic and glycinergic neurons (Figure 3A,B,E,F); they number ~700 in mice and ~2000 in rats (Ruffault et al., 2015; Ramanantsoa et al., 2011; Hernandez-Miranda et al., 2018; Dubreuil et al., 2009; Huang et al., 2012; Guyenet et al., 2016; Shi et al., 2017; Souza et al., 2020). All RTN neurons also contain transcripts for PACAP and Neuromedin B and most (>80%) contain mRNA coding for TASK-2 and GPR4, proteins that are required for their pH sensitivity (Figure 3C,E) (Shi et al., 2017). Co-localization of *Phox2b* and *Nmb*

mRNAs provides diagnostic identification of the rodent RTN (rats and mice) because *Nmb* is undetectable in neighboring Phox2b-expressing neurons such as facial motor neurons, catecholaminergic neurons (C1 and A5) and autonomic neurons (e.g., inferior salivary nucleus). Large inter-individual differences in mRNA levels encoding peptide transmitters (Neuromedin B, galanin, enkephalin) and many receptors have been observed among these neurons (Shi et al., 2017; Stornetta et al., 2009). This variability could denote the existence of distinct functional subgroups of RTN neurons, or differential regulation of particular genes under specific physiological circumstances.

In rodents, the RTN neurons are found at variable density under the entire extent of the facial motor nucleus (Figure 3F) with a major concentration (~ 2/3 of the total number) near the caudal end of the facial motor nucleus. RTN neurons have long (>1 mm) dendrites within 200 microns of the ventral medullary surface that commonly extend to the medial edge of the trigeminal tract (Figure 3D1,D2). Collectively, RTN neurons innervate the pontomedullary regions that generate the respiratory rhythm and pattern (caudal VRG, VRG, PreBötzinger and Bötzinger region, ventrolateral NTS, Kölliker-Fuse nucleus; locations shown in Figure 4a) (Bochorishvili et al., 2012). Whether each RTN neuron sends projections to all these regions or just a subset is unknown. RTN projections to the spinal cord, lower brainstem motor nuclei (hypoglossal, facial, trigeminal) or rostral to the pons have not been detected.

A neuronal cluster that could be the homolog of the rodent RTN has been identified in cats and in non-human and human primates in approximately the same brain region, i.e., below the facial motor nucleus (Levy et al., 2019; Rudzinski and Kapur, 2010; Bodineau et al., 2000b; Bodineau et al., 2000a). Its identification in primates was based on the presence of three markers expressed by the rodent RTN (immunoreactivity for Phox2b, the NK1R and *Gal* mRNA).

1.2. Physiological role of the mature RTN

a. Methodology: The first single-unit recordings of presumptive RTN neurons in vivo were probably obtained by Bodineau et al. in cats (Bodineau et al., 2000b; Bodineau et al., 2000a) although the phenotype of the recorded neurons could not be identified nor was it possible to test whether these neurons had respiratory chemoreceptor properties. By 2005, these limitations had been substantially overcome by combining single neuron recording and labeling in vivo, neuronal recordings in brain slices and various pharmacological tools to suppress synaptic transmission (Mulkey et al., 2004; Ritucci et al., 2005). This work, performed in rats, strongly suggested that the RTN region contains non-serotonergic glutamatergic respiratory chemoreceptors. In 2006, high levels of homeobox transcription factor Phox2b were identified in the RTN of the adult rat (Figure 3A) (Stornetta et al., 2006; Pattyn et al., 1997). Soon after, lentiviral vectors engineered with a Phox2b-responsive artificial promoter (PRSx8) were found to drive high levels of transgene expression predominantly in RTN neurons (Abbott et al., 2009; Marina et al., 2010; Hwang et al., 2001). The use of these vectors and the availability of several transgenic mouse strains (e.g., *Phox2b*-EGFP, *Phox2b*-Cre, and, more recently, *NMB*-cre) have been instrumental in

unraveling the structure and function of the RTN as defined in this review (Lazarenko et al., 2009; Li et al., 2020; Rossi et al., 2011).

b. RTN drives breathing in proportion to arterial pH: Under anesthesia in vivo, RTN neurons are typically silent when arterial pH (pHa) is above 7.4-7.5 and their activity increases linearly and sharply when pHa is reduced below that point (Guyenet et al., 2005; Mulkey et al., 2004). On average their discharge changes by 0.5 Hz per 0.01 pHa. In anesthetized rats, the effect of CO_2 on RTN neurons persists after pharmacological blockade of the respiratory pattern generator with a broad-spectrum glutamate receptor antagonist (Mulkey et al., 2004)whereas other respiratory related lower brainstem neurons become silent or unresponsive to CO_2(Mulkey et al., 2004). This unusual behavior suggested that the CO_2 sensitivity of RTN neurons might be caused by local acidification rather than by synaptic inputs. In unanesthetized rats, unilateral optogenetic activation of RTN neurons increases in breathing frequency and amplitude similarly to hypercapnia(Abbott et al., 2011; Souza et al., 2020). This effect requires the release of glutamate by the RTN neurons because the breathing stimulation is >95% reduced if vesicular glutamate transporter-2 (VGlut2) is selectively deleted from the optogenetically-stimulated RTN neurons (Holloway et al., 2015).

The firing of RTN neurons has not been directly recorded in unanesthetized rats but the overall respiratory drive contributed by these neurons has been inferred by measuring the instantaneous decrease in ventilation (ΔV_E) elicited by silencing these neurons optogenetically (Figure 5). Under respiratory alkalosis elicited by hypoxia (FiO_2 12%), RTN inhibition has very little effect on breathing (Figure 5)(Basting et al., 2015) but their optogenetic activation increases breathing robustly (Souza et al., 2020). Thus, moderate hypoxia (12-15% FiO_2) does not reduce the ability of RTN neurons to activate the rhythm and pattern generator but silences them. Based on this approach, the contribution of RTN to breathing in quietly resting unanesthetized rats has the same relationship to arterial pH as the RTN neuron firing rate in anesthetized animals, i.e., it is proportional to pH between pHa 7.45 and 7.25; above pHa 7.5 RTN neurons are silent (Basting et al., 2015). These properties are consistent with what would be expected of neurons that mediate the stimulatory effect of CO_2 on breathing.

c. RTN activates breathing frequency, inspiratory effort and active expiration: Optogenetic stimulation of RTN neurons increases breathing frequency, inspiration, and active expiration (Abbott et al., 2011; Abbott et al., 2009; Souza et al., 2020). Conversely RTN inhibition reduces all three parameters (Marina et al., 2010; Basting et al., 2015). These observations were made in rats and mice, conscious or anesthetized, and in an arterially perfused decerebrate preparation (working heart- brain preparation). The earlier results were obtained using opsin-expressing adeno-associated vectors that transduced RTN neurons as well as some of the neighboring C1 adrenergic neurons which also activate breathing in rodents (RTN:C1 ratio typically 3:1) (Souza et al., 2020; Abbott et al., 2014). More recently, RTN neurons ($Phox2b^+/NMB^+$) have been selectively stimulated in *Th*-cre rats in which the C1 cells located in the vicinity of RTN neurons had been selectively destroyed. In such rats, optogenetic stimulation of the RTN (>95% selectivity)

still robustly increases breathing frequency, tidal volume, phrenic nerve activity and active expiration (Figure 4B) (Souza et al., 2020). The circuitry through which RTN produces these various effects is not known precisely but plausible hypotheses can be proposed on anatomical grounds (Figure 4).

RTN neurons heavily innervate neurons located in the preBötzinger complex (Abbott et al., 2009; Bochorishvili et al., 2012), region that is most critical for breathing autorhythmicity (Gray et al., 1999; Del Negro et al., 2018). The fR increase elicited by RTN neurons could therefore result from a direct excitatory input to this rhythmogenic kernel (Figure 4A,C). However, according to Baertsch et al. (2018), the most effective way to increase the burst rate of the glutamatergic kernel is to reduce their refractory period and this is best produced by an increase in the activity of the inhibitory inspiration-related neurons of the preBötzinger complex. Thus, RTN could control the burst rate of the preBötzinger complex, hence fR, by two complementary actions: a decrease in refractory period mediated by an excitatory input to preBötzinger complex inhibitory interneurons and an acceleration of the “percolation phase” (Baertsch and Ramirez, 2019) via a direct excitatory input to the glutamatergic rhythm-generating core.

RTN also innervates the rVRG (identified in Figure 4A), a region of the ventrolateral medulla that contains phrenic and other inspiratory premotor neurons (Bochorishvili et al., 2012). The increased inspiratory amplitude elicited by RTN stimulation may therefore largely result from a direct excitatory input to the premotor neurons (Figure 4A). Finally, RTN also directly innervates the expiratory bulbospinal premotor neurons of the caudal ventral respiratory group (cVRG) (Figure 4A,D) (Souza et al., 2020). Thus, a direct excitatory input from RTN to the expiratory premotor neurons likely contributes to active expiration. In addition, RTN also innervates the Bötzinger region and the Kölliker-Fuse nucleus (Figure 4A) which, while not generating the breathing rhythm per se, regulate its frequency and the relative duration of the three phases of the respiratory cycle (Song and Poon, 2009; Dutschmann and Dick, 2012; Smith et al., 2013). RTN innervation of these regions could thus contribute to its effect on breathing frequency and may also facilitate the recruitment of expiratory muscles by reducing the strength of the late-expiratory inhibitory input to the cognate premotor neurons (Flor et al., 2020).

VGlut2 expression by RTN neurons is required for these cells to activate breathing (Holloway et al., 2015); thus RTN neurons probably signal on a fast time scale like the majority of the neurons that form the respiratory pattern generator. Indeed, breathing stimulation elicited by optogenetic activation of RTN reaches a maximum within a breath or two in anesthetized or unanesthetized rodents. However, RTN neurons also synthesize several neuropeptides. One of these, PACAP, is expressed massively at the time of birth and is required for optimal CO_2-stimulated breathing in neonatal and adult mice (Shi et al., 2021). Galanin, produced by at least 50% of RTN neurons, is generally inhibitory and may down-regulate the HCVR during sustained hypercapnia (Dereli et al., 2019; Stornetta et al., 2009). Finally, neuromedin-B (NMB) release by a subset of RTN neurons may trigger sighs (Li et al., 2020; Li et al., 2016b).

The respiratory stimulation elicited by optical stimulation of the RTN decays surprisingly slowly (~20s time constant) when end-tidal CO_2 is kept constant, e.g., in an artificially ventilated preparation (Abbott et al 2009). The phenomenon is not caused by a persistent increase in RTN action potentials beyond the stimulus period (Abbott et al 2009). Its kinetics are reminiscent of the "afterdischarge" (persistence of the increase in phrenic nerve activity) observed after electrical stimulation of the carotid sinus nerve or somatic afferents (Millhorn et al., 1981). The "afterdischarge" has its origin in the pontomedullary region but remains without definite cellular explanation. It could denote a primeval form of lower brainstem "arousal", perhaps dependent on the recruitment of neuromodulators with long-lasting effects on the respiratory pattern generator.

d. State-dependence of the HCVR: role of the RTN: Hypercapnia increases both fR and V_T; these effects are slightly more pronounced (larger increase per Torr) during quiet waking than during NREM sleep (Figure 6A) and least intense during anesthesia. As mentioned before (Figure 2), the enhancement during waking may be caused by the activation of cells such as the orexinergic or noradrenergic neurons that are silent during SWS and active to varying degree during waking(Carrive and Kuwaki, 2017; Huber et al., 2017; Li et al., 2016a; Dergacheva et al., 2016; Lazarenko et al., 2011; Carter et al., 2010; Adamantidis and L., 2009).

During REM sleep, the HCVR is also qualitatively different; the tidal volume increase is similar to quiet waking, but the most dramatic difference is that breathing frequency does not increase (Figure 6A). Although the frequency component of the reflex contributes more to the overall increase in minute ventilation in rodents than in larger species, the state-dependence of the HCVR has similar characteristics in rats (Figure 6A), cats (ΔT_{TOT} in ms/Torr: −34.7 in NREM sleep vs. +5.5 in REM sleep) (Netick et al., 1984) and humans (Douglas et al., 1982).

The breathing changes elicited by selective RTN stimulation in rats have the same state-dependence as those evoked by elevated CO_2 (Figure 6B). The difference between quiet waking and NREM sleep is merely quantitative, i.e., the effects of optogenetic RTN stimulation on fR and V_T are both moderately reduced during NREM sleep relative to quiet waking (Figure 6B). In striking contrast, RTN no longer exerts any control over fR during REM sleep although the nucleus still regulates inspiratory amplitude (Figure 6B)(Burke et al., 2015a). Likewise, optogenetic inhibition of RTN neurons fails to change fR during REM sleep but still reduces V_T (Burke et al., 2015a). In short hypercapnia and RTN can activate rhythm generator only when this network operates in auto-rhythmic mode (as during anesthesia, SWS and quiet resting (Burke et al., 2015a; Del Negro et al., 2018). A possible interpretation of this phenomenon is shown in Figure 6C1, C2). The scheme is based on two hypotheses. RTN innervates premotor neurons directly and its effect on the rhythm generator is gated out during REM sleep or active behaviors.

As mentioned above, RTN triggers active (a.k.a. abdominal) expiration (Burke et al., 2015a; Souza et al., 2020). In one study, active expiration was no longer evoked by RTN stimulation when the animals entered SWS or REM sleep (Burke et al., 2015a). Others have shown that hypercapnia ($FiCO_2$ 7%) can elicit active expiration during NREM as well as REM

sleep in rats (Leirao et al., 2017; Pisanski et al., 2020). This apparent discrepancy probably means that the excitability of the circuitry responsible for active expiration is lower during sleep than during the waking state. Therefore, activation of a small portion of the RTN ((equivalent to a low level of hypercapnia, as in (Burke et al., 2015a) is insufficient to trigger active expiration during NREM sleep but active expiration can still be produced during NREM sleep if $PaCO_2$ and, presumably, RTN stimulation is high enough.

In summary, RTN is a major regulator of breathing auto-rhythmicity and breathing amplitude, particularly during SWS. The contribution of the RTN to the HCVR is reduced during REM sleep because this nucleus does not control breathing frequency in that state. Although current understanding of breathing auto-rhythmicity has made considerable progress, very little is known about how it is regulated by the RTN and why this regulation is overridden during active behavior or REM sleep.

e. Interdependent control of breathing by the RTN and the carotid bodies: The carotid bodies and central chemoreceptors work cooperatively during hypoventilation: the increase in PCO_2 activates both structures and the decrease in PO_2 activates the carotid bodies. By contrast central and peripheral chemoreceptors work at cross purposes when arterial PO_2 is reduced, i.e., at altitude, because hypoxic hyperventilation causes respiratory alkalosis and the hypocapnia reduces RTN activity. Remarkably, in quietly resting rats, ventilation (V_E) is virtually unchanged during exposure to ambient gas mixtures varying in oxygen concentration between 65% FiO_2 (hyperoxia) and 15% FiO_2 (moderate hypoxia, equivalent to PO_2 present at an altitude of 2700 m) (Figure 5) (Basting et al., 2015). With hypoxia, the brief initial hyperventilation quickly leads to a new steady state characterized by a persistent and stable small reduction of both arterial PO_2 and arterial PCO_2 and a rise of pHa and, presumably, brain extracellular fluid (ECF) pH. Under these conditions, optogenetic activation of RTN stimulates breathing but optogenetic inhibition produces virtually no breathing reduction, indicating that RTN is silent and breathing, while unchanged from normoxia, is driven primarily by the carotid bodies (Figure 5A,B). Conversely, at 65% FiO_2, the activity of the carotid bodies is greatly reduced, $PaCO_2$ rises slightly and most of the drive to breathe originates from RTN neurons (Figure 5A,B) (Basting et al., 2015). Also, in rats under normoxic ambient conditions, the hypoventilation elicited by opto-inhibition of RTN neurons wanes quickly because arterial hypoxia sets in, triggering an increase in respiratory drive from the carotid bodies (Basting et al., 2016). In sum, based on experiments in resting rats, the RTN and the carotid bodies provide two sources of drive to the respiratory network that contribute unequally to breathing depending on FiO_2 (Figure 5C) but altogether maintain lung ventilation virtually constant over a large range of ambient PO_2 (Figure 5B). In resting rats, V_E rises only when FiO_2 is lowered below the level at which RTN is fully inhibited by the resulting alkalosis (between 15 and 12% FiO_2 in rats) and the drive to breathe contributed by the carotid bodies is no longer cancelled by the reduction of the breathing stimulus originating from the RTN. By contrast, when lung ventilation is reduced causing PaO_2 to decrease and $PaCO_2$ to rise concurrently, the two systems are recruited in parallel to stimulate breathing and maintain blood gas stability. The effect of more extreme hypoxia (<12% FiO_2) on RTN activity is unknown. This nucleus

could be reactivated if its excitatory input from the carotid bodies become strong enough to overcome the inhibitory effect of alkalosis(Figure 5C)(Takakura et al., 2006).

The way in which the carotid bodies and central chemoreceptors interact to activate breathing has been extensively examined from an input-output (black box) viewpoint. Depending on the species and the preparation, hypo-additive, additive or hyper-additive interactions were reported (Wilson and Day, 2013; Teppema and Smith, 2013) but the cellular basis of these interactions has not been worked out. In rats, RTN and the carotid bodies activate the respiratory pattern generator via largely separate pathways given that the HCVR survives the elimination of the carotid bodies and the HVR persists after lesioning the RTN (Guyenet et al., 2018; Souza et al., 2018; Guyenet et al., 2017). Also in rats, resection of the carotid bodies does not cause a noticeable breathing reduction, probably because RTN maintains ventilation at near normal levels albeit at the cost of a slight increase in steady-state $PaCO_2$ (Guyenet et al., 2018). In rats and mice, hypoxic stimulation of the carotid bodies attenuates considerably the hypoventilation that would otherwise be caused by the absence of the RTN (Ramanantsoa et al., 2011; Guyenet et al., 2017).

The reflex pathways are not totally independent, however. In anesthetized rats, carotid body stimulation operates in part by activating the RTN (Figures 1B, 5C) (Takakura et al., 2006), an effect that could contribute to the type of potentiative interactions between the reflexes that have been found in dogs and goats (Forster et al., 2008; Smith et al., 2010). Occlusive interactions have also been described in the perfused heart-brain preparation of rodents (Day and Wilson, 2009) but this type of interaction has not been seen in conscious animals or man (Smith et al., 2015). Also, the relative contribution of the carotid bodies and the central chemoreceptors to blood gas homeostasis seems to vary greatly between species (Smith et al., 2010). In resting dogs the carotid bodies are responsible for the bulk of the breathing stimulation elicited by low levels of hypercapnia and the gain of the central component of the HCVR increases with the degree of peripheral chemoreceptor input (Smith et al., 2010). In humans and in large animals in general, carotid body denervation produces a long-lasting and profound hypoventilation whereas the effect is minor and transient in rats (Forster and Smith, 2010; Souza et al., 2019). These species differences are unexplained in cellular or network terms and emphasize the limits of a “rodentomorphic” view of the human brain.

RTN has been relatively selectively ablated in mice using genetic manipulations affecting RTN neuron determination (*Phox2b*^{27alacki}*;;Egr2*^{cre} or *Egr2-Lbx1*^{FS} mice) (Ramanantsoa et al., 2011; Hernandez-Miranda et al., 2018) or using a neurotoxin in adult rats (Souza et al., 2018). The loss of RTN neurons varies depending on the model (85% in *Phox2b*^{27alacki}*;;Egr2*^{cre} mice, 92% in adult rats, possibly 100% in the *Egr2-Lbx1*^{FS} mice) and some collateral damage outside RTN has been documented in the *Phox2b*^{27alacki}*;;Egr2*^{cre} mice and in the rat model. Yet the results have been strikingly similar. In adult rats, RTN lesions cause hypercapnia (+10 mmHg $PaCO_2$) and hypoxia (Souza et al., 2018). These effects denote alveolar hypoventilation since resting metabolism is not changed (Souza et al., 2018). Similar effects presumably occur in *Phox2b*^{27alacki}*;;Egr-2*^{cre} and *Egr2-Lbx1*^{FS} mice, given their reduced resting V_E (Ramanantsoa et al., 2011; Hernandez-Miranda et al., 2018). Stimulation of breathing by CO_2 is massively decreased in all three models of RTN lesions (by 100% at birth in both

mouse genetic models and >80% in adult rats with neurotoxin lesions). Also, unlike in control animals, rodents with RTN lesions (*Phox2b*^{27alacki}*;;Egr-2*^{cre} mice and rat model) experience dramatic breathing reductions when exposed to hyperoxia, presumably because of a reduction in carotid body activity (Souza et al., 2018; Ramanantsoa et al., 2011). In sum, in the absence of RTN, rodents hypoventilate but otherwise appear normal, at least at rest. Their breathing relies heavily on a hypoxic/hypercapnic drive principally of carotid body origin. In both *Phox2b*^{27alacki}*;;egr-2* ^{cre} and *Egr2-Lbx1*^{FS} mice, born with greatly reduced numbers of RTN neurons, the ability of CO_2 to stimulate breathing is absent at birth but recovers to around 40% of control value in adulthood. This partial recovery of the HCVR could have multiple causes such as: carotid body hypertrophy resulting from chronic hypoxia, increased efficacy of the surviving RTN neurons or increased contribution of serotonergic or other putative central chemoreceptors. None of these possibilities have been explored and the long-term effects of RTN lesions in adult rodents have not been examined.

f. RTN and sleep apnea: Hypoxia-reoxygenation cycles are extremely detrimental to the brain and the cardiovascular system (Harper et al., 2013; Berssenbrugge et al., 1983). Such events occur in multiple diseases and are especially prevalent in obstructive sleep apnea and congestive heart failure (Javaheri and Dempsey, 2013; Dempsey et al., 2010; Berssenbrugge et al., 1983). More extreme hypoventilation or respiratory arrest is a major sign of other much rarer diseases such as sudden infant death (SIDS), congenital central hypoventilation syndrome (CCHS), sudden death in epilepsy (SUDEP) (Weese-Mayer et al., 2010; Garcia et al., 2013). Periodic breathing, the rapid crescendo-decrescendo alternation of hyperventilation and apnea during sleep, is usually attributed to augmentation of the hypoxic ventilatory reflex, which results in ventilation overshoot, excessive $PaCO_2$ reduction and, presumably, transient suppression of the respiratory drive contributed by central chemoreceptors (Javaheri and Dempsey, 2013). The problem occurs in healthy individuals at altitude because of low ambient PO_2 or in advanced stages of congestive heart failure (CHF) (Javaheri and Dempsey, 2013). Periodic RTN inactivity likely contributes to periodic breathing because this nucleus is silenced by acute hypoxic hyperventilation and acetazolamide, a drug given to reduce sleep-disordered breathing at altitude to acidify the plasma, activates RTN even during hypoxia (Basting et al., 2015). In CHF patients or healthy humans exposed to hypoxia, periodic breathing occurs during SWS but ceases during REM sleep (Orr et al., 2017; Berssenbrugge et al., 1983). This phenomenon likely occurs because RTN drives breathing auto-rhythmicity during SWS (hence the oscillations) but during REM sleep the respiratory rhythm is no longer regulated by chemoreceptors.

In rats, mild optogenetic stimulation of RTN neurons during non-REM sleep activates breathing without causing arousal or a change in blood pressure (Burke et al., 2015b). If ever practicable in humans, such an intervention could help maintain breathing in patients suffering from central sleep apnea without adversely affecting sleep or blood pressure. In addition, upper airway muscle tone, important for maintaining airway patency, is under central chemoreceptor control (Fregosi and Ludlow, 2014). Accordingly, RTN neuron stimulation could also conceivably alleviate sleep apnea of the obstructive type.

Congenital central hypoventilation syndrome (CCHS) is a developmental disease characterized by severe hypoventilation during sleep (central sleep apnea) and a greatly reduced central respiratory chemoreflex; the disease is generally caused by PHOX2B mutations or, more rarely, by frameshift mutation of transcription factor LBX1 (Carroll et al., 2014; Weese-Mayer et al., 2010; Hernandez-Miranda et al., 2018; Amiel et al., 2003). A Phox2b mutation that causes both severe sleep apnea and complete loss of the chemoreflex in humans (Phox2b27ala/+) prevents RTN development when introduced into mice (Goridis et al., 2010; Dubreuil et al., 2008). The specific *Lbx1* mutation described by Hernandez-Miranda et al. (2018) ((*Lbx1(FS)*) only interferes with a small subset of Lbx1 functions. The mutation selectively interferes with the ability of Lbx1 to cooperate with Phox2b, and thus also impairs the development of the RTN. These observations suggest that CCHS patients might also lack RTN at birth and that the loss of this nucleus could account for the sleep apnea as well as the loss of the chemoreflex. This theory seems only partially correct. Although the existence of an RTN-like structure is documented in humans and macaques (Rudzinski and Kapur, 2010; Levy et al., 2019), its absence in CCHS patients is not yet established. Moreover, selective loss of the RTN (*Phox2b*^{27alacki};; *Egr-2*^{cre} mice, *Egr2-Lbx1*^{FS} mice or toxin-treated adult rats) virtually eliminates the HCVR, but the hypoventilation is similar during waking and NREM sleep, and sleep apnea is not detectable in normoxia (Souza et al., 2018; Ramanantsoa et al., 2011). Therefore, additional defects besides the probable, but still unconfirmed, absence of the RTN must contribute to the sleep apnea of CCHS. Given that the carotid bodies compensate for the loss of RTN in rodents (Souza et al., 2018; Basting et al., 2016; Ramanantsoa et al., 2011), the severe hypoventilation experienced by CCHS patients during SWS could be caused by a simultaneous reduction of both homeostatic breathing reflexes – i.e., the central mechanisms for CO_2 regulation and peripheral mechanisms for O_2 regulation (Perez and Keens, 2013). Phox2b is indeed required for the development of the carotid bodies, their sensory innervation and the NTS, where their afferents terminate (Dauger et al., 2003).

g. RTN and CO_2-induced arousal: Hypercapnia produces arousal from sleep (Phillipson and Sullivan, 1978; Phillipson et al., 1977) but it is not a very potent arousal stimulus, at least in rats. Specifically, exposure to 3% $FiCO_2$ does not detectably perturb natural sleep (0/10) and most rats (7/ 10) are also able to cycle through periods of non-REM and REM sleep in the presence of 6% $FiCO_2$ despite a vigorous increase in ventilation (Burke et al., 2015a). Currently, arousal to hypercapnia is attributed primarily to the activation of the external-lateral nucleus of the lateral parabrachial complex; the key neurons are glutamatergic, express CGRP and project to the basal forebrain (Kaur and Saper, 2019). Activation of PBel neurons by CO_2 requires inputs from the dorsal raphe serotonergic neurons and from a variety of lower brainstem neurons that collectively encode the “inspiratory effort”; the latter are thought to originate from the medullary respiratory pattern generator, the carotid bodies, lung stretch afferents, airway receptors (Kaur et al., 2020; Kaur and Saper, 2019; Berry and Gleeson, 1997; Buchanan and Richerson, 2010) and, probably , central chemoreceptors such as RTN (Souza et al., 2019). In rats, optogenetic stimulation of the RTN produces brief arousals from SWS, and RTN lesions reduce the probability of arousal to a brief CO_2 exposure (Souza et al., 2019); RTN stimulation increases arousal probability but only if the breathing stimulation is substantial (>70% ΔV_E) (Souza et al.,

2020). Less intense RTN stimulation (~40% ΔV_E in rats) has no effect on BP or arousal (Burke et al., 2015b). Also, RTN lesions large enough to reduce the HCVR by over 75% only produce a relatively mild arousal deficit, at least compared to the deficit caused by lesions of the dorsal raphe or PBel in mice (Souza et al., 2019; Kaur et al., 2020).

CCHS patients who lack a central HCVR altogether, possibly because of the congenital absence of the RTN (Ramanantsoa et al., 2011; Amiel et al., 2009) also experience severe sleep apnea during SWS and failure to wake up (Marcus et al., 1991; Chen and Keens, 2004; Weese-Mayer et al., 2010). Yet, 85% of CCHS children on ventilator still wake up to CO_2 administration ($FiCO_2$ 60mmHg) (Marcus et al., 1991). Finally, mice with congenital selective deletion of RTN neurons survive and therefore do not have fatal sleep apnea (Ramanantsoa et al., 2011).

This evidence invites several conclusions. First, arousal to central chemoreceptor stimulation has a much higher CO_2 threshold than breathing stimulation and this finding is replicated by direct stimulation of the RTN central chemoreceptors. Second, observations in animal models (RTN lesion in rodents) suggest that the loss of the HCVR contributes to the failure to arouse from SWS but is not the sole factor. Finally, in healthy sleeping mammals, RTN may cause arousal by activating the PBel in two ways: via direct projections and indirectly by pathways responding to the activation of lung, chest, and airway afferents.

1.3. Early development of the RTN: embryonic parafacial oscillator and parafacial respiratory group—

The development of the RTN lineage relies on transcription factors Egr2, Phox2b, Lbx1 and Atoh1 (Ruffault et al., 2015; Ramanantsoa et al., 2011; Hernandez-Miranda et al., 2018; Dubreuil et al., 2009; Huang et al., 2012). RTN progenitors originate from the dB2 domain of rhombomere 5; these progenitors are Phox2b-positive, switch on Lbx1 at the post-mitotic stage, migrate ventrally and activate Atoh1 expression once they reach the region of the facial motor nucleus (Ruffault et al., 2015; Ramanantsoa et al., 2011; Hernandez-Miranda et al., 2018; Dubreuil et al., 2009; Huang et al., 2012). High levels of Phox2b expression persist in the adult RTN.

In brainstem-spinal cord preparations, the presumptive prenatal precursors of the mouse RTN (around 700 pH-sensitive neurons positive for VGlut2, Phox2b and NK1R and dependent on Egr2 and Atoh1 for their development) have a rhythmic discharge pattern that consists of a pre-inspiratory and a post-inspiratory burst of action potentials (Thoby-Brisson et al., 2009). These neurons have been called “embryonic parafacial oscillator” (ePF) because they fire synchronously in relative isolation i.e., in a thin transverse section of the brainstem in vitro; their group discharge is driven by $[H^+]$ and synchronized by gap junctions (Thoby-Brisson et al., 2009). The ePF is coupled with, and entrains, the inspiratory oscillator (preBötzinger complex) (Figure 7, left).

Years earlier, Onimaru and Homma (2003) had described the existence of neurons with similar biphasic pre- and post-inspiratory discharges (“pre-inspiratory” for short) in a brainstem spinal cord preparation from newborn rats; these cells, originally called parafacial respiratory group (pFRG) were also located beneath the facial motor nucleus (like the ePF) and were also thought to be coupled with, and to activate, the preBötzinger complex. The

pFRG neurons were subsequently found to be depolarized by [H^+] and to express both VGlut2 and Phox2b (Onimaru et al., 2014). Accordingly, there is now reasonably strong evidence that pFRG neurons are the neonatal precursors of the adult RTN as defined in this chapter (Onimaru et al., 2014). A substantial proportion of the acid-activated VGlut2/Phox2b+ neurons located beneath the facial motor nucleus do not have a biphasic discharge but fire tonically in brainstem preparations from early postnatal (P0-P1) rats (Onimaru et al., 2008). Based on these observations and the absence of bursts in adult RTN neurons in vivo (Mulkey et al., 2004; Guyenet et al., 2005), the bursting pattern that defines the pFRG (Onimaru et al., 2014; Onimaru and Homma, 2003) may be a neonatal carry-over of the late embryonic (ePF) properties of RTN neurons (Thoby-Brisson et al., 2009)(Figure 7). Gap junction-dependent synchronous discharges are present in many brain nuclei early in development and commonly disappear in the adult brain (e.g., locus coeruleus, sympathetic preganglionic neurons) (Patel and Joshi, 2015; Shen et al., 1994).

1.4. RTN, active expiration and the adult parafacial expiratory oscillator—The respiratory cycle consists of three phases (inspiratory, postinspiratory and late expiratory) (Richter and Smith, 2014) that may be orchestrated by three separate but coupled oscillators: the pre-Bötzinger complex (preBötC), the post-inspiratory complex (PiCO) and the parafacial expiratory oscillator (Del Negro et al., 2018; Anderson and Ramirez, 2017; Anderson et al., 2016; Janczewski and Feldman, 2006). Neural tissue can generate oscillatory activity by three mechanisms, often operating together: the first is via intrinsic properties of individual neurons (e.g., cell-autonomous membrane depolarization); the second relies on recurrent excitatory synaptic interactions or gap junctions within a group of similar neurons; the third and most common source of oscillatory activity results from synaptic interactions, often inhibitory, between two or more anatomically distant groups of neurons. The latter mechanism is probably the norm for the vast majority of neurons with on-off respiratory synchronous discharges. Oscillators also typically require tonic excitatory inputs to be active. For example, in vivo, the activity of the preBötzinger inspiratory oscillator require excitatory inputs from peripheral and central chemoreceptors.

Expiratory muscles are recruited for enhanced breathing, principally during exercise or when chemoreceptors are strongly activated (Marina et al., 2010; Forster et al., 2012). Judging from the result of various brain lesions, the “parafacial region” (region surrounding the facial motor nucleus and including the area where RTN neurons are located) contributes to the generation of this motor outflow, called active or abdominal expiration (Janczewski and Feldman, 2006; Pagliardini et al., 2011; Pisanski and Pagliardini, 2018). One view is that the parafacial region contains an expiratory “oscillator” distinct from, but coupled to, the preBötzinger complex (Janczewski and Feldman, 2006; Del Negro et al., 2018). The ventrolateral edge of the parafacial region (pFl) does indeed contain expiration-related glutamatergic neurons that are Phox2b-negative and not directly responsive to CO_2, hence clearly different from RTN (Huckstepp et al., 2016; Magalhaes et al., 2021). These neurons project to the cVRG and could thus be part of the expiratory oscillator postulated by Feldman and colleagues (Huckstepp et al., 2016; Janczewski and Feldman, 2006). However, evidence that selective elimination of the pFL neurons prevents active expiration is yet to be demonstrated.

In summary, RTN can undoubtedly activate an “expiratory oscillator” in the broadest sense of the word -- namely a lower medullary circuit that drives cVRG expiratory premotor neurons incrementally during a specific portion of the respiratory cycle, the E2 phase. A component of this presumably complex multi-neuronal circuit may reside in close proximity of the RTN chemoreceptors (Figure 4D) (Huckstepp et al., 2016; Magalhaes et al., 2021).

1.5. Four mechanisms contribute to the response of RTN neurons to CO_2—

The stimulatory effect of CO_2 on RTN neurons in vivo is at least partially attributable to the chemoreceptor properties of this nucleus, i.e., its ability to respond to a rise in local PCO_2, for which three mechanisms have been identified: intrinsic pH sensitivity of RTN neurons, paracrine actions of CO_2/H^+-stimulated astrocytes and an RTN-specific vascular response to CO_2. A portion of the stimulatory effect of CO_2 on RTN neurons in vivo involves synaptic inputs from CO_2-modulated neurons located elsewhere. The relative importance of these four mechanisms is uncertain.

a. Cell autonomous pH-sensitivity hypothesis: The pH-sensitivity of RTN neurons is at least partly an intrinsic property requiring expression of two molecular pH sensors, the two-pore domain potassium (K2P) channel TASK-2 and the G-protein coupled receptor (GPCR) GPR4 (Figures 8A,9) (Guyenet and Bayliss, 2015). The key evidence is as follows. RTN neurons from neonatal mice or rats respond to acidification in brainstem slices (Figure 8B,C) even when synaptic transmission is reduced (tetrodotoxin, blockers of glutamate receptors; low-Ca/ high-Mg) (Mulkey et al., 2004; Onimaru et al., 2014). The persistence of the effect of acid in low-Ca/ high-Mg medium suggests that vesicular exocytosis by either neurons or astrocytes (Gourine et al., 2010) is not essential and, indeed, the neurons remain acid-sensitive after acute dissociation (Figure 8D,E) (Wang et al., 2013b). In RTN neurons as elsewhere, TASK-2 mediates a pH-dependent background potassium current that decreases in amplitude with acidification through the physiological pH range (Bayliss et al., 2015; Reyes et al., 1998; Lesage and Barhanin). In cell culture, GPR4 stimulates cyclic AMP accumulation in a pH-dependent manner with pH_{50} of 7.4 (Guyenet et al., 2016; Kumar et al., 2015; Ludwig et al., 2003). TASK-2 and GPR4 transcripts are detectable histologically in >80% of RTN neurons (Figure 3C) and in ~90% by single-cell transcriptomics (Shi et al., 2017). In fact, GPR4 is the most highly transcribed GPCR in RTN (Shi et al., 2017). The pH sensitivity of RTN neurons in slices is absent or greatly reduced in mice with genetic deletion of *Kcnk5* (encodes TASK-2) or *Gpr4* (Figure 8F) (Wang et al., 2013a; Kumar et al., 2015). Incubation of brainstem slices from wild-type mice with a small molecule GPR4 receptor antagonist reduced the proportion of pH-sensitive RTN neurons (Kumar et al., 2015; Dong et al., 2017) and acute systemic treatment with NE 52-QQ57, another antagonist of GPR4 (but not of TASK-2), depressed CO_2-stimulated breathing in conscious rats and mice (by ~15%-25%) (Hosford et al., 2018). Moreover, mice in which either *Kcnk5* or *Gpr4* has been knocked out have a 65% reduction of their central respiratory chemoreflex and the double KO mice have virtually no residual reflex (95% reduction; Figure 8G) (Gestreau et al., 2010; Guyenet et al., 2016; Kumar et al., 2015). Finally, reintroducing GPR4 selectively into RTN neurons of *Gpr4* KO mice restores the respiratory chemoreflex (Figure 8H) and the ability of hypercapnia to elicit Fos expression in RTN neurons (Kumar et al., 2015).

The evidence supporting a key role of TASK-2 and GPR4 in pH sensing by RTN neurons is therefore considerable but not without limitations. For instance, although both TASK-2 and GPR4 have a highly restricted brain distribution, TASK-2 is not exclusively expressed by RTN neurons (Gestreau et al., 2010) and low levels of GPR4 transcripts are expressed by the brain vascular endothelium and by subsets of serotonergic neurons (Shi et al., 2017; Kumar et al., 2015; Hosford et al., 2018). Thus, the reduction of CO_2-stimulated breathing observed with global deletion of TASK-2 or GPR4 cannot be unequivocally attributed only to their absence in RTN neurons. Also, the reduced HCVR of GPR4-deleted mice could conceivably result from changes in blood flow because some GPR4 is expressed by endothelial cells (Hosford et al., 2018; Cleary et al., 2020). However, this interpretation overlooks that the central chemoreflex of GPR4$^{-/-}$ mice is restored by reintroducing the gene selectively into RTN neurons (Figure 8H) (Kumar et al., 2015). Moreover, although both TASK-2 and GPR4 are clearly sensitive to pH changes, it is formally possible that their contributions to RTN neuron chemoreceptor function reflect some other modulatory action. For example, TASK-2 is reportedly inhibited by G protein βγ subunits (Cid et al., 2013), leaving it potentially susceptible to receptor stimulation by other CO_2-dependent signaling molecules; likewise, GPR4 could have a yet-unidentified endogenous ligand that is released onto RTN neurons in a CO_2-dependent manner. GPR4 deletion could conceivably reduce the general excitability of RTN neurons and reintroducing this receptor selectively into RTN neurons of GPR4 KO mice may have restored the chemoreflex by increasing cellular excitability and responsiveness to other CO_2-dependent inputs (Kumar et al., 2015).

In sum, the notion that TASK-2 and GPR4 mediate the CO_2-sensitivity of RTN neurons in vitro and much of the central respiratory chemoreflex in vivo is generally well supported. Alternative interpretations remain plausible, but they require discounting the proven pH sensitivity of both TASK-2 and GPR4 in favor of some other purely theoretical modulatory effect of these proteins.

b. The paracrine hypothesis: The paracrine hypothesis (Figure 9) holds that astrocytes rather than neurons sense molecular CO_2 or [H^+] and are responsible for the activation of the respiratory pattern generator by hypercapnia (Gourine and Kasparov, 2011; Gourine et al., 2010). According to these authors, pH-sensitive astrocytes are present not only in the RTN but throughout the respiratory network and they depolarize RTN and other respiratory neurons by releasing adenosine triphosphate (ATP) (Gourine and Kasparov, 2011; Sheikhbahaei et al., 2018; Rajani et al., 2018). In essence, this hypothesis aligns with the concept that the central chemoreflex results from widely distributed effects of protons (or CO_2) on the respiratory pattern generator (Nattie and Li, 2012) and contradicts the view that the RTN or serotonergic neurons are privileged sites for CO_2 chemoreception (Sheikhbahaei et al., 2018).

One version of the astrocytic paracrine mechanism views CO_2 as operating via changes in pH (Figure 9). The central feature of the hypothesis is the sodium-bicarbonate exchanger (NBCe1). CO_2 would increase the activity of this electrogenic co-transporter via a direct effect of elevated extracellular bicarbonate (Turovsky et al., 2016) or indirectly by depolarizing the astrocytes; the latter effect would result from inhibition of a pH-sensitive inwardly-rectifying K channel (Wenker et al., 2010). NBCe1-mediated Na entry would then

stimulate sodium-calcium exchange, and the rise in intracellular calcium would cause ATP release via exocytosis (Turovsky et al., 2016). ATP would then activate neighboring neurons, notably RTN, via P2Y-type purinergic receptors (Kasymov et al., 2013; Gourine et al., 2010). Unfortunately, evidence that knocking out NBCe1 in brainstem astrocytes attenuates the central respiratory chemoreflex is not available. Also, the theory relies heavily on the observation that ChR2-driven astrocyte depolarization activates breathing (Gourine et al., 2010). This procedure may depolarize neighboring neurons by eliciting potassium release from astrocytes (Octeau et al., 2019).

The exocytotic release of transmitters by astrocytes, including ATP, is attenuated when these cells are transduced with dominant negative SNARE protein (dnSNARE) or tetanus toxin light chain (TeLC). In the pre-Bötzinger complex (rhythm generator), this procedure reduces the resting respiratory frequency of conscious rats, albeit modestly (11%) (Sheikhbahaei et al., 2018). Bilateral overexpression of a potent ectonucleotidase (TMPAP) by astrocytes had the same effect whereas activation of astrocytes transduced with a Gq-coupled DREAAD (receptor activated by administration of clozapine N-oxide), stimulated the resting breathing rate (+23%) (Sheikhbahaei et al., 2018). Based on these results RTN is not the only region of the breathing network that is regulated by astrocytes. However, expression of dnSNARE or TeLC by astrocytes also depressed (20-50%) the ventilatory response to every stimulus tested (hypoxia, hypercapnia and exercise) and reduced hypoxia-induced sighing (Sheikhbahaei et al., 2018). The uniformly depressant effect of dnSNARE and TeLC transduction on all ventilatory responses tested so far suggests that astrocytes may facilitate synaptic transmission within the preBötzinger complex regardless of the modality and source of these inputs. A similar non-specific facilitation could explain the contribution of RTN astrocytes to the CO_2 responses of RTN neurons.

A second version of the paracrine hypothesis posits that astrocytes detect molecular CO_2; in this conception, CO_2 opens connexin-26 expressed by astrocytes causing ATP release and subsequent neuronal activation via purinergic receptors (Figure 9) (Meigh et al., 2013; Huckstepp et al., 2010b). This interpretation is at odds with prior evidence that breathing is driven by extracellular $[H^+]$ (Pappenheimer et al., 1965) but both mechanisms could conceivably co-exist. Further supporting evidence is needed.

In summary, brainstem astrocytes are capable of activating various components of the respiratory pattern generator including RTN and the preBötzinger complex by releasing ATP and possibly other transmitters. Whether this glial mechanism selectively mediates the effects of CO_2/H^+ or broadly regulates synaptic responses or neuronal excitability remains to be seen. At present, the astrocyte theory does not explain satisfactorily why highly selective genetic lesions of RTN neurons abolish the central chemoreflex at birth in mice (Ramanantsoa et al., 2011; Hernandez-Miranda et al., 2018). Conceivably the astrocytic mechanism develops postnatally. In support of this assertion, ATP receptor antagonists have no detectable effect on the activation of RTN/pFRG neurons by CO_2 in the neonatal (P0-P4) brainstem spinal cord preparation (Onimaru et al., 2014). Also, purinergic P2 receptors modulate excitability of RTN neurons but do not mediate their pH sensitivity in P5-P7 rat brain slices (Mulkey et al., 2004; Mulkey et al., 2006). These negative results could mean that the paracrine mechanism has not yet developed. Another possibility however is that

astrocytes change RTN activity via changes in local blood flow (see next section for details), a mechanism that is inoperative in slices or en-bloc preparations. Also, the intensity of the hypercapnic ventilatory reflex increases markedly in rodents during the first two weeks of post-natal life and recovers to 40% of control in adult mice with genetic manipulations that eliminate the RTN (Ramanantsoa et al., 2011; Hernandez-Miranda et al., 2018; Putnam et al., 2005). These observations could also be interpreted as evidence of a late development of the paracrine mechanism of chemosensitivity, but many other interpretations are possible (plasticity of surviving RTN neurons, compensatory development of the carotid bodies, etc.). Finally and most importantly, the astrocyte-based version of the distributed chemosensitivity theory does not adequately explain the massive reduction of the central chemoreflex caused by RTN lesions in the adult rat nor the elimination of the reflex in adult TASK-2/GPR4 double knock-out mice (Souza et al., 2018; Guyenet et al., 2016; Kumar et al., 2015).

c. The microvasculature hypothesis: Brain hypercapnia typically produces vasodilation. If this were to happen in a region where CO_2 sensors are located, the resulting rise in blood flow would wash out locally produced CO_2 and reduce the ability of the chemoresponsive neurons (or glia) to detect changes in arterial $PaCO_2$ (Xie et al., 2006). A specialization within the RTN may prevent this potentially countervailing effect of CO_2; unlike in the cortex, acidification appears to constrict arterioles in the RTN and this vasoconstriction is reduced by application of ATP-receptor antagonists (Hawkins et al., 2017; Cleary et al., 2020). The source of this ATP is unclear. If ATP comes from astrocytes (as drawn in Figure 9), vascular constriction and subsequent CO_2 retention by the brain parenchyma could explain at least partly why optogenetic depolarization of RTN astrocytes ultimately activates RTN neurons and breathing (Gourine et al., 2010).

d. The synaptic input hypothesis: According to this hypothesis the activation of RTN neurons by hypercapnia is mediated by synaptic inputs from CO_2-activated neurons located elsewhere (Figure 1B). This hypothesis also has considerable support. As already mentioned, RTN neurons can be activated by stimulation of the carotid bodies, organs whose response to hypercapnia, albeit hypoxia-dependent, is indisputable (Takakura et al., 2006; Kumar and Prabhakar, 2012). The carotid body-RTN pathway operates via a direct Phox2b+ glutamatergic projection from the nucleus of the solitary tract to the RTN (Takakura et al., 2006). Also, the neuropil surrounding RTN neurons contains orexinergic, noradrenergic and serotonin terminals and RTN neurons express receptors for these transmitters; in slices, saturating concentrations of NE, serotonin, substance P, orexin or TRH activate RTN neurons as much as a severe (0.4) pH change (Rosin et al., 2006; Kuo et al., 2016; Sobrinho et al., 2016; Hawryluk et al., 2012; Mulkey et al., 2007a; Shi et al., 2016; Lazarenko et al., 2011). Subsets of CNS noradrenergic and serotonergic neurons respond to hypercapnia in anesthetized rats (Elam et al., 1981) or brain slices (Pineda and Aghajanian, 1997; Brust et al., 2014). Finally, lesions of orexin, noradrenergic or serotonergic neurons or administration of the appropriate receptor antagonists attenuate the central respiratory chemoreflex (Li and Nattie, 2010; Gonzalez et al., 2009; Biancardi et al., 2008; Kuo et al., 2016; Oliveira et al., 2016). According to Wu et al., the CO_2 response of RTN neurons in mouse brain slices (postnatal 13-20 days) or in neuronal co-culture, is predominantly driven by serotonin release and subsequent activation of 5-HT7 receptors (Wu et al., 2019).

RTN neurons also receive inhibitory inputs that may contribute positively or negatively to the effect of CO_2 on their activity. For example, RTN neurons receive feedback inhibition from the medullary respiratory pattern generator and from lung stretch receptors (Moreira et al., 2007; Takakura et al., 2007; Guyenet et al., 2005). These inputs are indirectly activated by hypercapnia and their effect is to limit the activation of RTN, possibly to protect the respiratory system from excessive activation. In vitro experiments suggest that RTN neurons receive input from nearby somatostatin-expressing inhibitory neurons whose activity is reduced by CO_2 and selective chemogenetic suppression of *Sst*+ parafacial neurons in vivo increases baseline breathing (Cleary et al., 2021).

In short, RTN neurons receive numerous conventional excitatory and inhibitory synapses. This fact, well established at the ultrastructural level (Lazarenko et al., 2009), highlights the obvious: RTN neurons are not mere CO_2/H^+ detectors but pH-modulated neurons subject to myriad synaptic inputs of still uncertain origin and function. Some inputs may originate from CNS neurons that are directly pH-sensitive, others from neurons that are indirectly activated by CO_2 because of behavioral and metabolic changes. Many of these CO_2-activated inputs are excitatory (e.g., serotonergic neurons, orexinergic neurons, glutamatergic input from the NTS relaying carotid body activation) but others are inhibitory (e.g., negative feedback from pulmonary stretch receptors and the central pattern generator) (Moreira et al., 2007; Takakura et al., 2006; Guyenet et al., 2005). Furthermore the inhibitory inputs to RTN are either activated (Moreira et al., 2007; Takakura et al., 2006; Guyenet et al., 2005) or inhibited (Cleary et al., 2021) by hypercapnia. Accordingly, the overall contribution of these CO_2-sensitive or CO_2-responsive inputs to the activation of RTN neurons by hypercapnia in vivo is not self-evident and is likely to vary according to the intensity of the hypercapnic stimulus and its effects on arousal and stress. This complex picture is still oversimplified because it does not consider the possibility that RTN contains multiple neuronal subtypes that may be differentially regulated.

1.6. Exercise hyperpnea and CRC—Exercise hyperpnea has a supraspinal component dubbed “central command” of uncertain brain origin; it also has a hormonal component and a peripheral sensory component that relies on the activation of muscle and joint afferents (muscle metaboreceptors and others) (Forster et al., 2012; Waldrop et al., 1986; Eldridge et al., 1981). Finally, the spinal locomotor network is also probably involved (Le Gal et al., 2014). In combination, these processes activate breathing and CO_2 excretion to an extent that closely matches, or even occasionally exceeds, the increase in the metabolic production of CO_2 such that at no time during exercise is $PaCO_2$ above resting level (Forster et al., 2012). Accordingly, authors have generally concluded that exercise hyperpnea could not be mediated by the activation of “central chemoreceptors” (Forster et al., 2012). Clearly, this interpretation depends on the definition of a central respiratory chemoreceptor. If the moniker is used restrictively to define a CO_2- or proton-activated signaling cascade (a receptor in the molecular sense), the statement is true but tautological. If the term chemoreceptor is used in the sense of a neuronal group that is activated by acidification and drives breathing (e.g., RTN, raphe or other putative nuclei), the statement that such cells are not involved in the hyperpnea of exercise is premature.

Exercise hyperpnea is extensively covered in this volume. In this section we simply review the few experimental results that may contradict the classic notion that central chemoreceptors could not mediate exercise hyperpnea. For example, c-Fos expression by RTN neurons ($Phox2b^{+}$-TH^{-}) is increased by exercise in rats (Barna et al., 2014) suggesting that both CO_2 and exercise may activate the RTN. Also, RTN neurons receive excitatory input from sensory afferents (sciatic nerve) and from the hypothalamus (e.g., lateral hypothalamus, perifornical region including orexinergic neurons) which could, at least in theory, convey information regarding locomotion or energy expenditure (Fortuna et al., 2009; Kanbar et al., 2016; Li et al., 2020; Lazarenko et al., 2011). Finally, in isolated neonatal rat brainstem-spinal cord preparations, the rise in respiratory rate observed during fictive locomotion is associated with an increase in the excitability of the pre-inspiratory neurons of the parafacial respiratory group (pFRG/Pre-I) and is prevented by bilateral lesion of the pFRG region (Le Gal et al., 2014). As mentioned before the pFRG/Pre-I neurons are neonatal RTN neurons.

On the other hand, exercise hyperpnea is preserved in CCHS patients in whom the HCVR is virtually absent (Paton et al., 1993; Shea et al., 1993). If the absence of RTN in CCHS can be verified, this evidence would argue against this nucleus playing an indispensable role in the hyperpnea of exercise. Yet, It would not exclude a major contribution of the RTN in normal individuals given the likely persistence or enhancement in CCHS patients of powerful alternate mechanisms such as the feedback from muscle afferents (see Chapter 8 for details).

In summary, the possibility that the hyperpnea of exercise could be partially mediated via synaptic activation of RTN or other central chemoreceptor neurons is supported by a growing, though admittedly incomplete, body of evidence.

2. Serotonergic neurons and CRC

The serotonergic neurons are currently classified according to their anatomical location, developmental lineage (e.g., primarily rhombomeric origin) and transcriptome (Okaty et al., 2020; Okaty et al., 2015). Each classically defined anatomical cluster (B1-9) contains serotonergic neurons derived from more than one rhombomere (Brust et al., 2014). The rhombomeric origin of the lower brainstem serotonergic cells probably specifies the function of the adult serotonergic neurons to a greater extent than the particular anatomical cluster in which they settle as mature neurons (Brust et al., 2014).

A somewhat different frame of reference is needed to analyze the contribution of serotonergic neurons vs. the RTN to breathing and the central chemoreflex. Whereas the mouse RTN consists of around 700 neurons that selectively target the lower brainstem regions harboring the respiratory pattern generator, the mouse brainstem contains around 26,000 serotonergic neurons (Ishimura et al., 1988) that, collectively, innervate the entire neuraxis and contribute to nearly every aspect of brain function, including autonomic regulation (cardiovascular system, thermoregulation, metabolism, food intake), motor activity including locomotion and breathing, the sleep-wake cycle, arousal and emotions (fear, anxiety). These widespread contributions of serotonin should be considered when interpreting the effects produced by lesioning or inhibiting large groups of serotonergic

neurons. Notably, the brief and typically severe hypercapnic stimuli (>6% $FiCO_2$) used to elicit the HCVR in experimental animals elicits arousal, aversive sensations and emotions that, in turn, increase VCO_2 hence V_E and therefore contribute substantially to the overall ventilatory reflex ($\Delta V_E/\Delta FiCO_2$). On the other hand, a small subset of lower brainstem serotonergic neurons may operate as true central respiratory chemoreceptors, i.e., neurons that mediate a portion of the breathing stimulation elicited by hypercapnia partly by virtue of their intrinsic sensitivity to changes in extracellular pH and axonal projections to the breathing rhythm and pattern generator (Brust et al., 2014).

2.1. Contribution of the serotonergic neurons to the HCVR—Serotonergic neurons have the potential to stimulate breathing via actions at all levels of the neuraxis including spinal cord, brainstem and above (Ptak et al., 2009; Bocchiaro and Feldman, 2004; Rasmussen and Aghajanian, 1990). The integrity of serotonergic neurons is important to the HCVR. The hypercapnic ventilatory response of adult Lmx1b(f/f/p) mice, in which virtually no serotonergic neurons survive, is decreased by 50% compared with wild-type mice (Hodges et al., 2008). A similar reduction was later found in mice subjected to a global pharmacogenetic inhibition of serotonergic neurons (Ray et al., 2011). Selective pharmacogenetic inhibition of the Egr2-Pet1 subgroup of serotonergic neurons reduces the HCVR of mice by a lesser amount (around 15%) (Figure 10) (Brust et al., 2014). These neurons are mildly excited in slices unlike those in the raphe obscurus neurons (*r6/7-Pet1*) (Figure 10C). Based on these results, the HCVR reduction caused by global inhibition of serotonergic neurons probably results from an accumulation of deficits including reduced excitability of the entire respiratory pattern generator (including, motor neurons and the RTN), reduced CO_2-induced arousal and perhaps reduced anxiety or stress (Figure 10D) (Buchanan and Richerson, 2010). Resting V_E is not detectably changed by inhibiting serotonergic neurons in mice, a somewhat surprising result given that metabolism (VO_2) is reduced (Ray et al., 2011). Resting V_E could be maintained by a slight increase in $PaCO_2$ that activates the carotid bodies or other central chemoreceptors is sufficient to restore ventilation. Serotonergic neuron impairment does not noticeably affect the hypoxic ventilatory reflex of mice. This selectivity is also not unprecedented because the HVR of rats is unaffected by massive RTN lesions (Souza et al., 2018). Like the lack of effect on resting V_E, the lack of effect of serotonin lesions on the HVR should be cautiously interpreted because a primary deficit of the HVR could be compensated by alterations of resting blood gases (lower resting PaO_2 and higher $PaCO_2$) that increase the intensity of the stimulus (carotid body activation elicited by a given FiO_2 reduction).

2.2. Serotonergic neurons as pH detectors—Dorsal raphe serotonergic neurons are acid-activated in slices and in cell culture (Severson et al., 2003). Acidification of the dorsal raphe via an implanted dialysis probe produces arousal from sleep in mice (Smith et al., 2018). Based on unit-recording experiments in unanesthetized cats, admittedly subject to sampling bias, 20% of presumptive serotonergic neurons of the dorsal raphe respond to hypercapnia (Veasey et al., 1997) and thus could theoretically be directly responsive to local pH changes in vivo. The same low proportion of responders was found in the medullary raphe (Veasey et al., 1995). However, given the huge number of raphe neurons, this small percentage still represents many thousands of CO_2-responsive or potentially CO_2-sensing

cells. The bulbospinal serotonergic neurons of the parapyramidal region are mildly inhibited by hypercapnia in rats under conditions when RTN neurons are robustly activated (Mulkey et al., 2004; Takakura and Moreira, 2013) further emphasizing that excitation in response to hypercapnia in vivo is not a general property of lower brainstem serotonergic neurons. This assertion has also been confirmed by lineage tracing studies. Lower brainstem serotonergic neurons derived from rhombomeres 3 and 5 are mildly activated by acidification in slices, but those derived from rhombomeres 7 and 8 are not (Figure 10) (Brust et al., 2014). The former populate portions of the raphe magnus and obscurus; their projection field includes the dorsal horn of the spinal cord and several lower brainstem regions implicated in respiratory control such as the ventral respiratory column, the solitary tract nucleus, and the RTN (Brust et al., 2014). The latter populate the raphe obscurus only.

In summary, most serotonergic neurons are activated by acidification in culture, they respond less commonly to acid in slices and least commonly in the whole animal. This variability may be partly related to the genetic heterogeneity of this class of neurons (Okaty et al., 2020). The molecular basis of the postulated direct effect of pH on serotonergic neurons remains to be identified. Many serotonergic neurons express the putative proton receptor GPR4 and acid-modulated channels such as TASK-1 and TASK-3 (encoded by Kcnk3 and Kcnk9) which could conceivably contribute to their pH sensitivity (Okaty et al., 2020; Bayliss et al., 2015; Ludwig et al., 2003; Mulkey et al., 2007b; Washburn et al., 2002) but no compelling evidence has surfaced yet for their role in vivo and combined genetic deletion of TASK-1 and TASK-3 knock out has no effect on the HCVR (Mulkey et al., 2007b) .

2.3. Serotonergic neurons and arousal to CO_2—CO_2-induced arousal seems largely mediated via the activation of glutamatergic neurons located in the external-lateral parabrachial nucleus, PBel (Figures 2 and 10) (Kaur et al., 2017; Buchanan et al., 2015; Buchanan and Richerson, 2010; Kaur and Saper, 2019). The critical parabrachial neurons express CGRP (Kaur et al., 2017; Kaur and Saper, 2019; Kaur et al., 2020). There is no evidence yet that they are directly pH-sensitive; their activation by hypercapnia is provisionally attributed to a convergence of excitatory inputs from central chemoreceptors such as the RTN and from NTS neurons that respond to the activation of peripheral chemoreceptors, pulmonary and airway afferents (Figure 2) (Souza et al., 2020; Kaur and Saper, 2019). CO_2-induced arousal requires an intact serotonergic system (Buchanan and Richerson, 2010). The serotonergic innervation of PBel neurons is assumed to facilitate their activation during hypercapnia and thus trigger arousal (Kaur et al., 2020). Systemic administration of a 5-HT2A receptor agonist, while not causing arousal by itself, is sufficient to restore CO_2-induced arousal in mice with global deletion of serotonergic neurons (Buchanan et al., 2015) or dorsal raphe lesions (Kaur et al., 2020). These observations suggest that CO_2-induced arousal requires the presence of extracellular serotonin in PBel but may not be triggered by a sudden increase in serotonin release. However, topical acidification of the dorsal raphe produces arousal; if one assumes that the degree of acidification matches the change occurring during hypercapnia, this evidence suggests serotonin release is in fact triggered by hypercapnia as a result of the intrinsic sensitivity of dorsal raphe neurons (Figure 10D) (Smith et al., 2018). Regardless of the mechanism,

this evidence demonstrates that serotonergic lesions reduce arousal and therefore reduce the behavioral component of the HCVR.

3. Other central respiratory chemoreceptor candidates

Many brain regions contain neurons that contribute to the HCVR in some capacity (Nattie and Li, 2012). The generic evidence includes one or more of the following observations: activating these neurons or regions stimulate breathing, destroying them attenuates the HCVR, subjecting the animals to hypercapnia activates these neurons as judged by c-Fos expression or, less frequently, unit recording in vivo. None of this evidence, singly or collectively, demonstrates unequivocally that their activation by hypercapnia or their contribution to the HCVR is caused by their ability to directly sense local acidification. First, neurons do not have to be activated by hypercapnia to contribute to the HCVR. Their resting activity could simply be permissive; for example, this explains adequately the effect of serotonin on PBel for CO_2-stimulated arousal (Kaur et al., 2020). Second, rather than via local acidification and direct or paracrine CO_2/H^+ sensing, the neurons could be activated by hypercapnia via a change in network activity resulting from CO_2-induced arousal, stress, dyspnea, etc. In the final analysis, the interpretation that such cells are central respiratory chemoreceptors depends on being able to parse out which portion of their activation by hypercapnia in vivo is mediated by their intrinsic response to acidification. In most cases, except RTN, only two pieces of supporting evidence have been produced. First, topical acidification of the brain region where the neurons of interest reside increases breathing (Nattie and Li, 2012). Second, a rise in CO_2/H^+ activates these neurons in brain slices or in culture. By our definition, this evidence is insufficient to demonstrate that such cells are respiratory chemoreceptors.

Many brainstem and hypothalamic neurons are inhibited or excited by acidification in acutely prepared tissue slices (Fukuda and Honda, 1976; Dean et al., 1989; Richerson, 1995; Yanovsky et al., 2012; Williams et al., 2007; Nichols et al., 2008). The observation is interpreted as suggesting that such neurons could be respiratory chemoreceptors if they reside in a region of the brain that controls breathing (e.g., NTS, ventrolateral medulla, hypothalamus). This evidence is not as straightforward as it seems. First, proving that the neurons of interest influence breathing in vivo can be quite difficult. The second issue is to determine what degree of acidification in vitro is relevant to CRC and where the realm of toxicology begins. A significant neuronal activation in response to a 0.1 to 0.2 pH change in vitro is viewed as potentially meaningful to CRC. Yet, to lower brain pH by 0.2 requires artificial ventilation with 11% $FiCO_2$ in an anesthetized rat (without the moderating effect of the HCVR) and this stimulus causes arterial PCO_2 to rise from 37 to ~90 mmHg (Katsura et al., 1994; Guyenet et al., 2005). The discharge of an RTN neuron in vivo increases by 0.5 Hz in response to a 0.01 pH change (~5% of its dynamic range) and a 0.08 arterial pH change has a massive impact on the contribution of RTN neurons to breathing in an unanesthetized rat (Figure 5A) (Basting et al., 2015; Guyenet et al., 2005). In addition, whether a given neuron would respond identically to acidification in the intact brain as in a slice is not a foregone conclusion. Neurons in slices or in culture have generally few active synaptic inputs and therefore tend to have a very high input resistance which makes them prone to develop large membrane potential changes and consequently a large increase in firing in

response to acid-induced membrane currents which could be totally ineffective in activating the same neurons in vivo. Finally, recordings in vitro are obtained in preparations in which astrocytes are absent (in cell culture) or disrupted by tissue slicing and, in any case, no longer normally communicating with the microvasculature, which must dampen their ability to buffer brain tissue pH (Theparambil et al., 2020).

The second extensively used approach to test for the presence of respiratory chemoreceptors in the brain is to determine whether topical acidification of a given structure via a dialysis probe stimulates breathing (Nattie, 2011). The perfusion fluid is usually a bicarbonate buffer equilibrated with 25% CO_2. Somewhat surprisingly, it has been estimated that such a high CO_2 concentration produces a local tissue acidification equivalent to a modest $PaCO_2$ rise of 6-7 mmHg (Li et al., 2013; Li and Nattie, 2002). This estimate was based on pH measurements “next to” the dialysis probe using a pH electrode. The tissue in direct contact with the dialysis probe, and thus exposed to 25% CO_2, would surely be far more acidic and the acidification would decrease sharply as a function of the distance from the semi-permeable membrane. Indeed, in ventilated rats exposed to 27% $FiCO_2$, arterial PCO_2 reaches 191 mmHg, arterial pH drops to 6.8, and brain pH decreases by 0.5 (Katsura et al., 1994); one would therefore assume that similar levels of PCO_2 and pH prevail in the tissue immediately adjacent to a dialysis probe perfused with a bicarbonate buffer equilibrated with 25% CO_2. In addition, the pH change elicited by the perfusion fluid could be exaggerated by the disruption of the buffering capacity of local astrocytes caused by the lesion (Theparambil et al., 2020).

Technical details aside, the breathing stimulation produced by local acidification with a dialysis probe is generally small, it can be elicited in the brainstem (ventral respiratory column including the pre-Bötzinger complex, NTS, raphe), the hypothalamus and the cerebellum (fastigial nucleus) and it can be supra-additive when two regions are simultaneously acidified (Dias et al., 2008; Li and Nattie, 2002; Nattie and Li, 2002; Li et al., 2013; Martino et al., 2007). These observations together with the plethora of neurons that are pH-sensitive in vitro form the basis of the “distributed chemoreceptor theory,” which argues that the HCVR is an emergent property of the breathing network at large, i.e., a reflex caused by the summation of small effects of acidification in myriad brain regions (Nattie, 2011). The repetitive evidence implicating these various regions in the HCVR will be omitted; instead, we focus next on the specific contribution of the orexin neurons and the locus coeruleus to the HCVR because these nuclei have also been most intensively examined in the context of stress and arousal.

Based on Fos expression, hypercapnia activates a portion of the orexin neurons in vivo (Sunanaga et al., 2009). Also, the HCVR is reduced after genetic deletion of these neurons (Li and Nattie, 2014) and almorexant, an orexin receptor antagonist, reduces the HCVR during the waking state (Li and Nattie, 2010). Thus, some orexin neurons are demonstrably activated during hypercapnic exposure, and the integrity of the orexin neurons is required for maximal expression of the HCVR. The interpretation of this evidence is less straightforward. One view is that these neurons are respiratory chemoreceptors. This statement implies that a local rise in PCO_2 is the main reason for their excitation by hypercapnia and their contribution to the HCVR. In support of this interpretation, orexin neurons are activated

by acidification in slices; however, as is usually the case, the mechanism by which acid activates these neurons is unknown (Gonzalez et al., 2009; Guyon et al., 2009). Another possibility is that hypercapnia activates the orexin neurons primarily or perhaps exclusively, via an increase in synaptic input, e.g., their activation by hypercapnia is a network effect associated with arousal or stress. In support of this theory, the orexinergic neurons are major contributors to the increased blood pressure and breathing elicited by stress caused by other factors than CO_2 (Carrive and Kuwaki, 2017) and their activity is also increased by arousal (Lee et al., 2005). Clearly both stress and arousal are elicited by hypercapnia (Kaur and Saper, 2019; Carrive and Kuwaki, 2017). In summary, the reason why orexin neurons are activated by hypercapnia and facilitate the HCVR in rodents could be a mix of direct effects of parenchymal acidification and synaptic activation caused by stress and arousal. The relative contribution of these two factors is simply unknown. Finally, the relevance of these animal findings to the chemoreflex in humans is not a foregone conclusion. People with narcolepsy caused by degenerating orexin neurons have a normal hypercapnic ventilatory reflex while awake (Han et al., 2010).

The notion that locus coeruleus neurons are central respiratory chemoreceptors rests on similar lines of evidence. In slices, these neurons fire more slowly when the pH of the perfusion fluid is alkalized by lowering CO_2 and they are activated by increasing CO_2 and bath acidification (Pineda and Aghajanian, 1997). In vivo (anesthesia) high levels of $FiCO_2$ increase their activity (Elam et al., 1981). Finally, locus coeruleus lesions attenuate the HCVR whereas activation of this nucleus increases breathing (Gargaglioni et al., 2010; Liu et al., 2021). The locus coeruleus, like the orexin neurons, is an important executive center of the waking state which is strongly activated by arousal and stress (Foote et al., 1980; Adamantidis et al., 2010; Aston-Jones and Cohen, 2005). Also, the locus coeruleus receives a powerful excitatory orexinergic input and its activation produces arousal and behavioral activation (Ivanov and Aston-Jones, 2000; Carter et al., 2010). Thus, while both the orexin system and the locus coeruleus are clearly necessary for full expression of the HCVR, it is uncertain whether their activation by hypercapnia in vivo is primarily caused by their pH sensitivity or is a network effect mediated by increased synaptic inputs related to arousal and stress. If a network effect is primarily responsible for the activation by CO_2 of orexinergic neurons and the locus coeruleus, the obvious question is how CO_2 initiates the process of network activation. One possibility was already discussed: activation of the lateral parabrachial nucleus (Kaur et al., 2020; Kaur and Saper, 2019; Kaur et al., 2017).

In summary, as already pointed out by Erlichman (2009), the activation of locus coeruleus, orexin and myriad other brain neurons by hypercapnia could be largely network driven i.e., the result of a change in behavior or brain state rather than a direct effect of local PCO_2/H^+ on these neurons.

4. Relative contribution of $[H^+]$, molecular CO_2 and bicarbonate to CRC

CO_2 is in equilibrium with bicarbonate and $[H^+]$ in the brain as elsewhere. Which of these three molecular entities is responsible for breathing stimulation is a debate that is regularly being resurrected in the field of CRC with modest progress towards full resolution (Huckstepp and Dale, 2011). The problem is difficult to solve. Changes in PCO_2/H^+ alter

blood flow and can produce arousal from sleep, anxiety, seizures. Titratable proteins are legion and protons also serve as synaptic transmitters (Du et al., 2014). The dominant view, often called the “reaction theory” (Loeschcke, 1982) holds that the stimulatory effect of CO_2 on breathing is mediated by changes in proton concentration somewhere in the brain (for reviews see (Fencl et al., 1966; Nattie and Li, 2012; Huckstepp and Dale, 2011). For example, Loeschcke in 1958 ((reviewed in (Loeschcke, 1982) showed that breathing was powerfully activated by perfusing the fourth ventricle and subarachnoid space of anesthetized cats with acidic solution while arterial PCO_2 was kept constant and Pappenheimer et al. (Fencl et al., 1966) showed that the breathing stimulation elicited by hypercapnia in awake goats tracks the cerebrospinal fluid proton concentration rather than PCO_2 when the bicarbonate concentration is artificially manipulated. Other observations in anesthetized mammals have been interpreted as evidence that CNS changes of CO_2 and $[H^+]$ could have independent actions on breathing (Eldridge et al., 1985; Shams, 1985). However, this interpretation relies on an unverifiable assumption, which is that the pH changes measured at the ventral medullary surface are the same as those to which the underlying respiratory chemoreceptors are exposed during metabolic or respiratory acidosis. Regardless, while the reaction theory is eminently plausible, it is still not proven. The field has not yet reached a consensus regarding the type of cells that sense brain $[H^+]$ (arguably the “true” respiratory chemoreceptors) and how this signal triggers a change in breathing, nor is there agreement about the relevant proton receptors.

In vitro, the effect of CO_2 on neurons or glia is usually attributed to changes in proton concentration because changing the bicarbonate concentration of the superfusion fluid under constant PCO_2 produce results consistent with the pH changes expected from the Henderson-Hasselbalch equation. For example, based on this type of evidence, CO_2 activates juvenile or postnatal RTN neurons (a.k.a. pfRG) in brain slices via changes in pH (Mulkey et al., 2004; Kawai et al., 2006). The activation of serotonergic neurons by CO_2 in vitro, putatively also an intrinsic property, is attributed to changes in the intracellular proton concentration (Wang et al., 2002). At least some of the effect of CO_2 on astrocytes also result from the protonation of a potassium channel ($K_{IR}4.1$ or 5.1) (Wenker et al., 2010). As detailed above the activation of RTN neurons by acid is caused by the protonation of the channel TASK-2 and the G-protein coupled receptor GPR4 (Mulkey et al., 2004; Guyenet et al., 2019; Bayliss et al., 2015; Kumar et al., 2015; Onimaru et al., 2014). RTN is the only example of central respiratory chemoreceptor whose pH sensitivity in vivo and in vitro is reasonably well understood at the molecular level. The proton receptors responsible for the activation of other putative respiratory chemoreceptors are unidentified. A litany of ion channels has been proposed to mediate the effects of pH on breathing (TASK-1, TASK-3, Kir channels, various calcium channels, ASIC channels etc.). Although protonation can undoubtedly alter the activity of these proteins in vitro, there is little evidence yet that this effect plays a role in CRC (Wemmie et al., 2002; Bayliss et al., 2015).

Molecular CO_2 may also contribute to respiratory chemoreception in the following way. Several connexins (Cx26, 30 and 32, but not 31) can be activated when exposed to moderate levels of PCO_2 at constant pH (Huckstepp et al., 2010a). The authors hypothesize that CO_2 carbamylates in a pH-independent manner a lysine residue of connexin 26 hemi-channels expressed by astrocytes; this non-enzymatic reaction, thought to be analogous

to that of CO_2 with hemoglobin, would increase channel opening and allow ATP release (Huckstepp et al., 2010b; Huckstepp et al., 2010a; Meigh et al., 2013). Carbamylation was not directly observed but insertion of the presumptive carbamylation motif of Cx26 into Cx31, a CO_2-insensitive connexin, creates a CO_2-sensitive mutant hemichannel (Meigh et al., 2013). Finally, expression in glial cells of a dominant negative form of Cx26 (Cx26DN), which presumably removes the CO_2-sensitivity of endogenously expressed wild type Cx26, reduced the chemoreflex in mice (van de Wiel et al., 2020). However, this effect was transient, consisted of a selective reduction of V_T (no effect on fR) and was observed when mice were exposed to 6% but neither 3% nor 9% $FiCO_2$. Despite these shortcomings, this observation was nonetheless interpreted as evidence that molecular CO_2 contributes to CRC. Another unusual feature of the work is that the transduced glial cells were located in the caudal parapyramidal region, the presumed location of the caudal chemosensitive area described by early investigators in cats and dogs (Loeschcke, 1982; Mitchell et al., 1963). In sum, the existence of pH-independent stimulatory effects of CO_2 on breathing is a viable hypothesis in need of further evidence. ATP release by astrocytes has also been invoked by Gourine and his colleagues as a mechanism by which CO_2 triggers the HCVR (Turovsky et al., 2016) but the release mechanism proposed by these investigators is vesicular exocytosis and the initial event that causes vesicular release is depolarization, possibly via protonation and closure of a pH sensitive potassium channel. Protons and molecular CO_2 could conceivably both contribute to ATP release by brainstem astrocytes, hence to the HCVR.

Finally, bicarbonate may regulate RTN neurons activity independently of its effect on pH (Goncalves and Mulkey, 2018). The mechanism is not elucidated.

Conclusions. CRC: facts, speculations and potential therapeutic applications

CRC regulates breathing via feedback and via changes in state or behavior. The feedback mechanism has the lowest CO_2 threshold and up- or down-regulates breathing roughly in proportion to the intensity of the stimulus ($\Delta PaCO_2$); this process is presumably responsible for the entirety of the CNS effect of CO_2 on breathing during non-REM sleep and for most, perhaps all, the HCVR elicited by small increases in $PaCO_2$ at rest during waking. This feedback operates without producing any conscious aversive sensation (dyspnea), stress, or arousal, and works by activating the autorhythmic lower brainstem circuitry that maintains breathing at rest during wake and during NREM sleep or anesthesia. The principal nodal point of this feedback mechanism may be the retrotrapezoid nucleus.

Higher levels of $PaCO_2$ further enhance the feedback mechanism and trigger the behavioral component of the HCVR. The latter component is probably the result of dyspnea, stress, arousal, locomotion, or increased muscle tone and their associated effects on breathing. It could also result from direct effects of higher levels of acidification in the hypothalamus and elsewhere (distributed chemoreceptor theory). The behavioral component has a higher PCO_2 threshold than the homeostatic one and, like any behavioral response to an aversive stimulus, its specific manifestations are presumably species dependent. Unlike the homeostatic

component, the behavioral component is not linearly related to the stimulus intensity. This is especially clear of CO_2-induced arousal from sleep, an all-or-none event triggered when $FiCO_2$ reaches a particular threshold. The triggering event is probably defined by the convergence on the PBel-CGRP of multiple excitatory pathways whose intensity increases with PCO_2 (lung afferents, efferent copy from the pattern generator, RTN and serotonergic influences) (Kaur and Saper, 2019).

In this review, we have raised the possibility that the activation of the orexin system and the locus coeruleus by hypercapnia could be network driven rather than the result of direct CO_2/H^+ sensing by these neurons as postulated by proponents of the distributed chemoreceptor theory (Nattie, 2012; Nattie, 2011). Both types of neurons are sensitive to acid in vitro and activated by hypercapnia in vivo but this does not establish causality. How much of their intrinsic pH-sensitivity contributes to their activation by hypercapnia in vivo is simply unknown.

RTN (called pFRG in the newborn) can be objectively identified and distinguished from surrounding neurons by RNA expression profile and cell lineage; co-expression of *Phox2b* and *Nmb* transcripts is currently the most diagnostic criteria in rodents. RTN lesion or simultaneous deletion of TASK-2 and GPR4 reduces the central respiratory chemoreflex to an extent (>90%) that no other type of lesion or pharmacological treatment compatible with life has heretofore produced. In rodents, near complete RTN lesions produce moderate hypoventilation (around 10 mmHg rise in $PaCO_2$ in adult rats). This hypoventilation is limited by a compensatory increase in the contribution of the carotid bodies to breathing. The effect of RTN lesions on the HCVR is selective in the sense that they have no detectable effects on body temperature, metabolic rate and the hypoxic ventilatory reflex, they do not produce noteworthy breathing irregularities at rest, and they do not disrupt the orderly recruitment of pump and airway muscles. The consequences of RTN lesions are thus different from those caused by destroying the preBötzinger complex or dorsolateral pons (McKay et al., 2005). The effects of RTN lesions are also different from those caused by destroying serotonergic neurons. For instance, the HCVR deficit is much larger after RTN lesions, hypoventilation at rest is observed only after RTN lesion and RTN lesions have no effect on metabolism and thermoregulation.

Four non-exclusive mechanisms have been proposed to explain how RTN responds to hypercapnia *in vivo*: intrinsic neuronal sensitivity to protons mediated by TASK-2 and GPR4, paracrine activation of RTN neurons by CO_2-responsive astrocytes, CO_2-induced vascular contraction and synaptic inputs (excitatory inputs from other CO_2-activated neurons, especially serotonergic cells, and reduction of inhibitory input from CO_2-inhibited neurons). The paracrine effect of astrocytes is mediated by ATP release and possibly triggered by a direct effect of protons or molecular CO_2 on these cells. We do not know at present whether the coexistence and cooperation of these four mechanisms are unique characteristics of the RTN, but this possibility could explain why these neurons and the breathing network are so sensitive to pH changes in vivo (threshold < 0.01 pHa) and why RTN is so critical to CRC.

The relative importance of these four mechanisms has not been precisely quantified. The cell autonomous mechanism could be viewed as the most critical because deleting the molecular proton detectors, TASK-2 and GPR4, the two proteins responsible for the activation of RTN neurons by acid, practically eliminates the HCVR. Yet, alternative interpretations of these experiments have not been eliminated, for example the possibility that these proteins have other critical functions in RTN neurons besides proton sensing. The relative contribution of the four mechanisms could also depend on the level of hypercapnia and the degree to which the behavioral component of the reflex is triggered. The massive HCVR reduction caused by RTN lesions suggests that this nucleus could also mediate a substantial portion of the behavioral component of the reflex. Indeed, RTN receives a strong orexin, serotonergic and noradrenergic input, and evidence suggests that it may contribute to the hyperpnea of exercise.

RTN neurons regulate alveolar ventilation by activating the respiratory rate, inspiratory amplitude, and active expiration. How this is accomplished at the network level is not yet clear since current understanding of the connectivity of RTN neurons relies exclusively on anatomical studies. RTN neurons are definitely not premotor neurons but probably innervate the latter along with many other targets. RTN regulates the respiratory pattern generator via excitatory axonal projections to the ventral respiratory column, the lateral parabrachial complex and selected regions of the NTS.

Rate control by RTN operates under anesthesia, quiet resting and SWS but it is non-functional during REM sleep. Accordingly, this nucleus selectively regulates breathing auto-rhythmicity. Presumably, RTN has no control over the breathing rhythm during sniffing, vocalization, or any form of cortical control of breathing, but this hypothesis needs testing.

RTN regulates tidal volume by controlling inspiratory amplitude and active expiration. Both effects are likely mediated in part by direct excitatory projections to inspiratory premotor neurons located in the rVRG (inspiration) and the cVRG (abdominal muscle control). However, the membrane trajectory of inspiratory and expiratory premotor neurons is also sculpted by inputs from inhibitory neurons (expiratory, inspiratory and post-inspiratory) that could be influenced by RTN projections to other portions of the pontomedullary respiratory network (Flor et al., 2020).

The mechanism by which RTN elicits active expiration is surprisingly controversial. The divergence of views is undoubtedly exacerbated by the elastic definition of what constitutes a respiratory oscillator, by the unusual oscillator-like properties of late embryonic RTN neurons (Thoby-Brisson et al., 2009) and by the proximity of RTN neurons with unrelated expiratory-related neurons that probably contribute to active expiration (Magalhaes et al., 2021).

Although the RTN contains a mere 700 neurons in mice, and 2000 in rats, it could consist of several neuronal subtypes each dedicated to the control of a particular aspect of breathing (rate, inspiration, active expiration, sighing, CO_2-induced arousal). This hypothesis has yet to be convincingly documented but a few clues exist. For example, RTN neurons display several types of respiratory modulation (Guyenet et al., 2005). Also, a subgroup of RTN

neurons that expresses particularly high levels of neuromedin B may selectively control emotional sighing (Li et al., 2020). Yet, RTN does not appear essential to hypoxia-induced sighing (Souza et al., 2018). An important future research objective will be to monitor the discharge pattern of various subsets of chemically identified RTN neurons in intact unanesthetized laboratory animals under various physiological conditions (rest, exercise, sleep, hypercapnia, hypoxia etc.).

Serotonergic neurons facilitate breathing and enhance the HCVR. This facilitation relies prominently on the Egr-2 derived lower brainstem serotonergic neurons and on the dorsal raphe. The dorsal raphe appears particularly responsible for the portion of the HCVR that depends on arousal (Kaur et al., 2020). The Egr-2 derived serotonergic neurons, may fit the strict definition of a central respiratory chemoreceptor (Brust et al., 2014). Under certain conditions (slices, culture) most serotonergic neurons clearly respond to extracellular acidification but this is not the case in vivo. According to currently available evidence, 80% of the putative serotonergic neurons recorded in the dorsal raphe or the midline medullary raphe of intact unanesthetized animals are unresponsive to hypercapnia (Veasey et al., 1997; Veasey et al., 1995) and mechanisms other than an intrinsic sensitivity to pH could account for those that are activated. The parapyramidal group of serotonergic neurons are inhibited by hypercapnia in rats (Takakura and Moreira, 2013; Mulkey et al., 2004) and the activity of raphe obscurus serotonergic neurons of anesthetized mice is unaffected by hypercapnia (Depuy et al., 2011). In the final analysis whether serotonergic neurons are respiratory chemoreceptors will require definite and currently unavailable answers to the following questions. Which subset of neurons respond to hypercapnia in the unanesthetized animal (as opposed to in vitro)? is their activation network driven or caused by their intrinsic sensitivity? and, if the latter is correct, what is the molecular basis of their pH sensitivity?

The role of the RTN is probably not limited to the feedback regulation of breathing by CO_2 although this assertion rests on limited evidence. RTN/pFRG may contribute to the hyperpnea of exercise, perhaps via inputs from the spinal locomotor network or muscle afferents (Barna et al., 2014; Le Gal et al., 2020; Le Gal et al., 2014; Kanbar et al., 2016). Also, RTN receives excitatory inputs from orexinergic neurons which activate breathing when metabolism increases (as during waking or stress). In sum, RTN probably integrates multiple interoceptive signals besides brain pH, along with CNS information related to exercise, metabolic conditions, and the state of vigilance. The integration of all these inputs by RTN may be the elusive mechanism by which lung ventilation matches the metabolic rate and thus maintain PCO_2 stability. The homeostatic function of the RTN may be formally more comparable to that of the hypothalamic arcuate nucleus whose output defines the amount of food to be ingested to maintain body weight constant (Chen et al., 2015).

Based on mouse genetic studies, the congenital absence of RTN is a likely cause of the drastically reduced HCVR of CCHS patients. However, the anatomical characterization of this nucleus in primates is still sketchy (Levy et al., 2019; Rudzinski and Kapur, 2010) and evidence of its absence or hypoplasia in CCHS is still unconfirmed. In rodents at least, genetic, or more conventional manipulations that eliminate RTN do not cause sleep apnea, one of the cardinal signs of CCHS (Ramanantsoa et al., 2011; Souza et al., 2018). Thus, CCHS patients likely have additional lesions that impact the homeostatic control of

breathing during sleep. These defects probably involve structures whose development or function in adulthood also depends on Phox2b expression such as the carotid bodies and the lower brainstem circuits responsible for the HVR (Pattyn et al., 1997; Stornetta et al., 2006; Perez and Keens, 2013).

The postnatal development of the RTN is also of both theoretical and practical interest. Early childhood is associated with breathing instability and pathologies such as neonatal sleep apnea and sudden death which could be caused, or exacerbated, by disruption of the metabolic control of breathing (Darnall, 2010). The gain of the HCVR increases notably during the first two weeks after birth (in rats) (Davis et al., 2006). These observations have several possible interpretations that need to be disambiguated. Rodents are born at a very early stage of brain development; the postnatal increase in HCVR gain could denote the maturation of the behavioral component of the reflex. Alternatively, the HCVR gain could be increasing because an important component of the lower brainstem CO_2 sensing mechanism, perhaps the astrocyte-dependent paracrine mechanism, only becomes fully functional during this neonatal period. Finally, the postnatal increase of the HCVR gain could have nothing to do with the CO_2-sensing mechanism proper but could owe to the maturation of the pattern generator and air pumping muscles.

Periodic breathing caused by hypoxia at altitude is presumably caused by cyclical inhibition of the RTN. Indeed, the RTN is silenced when conscious rats are exposed to mild hypoxia (12-15% FiO_2) and this is the direct consequence of respiratory alkalosis because the activity of this nucleus is restored by acidifying the blood with CO_2 or acetazolamide (Basting et al., 2015). By extension periodic breathing in humans likely results from periodic inactivity of the RTN. Other putative central respiratory chemoreceptors could certainly also contribute to cyclical breathing during hypoxia, but evidence is lacking so far.

In rats, mild optogenetic stimulation of RTN neurons during SWS sleep activates breathing without causing arousal or a change in blood pressure, consistent with the fact that arousal from SWS requires a substantial increase in $FiCO_2$ (Burke et al., 2015b). Thus, if ever practicable in humans, mild RTN stimulation could help maintain breathing in patients suffering from central sleep apnea without adversely affecting sleep or blood pressure. Regrettably, this procedure might be ineffective in CCHS patients given that they probably lack the RTN (Amiel et al., 2009). Finally, RTN stimulation is likely to increase airway patency since the tone of upper airway muscles is also under central chemoreceptor control(Fregosi and Ludlow, 2014). Accordingly, mild RTN stimulation could also conceivably help relieve the most common form sleep apnea, the obstructive type, also without impacting sleep or blood pressure.

In conclusion, the critical importance of the rostral chemosensitive area to CRC has been confirmed by modern research, key neurons such as the RTN have been identified and the molecular mechanisms by which CO_2 stimulates breathing are much better understood. Based on the study of this nucleus, respiratory chemoreceptors may be multicellular sensors. This is not unique. Sodium and osmotic pressure detection by the organum vasculosum laminae terminalis (OVLT) also rests on intrinsic properties (TRPV1) of the principal neurons and paracrine effects of sodium-sensing specialized astrocytes (Guyenet, 2019;

Ciura et al., 2011). In the case of sodium, the key biological variable is sensed only in very few brain locations and the neuronal output of the sensor is regulated by synaptic inputs from neurons that anticipate or react to changes in the regulated variable (Zimmerman et al., 2016). Substituting Na with CO_2, this general model (feedback and anticipatory regulation of the output of the sensor) applies to the RTN. Feedback regulation originates from cardiopulmonary receptors and anticipatory regulation likely from inputs related to exercise, the state of vigilance and hypothalamic circuits encoding energy expenditure. The existence of central respiratory chemoreceptors other than the RTN is eminently plausible but the question needs to be investigated further using the strict defining criteria enunciated in the introduction to this chapter.

Glossary:

Bötzinger region
VRC segment #1 (Figure 3B). Controls breathing and the circulation

caudal VRG
VRC segment #4 (Figure 3B). Controls abdominal muscles implicated in active expiration

CCHS
congenital central hypoventilation syndrome. Developmental disease. Main signs are absence of HCVR, reduced HVR, sleep apnea and lack of arousal to asphyxia

Central command
feed-forward regulation of breathing for example via input from a locomotor pattern generator to the brainstem respiratory network

Central respiratory chemoreceptor
brain neuron that is activated or inhibited by acidification **and** capable of increasing breathing as a result. By definition, effect of $[H^+]$ must be an intrinsic neuronal property or a local paracrine mechanism (e.g., astrocyte- or vasculature-dependent)

CRC
central respiratory chemoreception: detection of changes in CO_2/H^+ within the brain and the associated effects on breathing

Embryonic parafacial oscillator (ePF)
synonym for prenatal RTN/pFRG

$FiCO_2$
% CO_2 in inspired gas

fR
breathing frequency

HCVR
hypercapnic ventilatory reflex; breathing stimulation elicited by high $FiCO_2$

HVR
hypoxic ventilatory reflex: elicited by hypoxia (reduced FiO_2) this reflex is initiated by the carotid bodies

NTS
nucleus tractus solitarius. Receives sensory input from the carotid bodies, lungs and airways

PaO_2
arterial partial pressure of O_2

$PaCO_2$
arterial partial pressure of CO_2

Parafacial
adjective referring to the reticular formation surrounding or below the facial motor nucleus (Figure 3B)

preBötzinger complex
VRC segment #2 (Figure 3B); region required for breathing auto-rhythmicity

pFL and pFV
lateral and medial half of the brain region that contains the RTN neurons (definition after (Huckstepp et al., 2015)). pFL and pFV contain other neurons beside RTN. pFL may also contain an expiratory oscillator

pFRG
parafacial respiratory group. Word originally coined by Onimaru et al. (2003) to describe a neuronal group with pre-inspiratory bursts located rostral to the Bötzinger region in neonate rats. pFRG neurons express Phox2b and other markers of the RTN and are almost certainly the neonatal precursors of the latter (Guyenet et al., 2019; Shi et al., 2017; Onimaru et al., 2014). The RTN is thus often, and appropriately, called RTN/pFRG. Unfortunately, the moniker pFRG is also used as a generic term to describe a vaguely defined region of the pontomedullary reticular formation that surrounds the facial motor nucleus

Phox2b
transcription factor required for development of visceral afferents and selected lower brainstem neurons, including RTN. Phox2b mutations are the principal cause of CCHS

Respiratory neuron
term describing any neuron whose discharge is phase-locked with the respiratory outflow. Such neurons may or may not play a role in breathing

Rostral VRG (rVRG)
VRC subdivision (#3 in Figure 3B) containing inspiratory premotor neurons

Sleep apnea
breathing cessation during sleep

Ve

minute ventilation; product of tidal volume (volume of air inhaled during a single breath) x fR

VRC

ventral respiratory column: network that generates the respiratory rhythm and pattern; located in the ventrolateral medulla; includes (from rostral to caudal) Bötzinger region, preBötzinger complex, rostral VRG, caudal VRG

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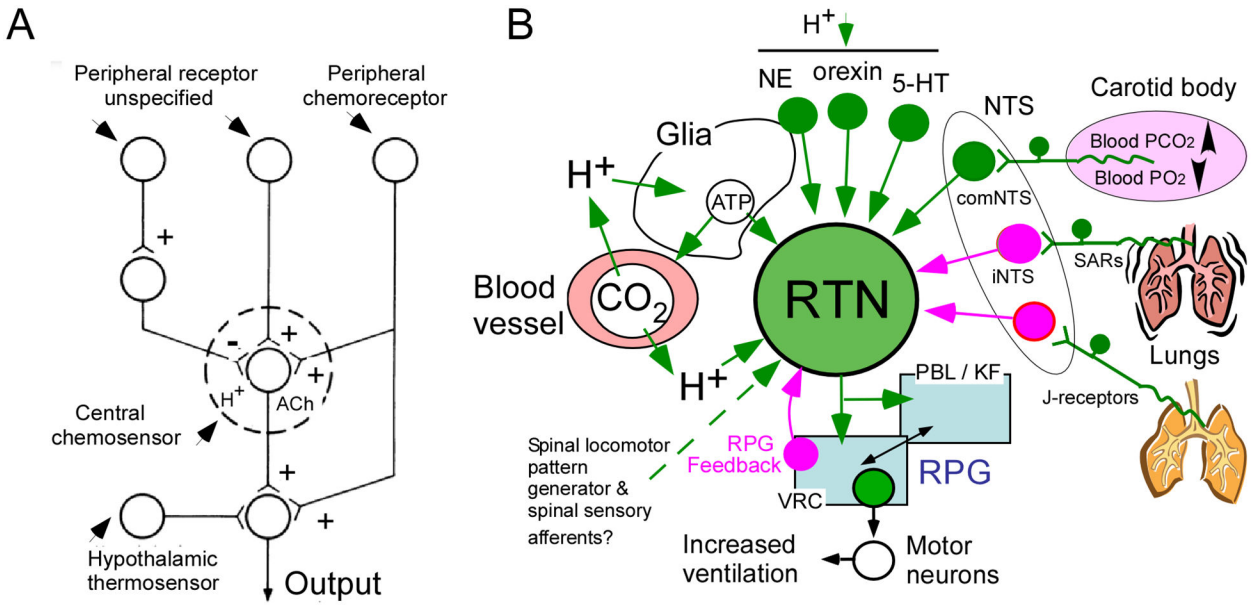

Figure 1: Respiratory chemoreceptor neurons

A. Loeschcke's concept of a ventral medullary surface respiratory chemoreceptor (1982), reproduced with permission from Wiley. The neurons and all their connections were inferred from the study of respiratory reflexes in anesthetized cats, dogs or goats, the deficits caused by various lesions of the ventral surface of the medullary oblongata (VMS) and the effects produced by topical application of drugs or acidic solutions to the VMS. Based on pharmacological evidence Loeschcke (1982) postulated that "the chemosensitive mechanism is a cholinergic synapse on which H+ acts like ACh and can be replaced by it". This particular mechanism has not been verified but Loeschcke's view that the VMS pH sensing mechanism is complex and that the lynchpin of the system consists of H+ activated neurons that are subject to myriad synaptic inputs is well supported by recent evidence.

B. Current concept of a ventral medullary surface respiratory chemoreceptor ((from (Guyenet and Stornetta, 2022) copyright by Elsevier): the retrotrapezoid nucleus (RTN). The chemosensor is probably a multicellular structure. [H+] sensing is mediated by the intrinsic pH sensitivity of the output neurons (RTN neurons) and by paracrine effects of astrocytes and blood vessels on these neurons. RTN neurons are identified by their gene expression pattern (*Phox2b*, *Nmb*, *VGlut2*). Their structure, axonal projections and inputs have been examined by histological and electrophysiological methods and their effect on breathing has been determined using selective optogenetic loss- or gain of function experiments and a variety of lesions. The existence of excitatory input from peripheral chemoreceptors and inhibitory inputs activated by lung stretch afferents were postulated by Loeschcke and have been confirmed.

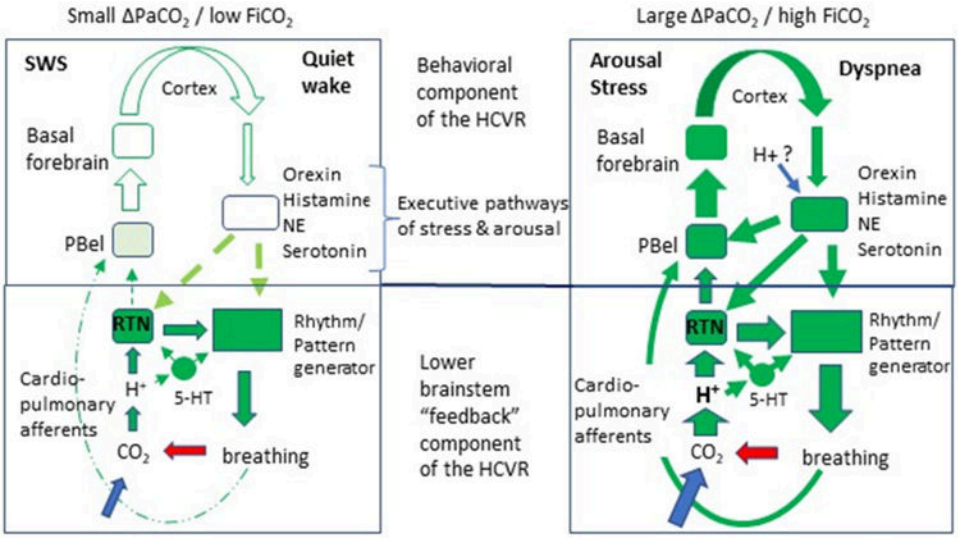

Figure 2: Feedback vs. behavioral components of the hypercapnic ventilatory reflex

Left side: in the conscious resting state and during slow-wave sleep, small changes in $PaCO_2$ activate breathing via pH sensors and circuits that are largely, perhaps exclusively confined to the pontomedullary region. The effect of CO_2 operates as simple feedback. [H+] is sensed primarily by the RTN and, presumably a subset of serotonergic neurons.

Right side: exposure to higher levels of $FiCO_2$ triggers the behavioral component of the reflex, the result of arousal from sleep, stress and dyspnea. Arousal is triggered via the convergence on the lateral parabrachial region (external lateral nucleus, PBel) of multiple pathways activated by the rise in CO_2 including central and peripheral chemoreceptors and pathways activated by the rise in breathing (pulmonary and airway afferents, respiratory pattern generator). Arousal, stress or dyspnea engage basal forebrain, cortical and hypothalamic structures. Executive pathways of arousal and stress such as the orexin system and the locus coeruleus are activated as a result and further enhance breathing via their excitatory connections to the pontomedullary respiratory pattern generator and chemoreceptor neurons such as the RTN. The locus coeruleus, subsets of lower brainstem serotonergic neurons and the orexin system may also respond directly to acid.

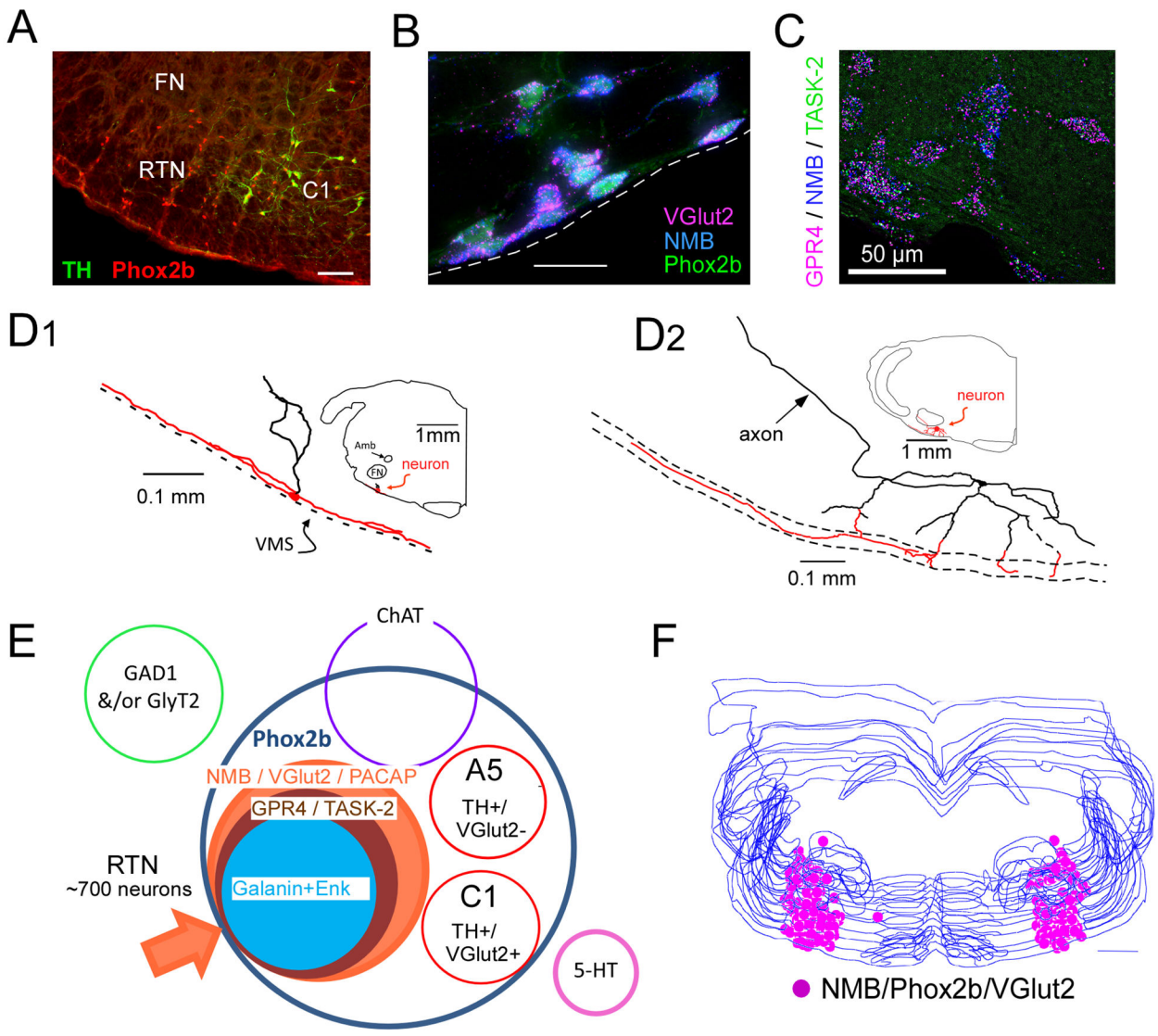

Figure 3: Anatomy and transcriptome of the RTN

A: RTN identified in a rat by the presence of Phox2b-immunoreactive nuclei and the absence of tyrosine hydroxylase (transverse section, left side). C1, tyrosine-hydroxylase-positive neurons which also express Phox2b. FN, facial motor nucleus (Phox2b-negative in the adult rodent); after (Guyenet and Bayliss, 2015). **B.** Transverse section from *Phox2b*-EYFP mouse brain showing co-expression of Phox2b and mRNA transcripts for *Neuromedin B* (*NMB*) and *VGlut2. NMB* mRNA is absent from the C1 cells (not shown). **C.** co-localization of mRNA transcripts for *NMB, GPR4 and TASK-2* in RTN (mouse brain). GPR4 and TASK-2 are putative proton receptors; after Shi et al. (2017). **D1, D2.** Dendritic structure of two representative RTN neurons identified by unit recording in anesthetized rats and labeled juxtacellularly with biotinamide. Long dendrites (highlighted in red) confined to within 200 microns of the ventral medullary surface (dotted line) are a characteristic feature which suggests that RTN neurons may sense both cerebrospinal and extracellular fluid [H^+]. **E.** Venn diagram depicting the biochemical features that distinguish

the mouse RTN from neighboring neurons. RTN is defined as a cluster of neurons positive for *NMB*, *PACAP* and *VGlut2*. *GPR4* and *TASK-2* transcripts are detectable in ~ 90% of these neurons; after Shi et al. (2017). **F.** Anatomical location of the mouse RTN defined as neurons (n = ~700) that co-express *NMB*, Phox2B and *VGlut2*; neurons plotted on a series of equidistant transverse sections (calibration 0.5mm); after Shi et al. (2017).

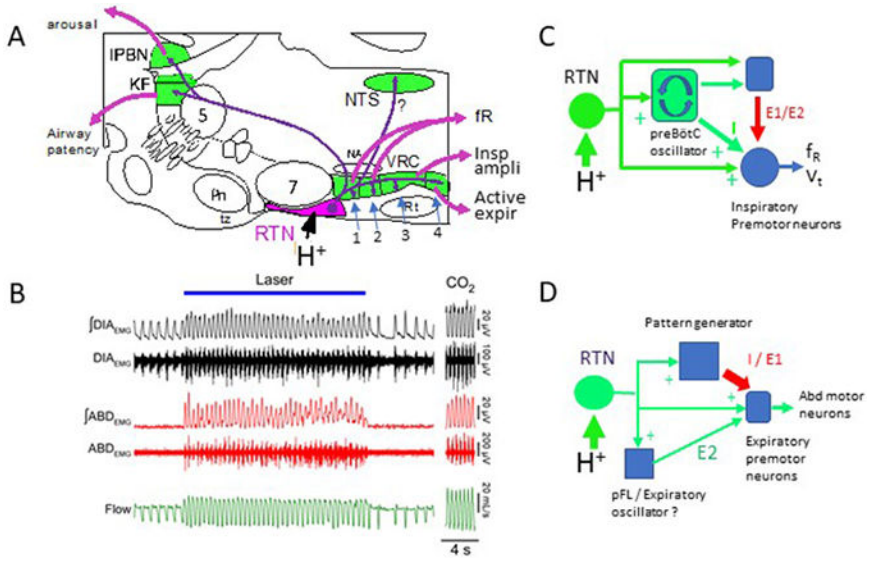

Figure 4: RTN neurons activate breathing frequency, inspiratory activity and abdominal expiration

A. RTN innervates the lateral parabrachial region (lPBN), the Kölliker-Fuse nucleus (KF), all four subdivisions of the ventral respiratory column (identified by numbers 1-4 from rostral to caudal: Bötzinger region, preBötzinger complex, rVRG and cVRG). RTN also innervates the NTS; NTS targets are unidentified; they may include inspiratory premotor neurons (dorsal respiratory group). Magenta arrows indicate the presumed function of the various RTN projections. **B.** unilateral selective optogenetic stimulation of the RTN in an unanesthetized rat; the effect is compared to that of 10% $FiCO_2$ (right column labelled "CO_2") (after (Souza et al., 2020). **C.** RTN connections that may underlie the effect of RTN on breathing frequency and inspiratory amplitude. The blue square represents inputs from early and late expiratory neurons located in the ventral respiratory column or the dorsolateral pons. **D.** RTN connections that may underlie the effect of RTN on active expiration. A presumptive expiratory oscillator, pFL, is located lateral to the RTN neurons and may receive synaptic inputs from the latter.

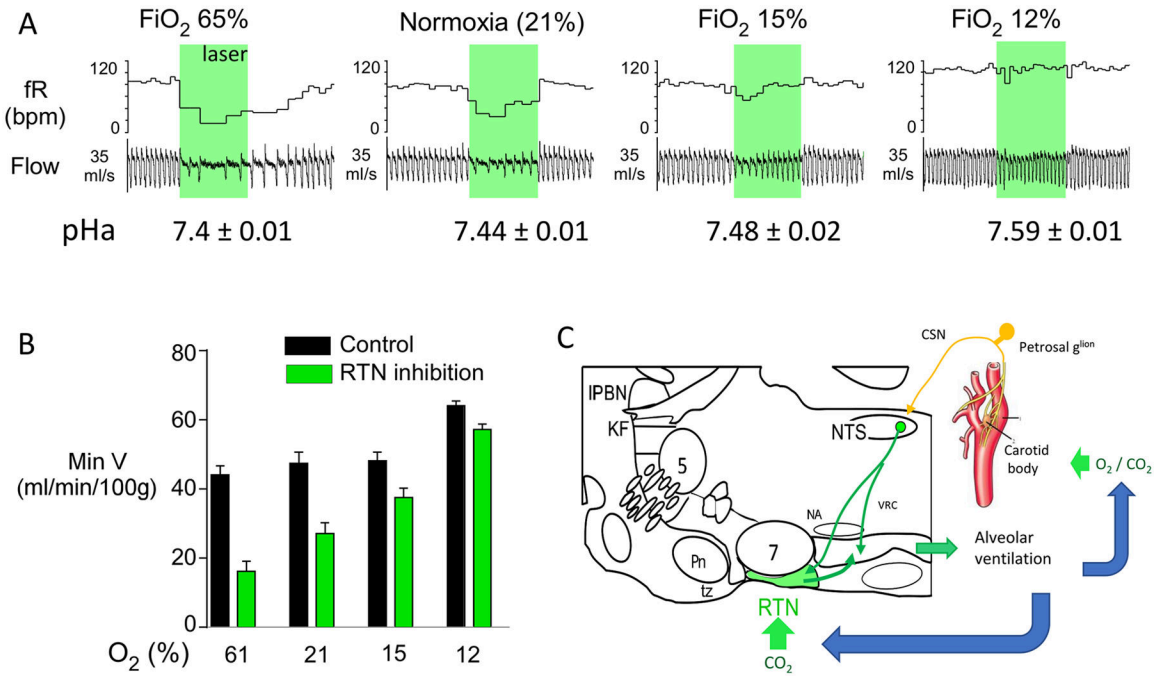

Figure 5: Interactive feedback regulation of breathing by the RTN and carotid bodies

A. Respiratory acidosis and alkalosis elicited respectively by hyperoxia or graded hypoxia in a conscious rat. The breathing stimulus contributed by RTN (gauged by the instant breathing reduction elicited by bilateral optogenetic inhibition) increases markedly with acidosis and decreases with alkalosis becoming virtually nil at 12% FiO_2. **B.** At steady-state, minute ventilation (V_E) is constant in conscious rats exposed to FiO_2 from 65% down to 12% (equivalent to 2700m altitude) presumably because the hypoxia-derived stimulatory effect of the carotid bodies on breathing is exactly compensated by the reduction in RTN activity elicited by alkalosis. Hypoxia starts increasing V_E only after RTN is fully inhibited (FiO_2 12% and below). **C.** schematic interpretation of the dual control of breathing by RTN and the carotid bodies. Both chemoreceptors can activate the respiratory pattern generator independently of each other. Under anesthesia, the carotid bodies can also excite RTN (Takakura et al., 2006). This connection has not yet been demonstrated in unanesthetized mammals.

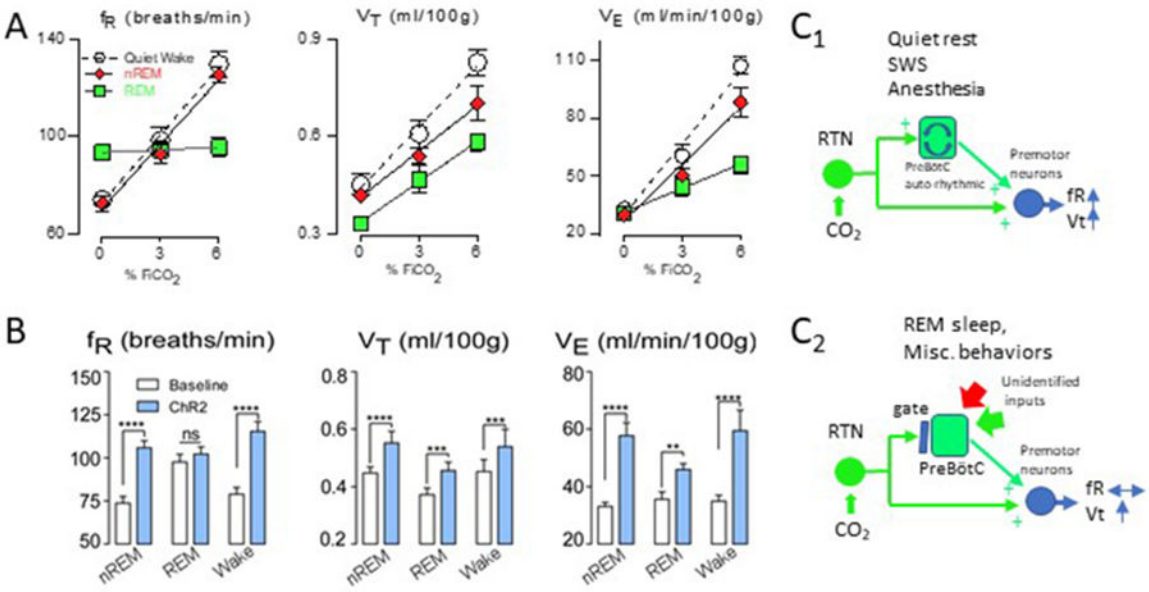

Figure 6: state-dependence of CRC and implications for periodic breathing

A. Breathing frequency, tidal volume and minute volume in unanesthetized rats exposed randomly to room air, 3% and 6% $FiCO_2$ during slow-wave sleep (SWS), REM sleep and quiet waking. V_T increases regardless of the state of vigilance, albeit somewhat more during waking than during SWS or REM. CO_2 increases fR during quiet waking and SWS but has no effect on fR during REM sleep. **B.** the breathing stimulation elicited by unilateral optogenetic stimulation of RTN has the same state dependence as that evoked by CO_2 (A-B after (Burke et al., 2015a) with permission from Wiley). **C1 and C2.** Schematic interpretation: RTN activates fR via its projections to the preBötzinger complex and V_T via projections to premotor neurons. fR is controlled by RTN only when the preBötzinger complex is autorhythmic. During REM sleep or behavioral control of breathing the preBötzinger complex is either bypassed or under the control of external inputs and the effect of RTN on fR is gated out. The effect of RTN on V_T, largely mediated by excitatory inputs to the premotor neurons persists.

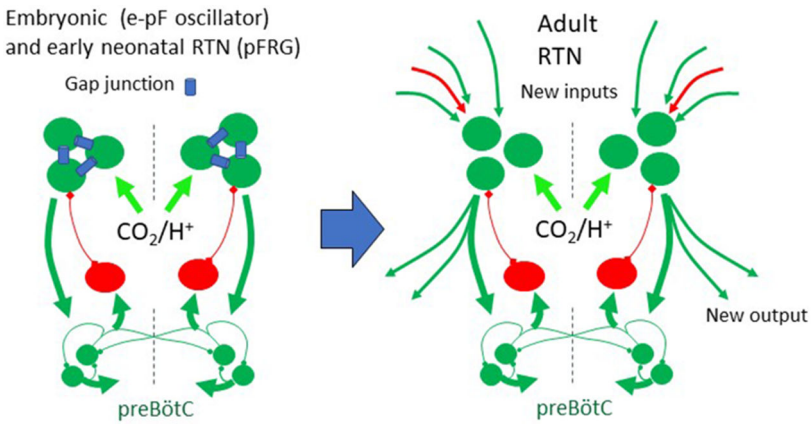

Figure 7: ePF, pFRG and RTN, same neurons, different development stage.

Left: schematic representation of the prenatal precursor of the RTN, the embryonic parafacial oscillator (Thoby-Brisson et al., 2009) as interpreted by Guyenet and Bayliss. The discharge of these neurons occurs in bursts; it is [H^+]-driven, amplified by gap junctions and synchronized with the preBötzinger oscillator via inhibitory synaptic feedback (in red). During a brief period after birth the rodent RTN, a.k.a. pFRG (Onimaru et al., 2012; Onimaru and Homma, 2003) retains its late embryonic firing characteristics.

Right: the adult RTN acquires multiple new synaptic inputs and innervates every portion of the rhythm and pattern generator. The neurons are still driven by the local [H+]. They do not burst synchronously, perhaps because they are no longer connected through gap junctions or because the feedback from the preBötzinger complex plays a lesser role.

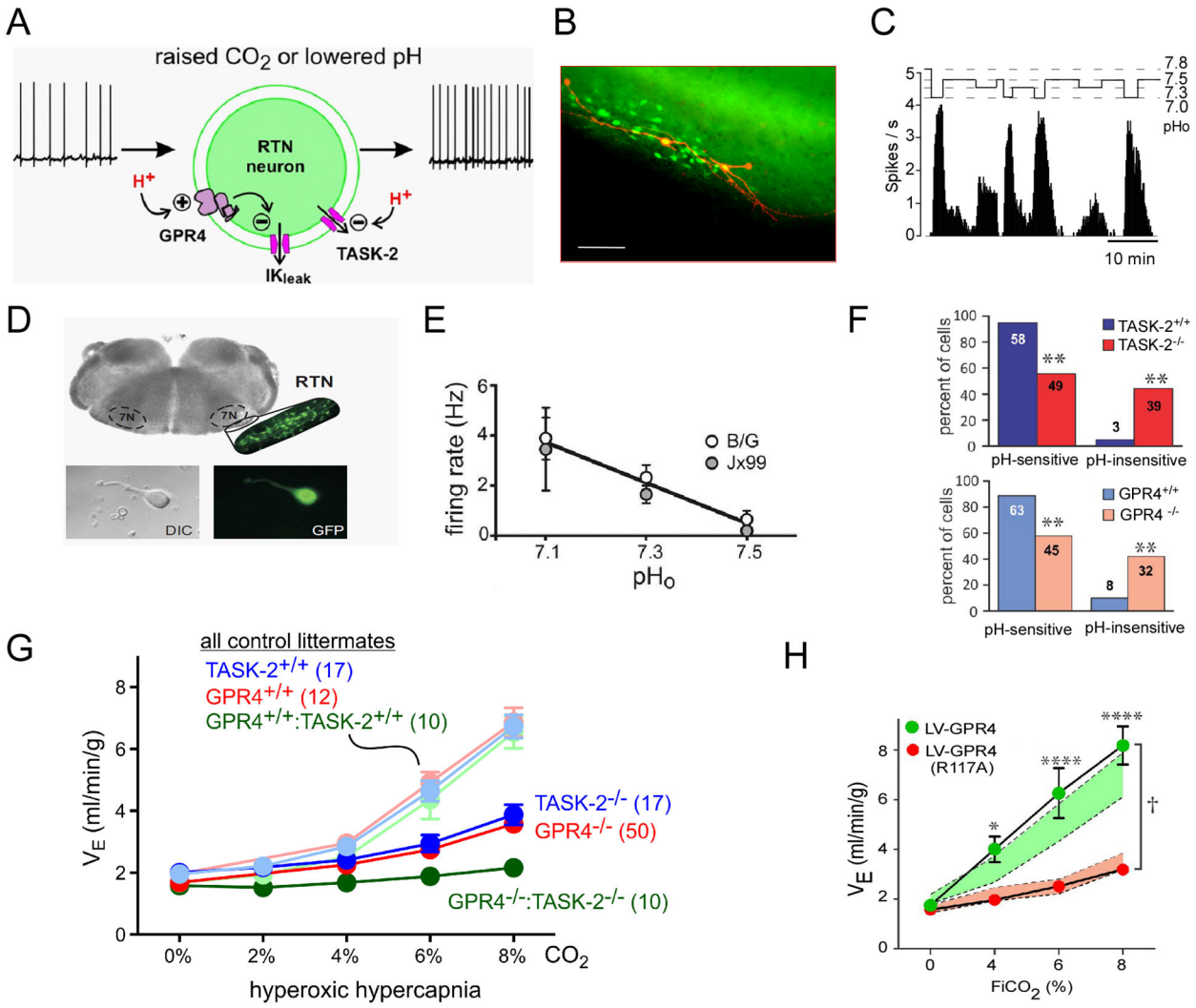

Figure 8: RTN chemosensitivity; the cell autonomous pH-sensitivity hypothesis.

A. Intrinsic pH-sensitivity of RTN neurons relies of two proton receptors: the potassium channel TASK-2 which is closed by acidification and the G-protein coupled receptor GPR4 which closes an unidentified potassium channel.

B. Intracellular fills (biotinamide) of two RTN neurons recorded in a transverse brain slice (neonatal *Phox2b-EGFP* JX99 mouse; scale bar: 50 μm). Note the extensive superficial dendrites. After Guyenet et al. (2016).

C. Effect of acidification on the discharge rate of a single RTN neuron (neonatal Phox2b-EGFP JX99 mouse); after Lazarenko et al.(2009).

D. Dissociation and isolation of RTN neurons from the Phox2b-EGFP JX99 mouse; after Wang et al., (2013b)

E. Relationship between mean discharge rate and bath pH in RTN neurons isolated from two different transgenic strains of Phox2b-EGFP mice; after Wang et al. (2013b).

F. A substantial population of pH-insensitive RTN neurons is evident in recordings from brain slices from either TASK2−/− or GPR4−/− mice; after (Kumar et al., 2015) and (Wang et al., 2013a).

G. The central respiratory chemoreflex of TASK2−/− and GPR4−/− mice (breathing response to CO_2 in hyperoxia) is ~65% reduced relative to control mice. The reflex of the double KO mice is virtually absent; after Kumar et al.(2015).
H. Depressed chemoreflex of GPR4−/− mice is restored to control level by re-expressing wild-type GPR4, but not a signaling-deficient GPR4(R117A) mutant, in RTN neurons with a lentiviral vector; after Kumar et al.(2015). The entire figure is reproduced from

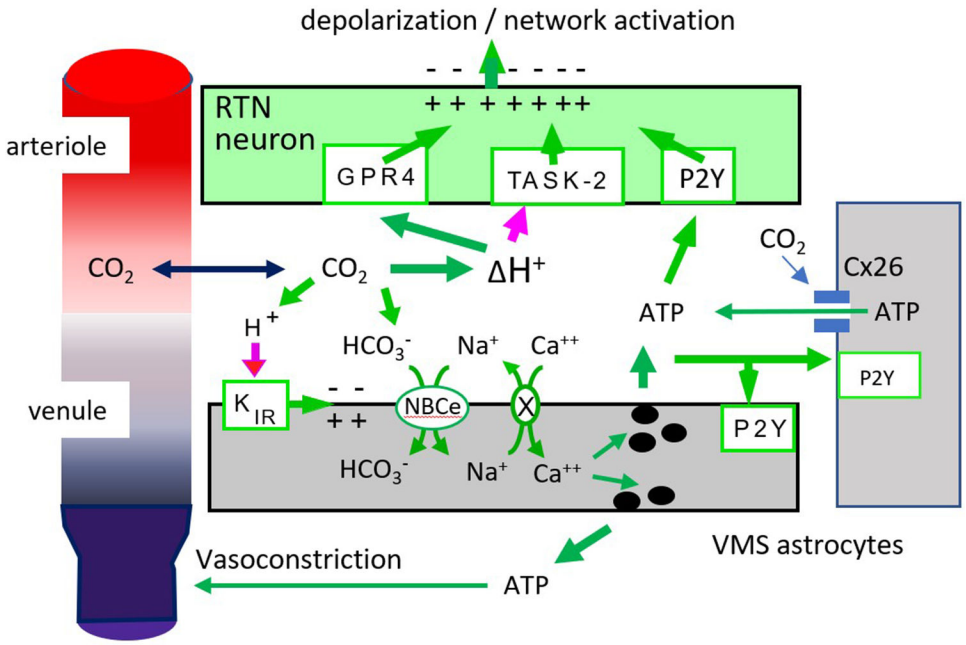

Figure 9: RTN chemosensitivity; cell-autonomous, paracrine and microvascular hypotheses

Cell autonomous pH sensitivity: CO_2 diffuses freely between blood vessels and brain parenchyma. Hypercapnia increases [H+] which depolarize RTN neurons by closing TASK-2 potassium channel and by activating the proton receptor GPR4.

Paracrine hypothesis after Gourine et al. (2010): Hypercapnia increases [H+] which depolarizes astrocytes by inhibiting potassium channels Kir(4.1/5.1)(Mulkey and Wenker, 2011). The membrane depolarization drives bicarbonate inside astrocytes via the electrogenic sodium-bicarbonate exchanger (NBCe1). Bicarbonate exchanges with sodium which in turn drives calcium influx via the sodium/calcium exchanger. The rise in intracellular calcium elicits the vesicular release of ATP which activates RTN neurons and possibly other glial cells via P2Y receptors. An alternative hypothesis posits that molecular CO_2 reacts non-enzymatically with connexin 26 causing its opening and the release of ATP from astrocytes by simple diffusion through the open hemichannels (Huckstepp et al., 2010a; Huckstepp et al., 2010b).

The microvascular hypothesis after Cleary et al. (2020): CO_2 constricts the RTN vasculature by releasing ATP, reducing washout of CO_2 derived from brain metabolism and enhancing [H+] in the vicinity of the chemoreceptors (RTN or astrocytes). Astrocytes are shown here as the source of the ATP, but this point is hypothetical.

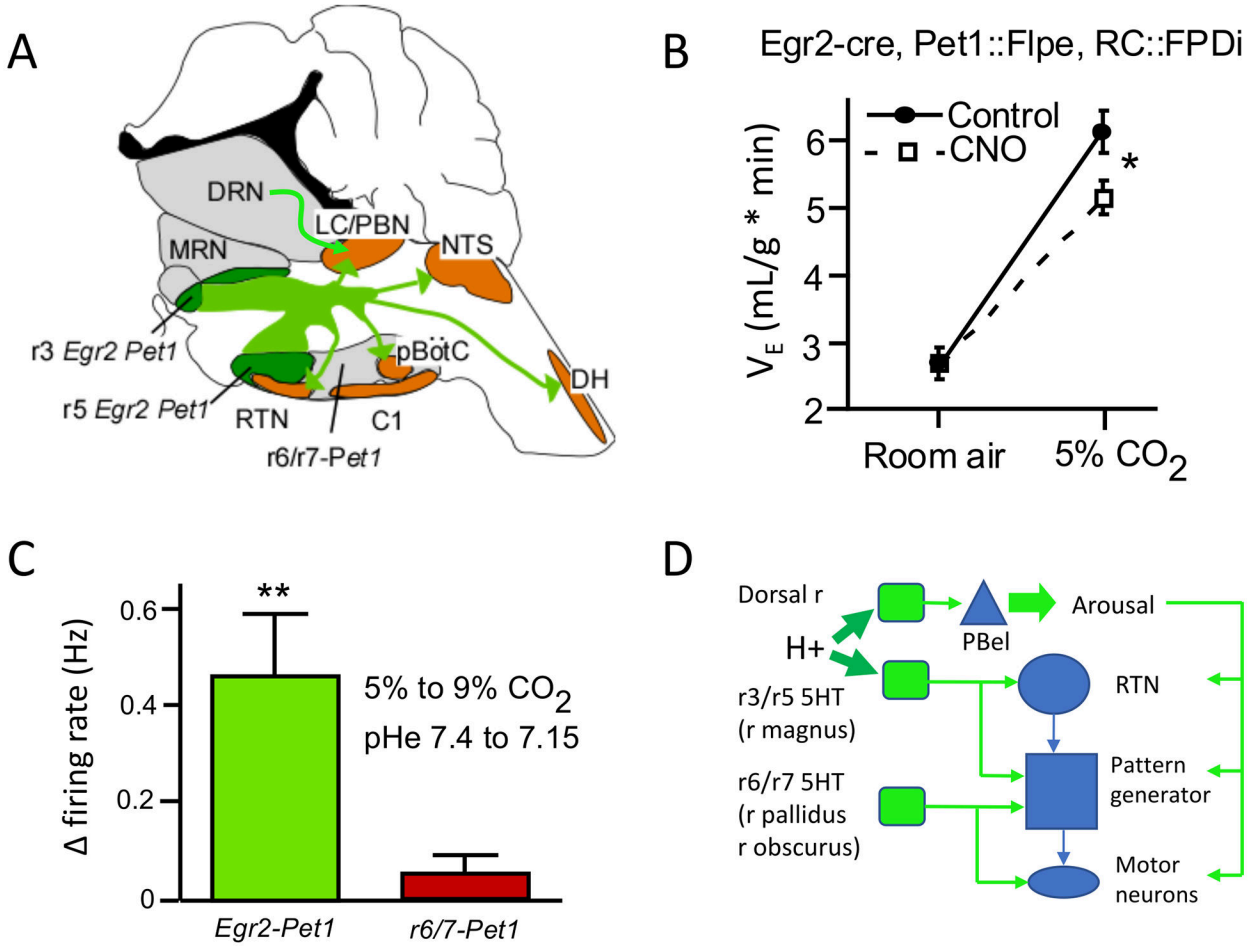

Figure 10: contribution of serotonergic neurons to CRC

A. The dorsal raphe (DRN) serotonergic projection to the external lateral subnucleus of the lateral parabrachial complex (PBN) enables CO_2-induced arousal. The dorsal raphe may be directly responsive to acid. Serotonergic neurons of rhombomere 3/5 lineage (*Egr-2* dependent) populate parts of raphe magnus and obscurus and innervate selected portion of the spinal cord (dorsal horn) plus portions of the medulla oblongata that regulate breathing, including the RTN. **B.** Pharmacogenetic inhibition of Egr-2-derived serotonergic neurons reduce the HCVR by around 15%. **C.** *Egr-2*-derived serotonergic neurons are mildly activated by CO_2 in slices whereas those derived from rhombomere 6 and 7 do not respond. **D.** Interpretation by Guyenet and Bayliss of the contribution of the serotonergic neurons to CRC. A-C from (Brust et al., 2014) with slight modifications. Letter “r”, as in Dorsal r, stands for raphe.