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Review

Neural Control of Breathing and CO_2 Homeostasis

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Recent advances have clarified how the brain detects CO_2 to regulate breathing (central respiratory chemoreception). These mechanisms are reviewed and their significance is presented in the general context of CO_2/pH homeostasis through breathing. At rest, respiratory chemoreflexes initiated at peripheral and central sites mediate rapid stabilization of arterial PCO_2 and pH. Specific brainstem neurons (e.g., retrotrapezoid nucleus, RTN; serotonergic) are activated by PCO_2 and stimulate breathing. RTN neurons detect CO_2 via intrinsic proton receptors (TASK-2, GPR4), synaptic input from peripheral chemoreceptors and signals from astrocytes. Respiratory chemoreflexes are arousal state dependent whereas chemoreceptor stimulation produces arousal. When abnormal, these interactions lead to sleep-disordered breathing. During exercise, central command and reflexes from exercising muscles produce the breathing stimulation required to maintain arterial PCO_2 and pH despite elevated metabolic activity. The neural circuits underlying central command and muscle afferent control of breathing remain elusive and represent a fertile area for future investigation.

Introduction

All cellular functions of the brain and body are influenced by the prevailing pH and only small pH variations are compatible with life. Because metabolically produced CO_2 is in rapid equilibrium with H^+, and can be removed via lung ventilation, dynamic control of breathing by CO_2 provides a major homeostatic mechanism for acute regulation of acid-base status. The molecular, cellular, and neural bases for this critical interoceptive chemosensory control system have been greatly clarified in recent years.

Three classes of neural mechanisms are implicated in matching the metabolic production of CO_2 to its elimination by the lungs: the chemoreflexes, central command, and neural feedback from muscles (Forster et al., 2012). The central respiratory chemoreflex is the breathing stimulation elicited by elevated brain PCO_2 (CNS hypercapnia); the peripheral chemoreflex is the breathing stimulation elicited by activation of the carotid bodies and related organelles (aortic bodies) (Dempsey et al., 2012; Kumar and Prabhakar, 2012). The carotid bodies are activated by arterial hypoxemia in a pH-dependent manner (i.e., blood acidification enhances the stimulatory effect of reduced PaO_2), by blood flow reduction and by increased blood concentration of lactate, potassium, and catecholamine (Kumar and Prabhakar, 2012). The chemoreflexes minimize $PaCO_2$ fluctuations by making corrective changes in lung ventilation and thus CO_2 elimination. This regulation operates continuously because chemoreceptors provide a tonic stimulus to breathe (e.g., Blain et al., 2009; Dempsey et al., 2012). The chemoreflexes are state dependent and, conversely, chemoreceptor stimulation produces arousal. The neural mechanisms that underlie these reciprocal interactions are important because many sleep-related pathologies are manifest as breathing disorders (Javaheri and Dempsey, 2013). In this review, we focus on the cellular sensors and molecular detectors underlying central respiratory chemosensitivity and the neuronal networks they activate to stimulate breathing or to cause arousal. The central pathways that integrate information from carotid bodies and central respiratory chemoreceptors will also be considered but the reader is directed to more extensive reviews on the carotid bodies and oxygen sensing (e.g., Nurse, 2014; Prabhakar, 2013).

$PaCO_2$ and PO_2 do not change significantly during light to moderate aerobic exercise (Forster et al., 2012) ruling out chemoreceptor stimulation as the cause of the increased breathing (hyperpnea). Instead, exercise hyperpnea and $PaCO_2$ stability depend primarily on feedback from skeletal muscle afferents and on central command (Forster et al., 2012; Kaufman, 2012; Waldrop and Iwamoto, 2006). Central command refers to the influence of brain structures involved in locomotion on the respiratory network during physical exercise (Eldridge et al., 1981; Forster et al., 2012). We will also briefly summarize current understanding of central command and muscle afferent mechanisms for exercise hyperpnea.

Respiratory Chemoreflexes: General Considerations

During normal unlabored breathing (eupnea), $PaCO_2$ is maintained within a few millimeters of mercury of a physiological set-point (~35 mmHg) (Duffin et al., 1980); small fluctuations around this set-point are not consciously perceived and have no impact on the state of vigilance. By contrast, large acute increases in $PaCO_2$ (e.g., from airway blockade, diving, sleep apnea, bronchial disease, and accidental or experimental exposure to CO_2) produce noxious sensations in awake subjects (dyspnea, urge to breathe, panic) and arousal from sleep (Kaur et al., 2013; Parshall et al., 2012). Some of the responses to high PCO_2 are adaptive, e.g., CO_2-induced arousal protects against accidental asphyxia by enabling postural changes that alleviate airway obstruction. Arousal, negative emotions, and (in rodents) olfactory sensation can, in turn, stimulate breathing and contribute to the ventilatory response to CO_2 (Hu et al., 2007; Kaur et al., 2013; Taugher et al., 2014).

The high gain of the hypercapnic ventilatory chemoreflex (breathing stimulation caused by a rise in $PaCO_2$, Figure 1A) requires a sensitive CO_2/H^+ detection mechanism and a specialized neural circuit capable of converting changes in sensor

activation into a powerful breathing response. The fundamental, open questions related to respiratory chemoreception are as follows: Does the process rely on specialized CO_2 or proton detectors or on protonation of broadly distributed CNS channels, receptors, or enzymes? If specialized CO_2 or proton detectors exist, where are they located (neurons, glia, vasculature)? Are they expressed throughout the respiratory pattern generator (RPG) or is this circuitry CO_2-insensitive and regulated by specialized clusters of CO_2-responsive neurons? Finally, given that respiratory chemoreflexes rely on sensory information from both peripheral and central chemoreceptors, how is that information integrated?

Is Central Respiratory Chemoreception an Emergent Property of the CNS?

Since the 1980s, the dominant view has been that the central respiratory chemoreflex derives from direct effects of $[H^+]$ distributed throughout the CNS (Nattie, 2011). One supportive argument is the huge variety and ubiquitous presence of pH-sensitive proteins (channels included) in the brain (Holzer, 2009). Two types of experimental results are also invoked. First, acidification commonly excites or inhibits neurons in vitro and dialyzing CO_2-enriched artificial CSF in many brain regions alters breathing, albeit weakly (Nattie, 2011). Second, the hypercapnic ventilatory chemoreflex is attenuated by lesioning structures that are clearly not part of the respiratory pattern generator (e.g., serotonergic neurons, orexinergic neurons, locus coeruleus) (Nattie, 2011). Neither line of evidence, singly or in combination, is conclusive (Guyenet, 2014). The level of acidification applied in such experiments (up to 0.5 pH) may occur only during severe hypercapnia, asphyxia, or brain ischemia and the in vitro evidence typically does not demonstrate that the neurons under study regulate breathing. Interpreting effects of brain lesions on the chemoreflex is also problematic (reviewed in Guyenet, 2014; Nattie, 2011). In such experiments, the chemoreflex is measured by exposing animals acutely to CO_2 levels that produce arousal and elicit behavioral effects (Kaur et al., 2013; Taugher et al., 2014). The effective lesions could have reduced ventilatory responses by any of three mechanisms: the lesioned neurons could indeed be respiratory chemoreceptors; they could mediate the emotional, behavioral, or arousal effects of hypercapnia (Kaur et al., 2013; Taugher et al., 2014); or they could simply bias the chemoreflexes at multiple brain sites.

Intense carotid body stimulation is aversive in intact mammals (Marshall, 1994) and hypoxia causes arousal, supporting a role of these particular chemoreceptors in eliciting emotional and other non-cardiorespiratory effects of CO_2 and/or hypoxia. Central respiratory chemoreceptors likely trigger non-respiratory effects of CO_2 because hypercapnia evokes no adverse sensation, emotional or otherwise, in patients with congenital central hypoventilation syndrome (CCHS) who lack a respiratory chemoreflex but have normal intellect and sensory perception (Shea et al., 1993; Weese-Mayer et al., 2010). Yet, supporting a more widespread effect of high PCO_2, freezing and conditioned place avoidance in mice elicited by exposure to 10% CO_2 requires expression of acid-sensitive ASIC channels in specific forebrain nuclei (e.g., Taugher et al., 2014). Thus, these aversive, behavioral, and arousal effects elicited by large acute increases in PCO_2 must recruit broad regions of the brain, which may be either directly sensitive to severe acidification or recruited synaptically when lower brainstem chemoreceptors and the carotid bodies are strongly stimulated.

For central respiratory chemoreception, the most compelling argument against the notion of a highly distributed brain property is that genetic lesion of a very small cluster of lower brainstem neurons, the retrotrapezoid nucleus (RTN), or mere deletion of two proton detectors expressed by RTN neurons (TASK-2 and GPR4) nearly eliminates the hypercapnic ventilatory reflex (Guyenet, 2014; Kumar et al., 2015; Ruffault et al., 2015). This review's leitmotif will be that, contrary to prevalent opinion, the breathing stimulation elicited by low level hypercapnia is probably largely mediated by a direct effect of protons on the carotid bodies and very few brainstem structures, among which the RTN and a subset of serotonergic neurons are preeminent.

The Retrotrapezoid Nucleus

The RTN moniker was coined to describe neurons located under the facial nucleus in a region suspected since the 1960s to contain respiratory chemoreceptors (Smith et al., 1989; Guyenet, 2014 for review) (Figure 1B). Early experiments (1989–2003) revealed that this region contains neurons that innervate caudal portions of the ventral respiratory column, are activated by hypercapnia, and increase breathing when stimulated with glutamate or bicuculline (Guyenet, 2014 for review). The relevant neurons were later shown to express VGlut2 and to encode $PaCO_2$ linearly in vivo with a 0–10 Hz dynamic range of action potential discharge between 35 and 76 mmHg $PaCO_2$ (Figures 1D and 1E) (Mulkey et al., 2004). Unlike other respiratory neurons, RTN neurons are still activated by hypercapnia after glutamate receptor blockade in vivo, suggesting that they respond to changes of local PCO_2 (Figure 1D) (Mulkey et al., 2004). Consistent with this hypothesis, RTN neurons are activated by hypercapnia or acidification ex vivo, including after isolation (Figure 2B) (Mulkey et al., 2004; Wang et al., 2013b). In 2003, mutations of homeodomain transcription factor Phox2b, were shown to cause CCHS (Amiel et al., 2003) and, in 2006, Phox2b was identified in all RTN CO_2-responsive neurons (Figures 1C–1F_1) (Stornetta et al., 2006). Finally, these particular Phox2b-positive neurons are selectively missing in newborn mice that express a CCHS-causing Phox2b mutation (*Phox2b*$^{27ala/+}$) (Dubreuil et al., 2008); these mice respond poorly to CO_2 and suffer fatal central apneas at birth, recapitulating key features of this disease.

Within the medullary reticular formation "nuclei" can only be defined as collections of neurons with closely related biochemical characteristics, connectivity, function and, ideally, genetic lineage. CO_2/H^+-sensitive RTN neurons can be defined histochemically as a bilateral cluster of ~2,000 Phox2b-immunoreactive (in rats, ~800 in mice) located under the facial nucleus (Lazarenko et al., 2009). These neurons have a shared genetic lineage (i.e., derived from neurons that express Phox2b, Atoh-1, and Egr-2) and unique phenotype (express Phox2b, NK1 receptors, VGlut2, TASK-2, GPR4, and galanin, but not GABA, glycine, acetylcholine, or catecholamines) (Goridis et al., 2010; Guyenet, 2014; Lazarenko et al., 2009; Ruffault et al., 2015; Wang et al., 2013b). Differential expression of galanin, TASK-2, and GPR4 defines subsets of RTN neurons (Kumar

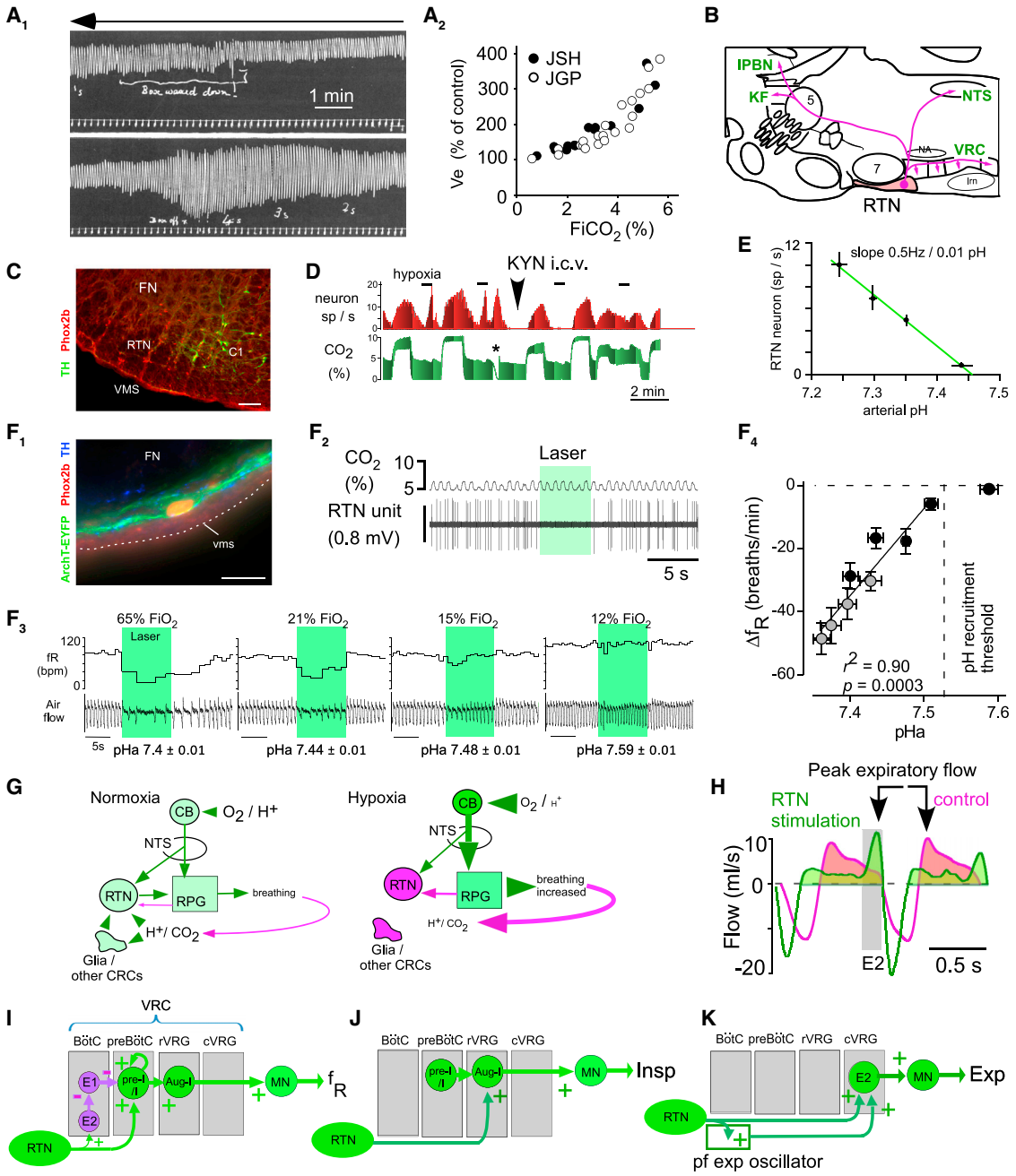

Figure 1. The Retrotrapezoid Nucleus

(A_1) the hypercapnic ventilatory reflex in humans (smoked drum recording to be read from right to left, top to bottom). Fraction inspired CO_2 ($FiCO_2$) was gradually increased by rebreathing air from box placed around head (reproduced from Haldane and Priestley, 1905). Amplitude of signal represents Vt (tidal volume).
(A_2) Plot of % increase in Ve (Vt x fR) versus $FiCO_2$ from Table on page 249 of Haldane and Priestley, 1905.
(B) Location and projections of the rodent RTN (parasagittal section). KF, Kölliker-Fuse nucleus; lPBN, lateral parabrachial nuc.; lrn, lateral reticular nuc.; NTS, solitary tract nuc.; VRC, ventral respiratory column; 5, trigeminal motor nuc.; 7, facial nuc.
(C) Transverse section at bregma level −11.5mm of an adult rat (left side) showing RTN neurons (FN: facial motor nucleus). The $Phox2b^+$:tyrosine-hydroxylase $(TH)^-$ neurons are RTN neurons. The $Phox2b^+$:TH^+ are C1 adrenergic neurons. Cal: 0.1 mm. From Guyenet (2008).
(D) Single RTN neuron recorded in an anesthetized rat. The firing frequency of the neuron (upper trace) is increased both by raising $FiCO_2$ in a background of hyperoxia (selective central chemoreceptor activation), by brief hypoxia (selective carotid body stimulation) or by short asphyxia (asterisk); the top of the CO_2 trace (CO_2 concentration at end expiration) approximates arterial PCO_2 measured in percentage of atmospheric pressure. Blocking excitatory transmission with kynurenic acid i.c.v. eliminates the input from the carotid body but the effect of hypercapnia is unchanged (adapted from Mulkey et al., 2004).
(E) Relationship between discharge rate of RTN neurons (n = 11) and arterial pH in anesthetized rats (glutamatergic transmission blocked with kynurenate as in D; adapted from Guyenet et al., 2005). Error bars represent SEM; 11 rats/point.
(F_1) Rat RTN neuron transduced with archaerhodopsin-eYFP (ArchT). Cal: 20 μm.

(legend continued on next page)

et al., 2015). A separate terminology, either RTN/pfRG (parafacial respiratory group) or ''parafacial nuclei,'' includes RTN and additional unidentified neurons that contribute to active expiration (see section on RTN development) (Feldman et al., 2013; Huckstepp et al., 2015). Active expiration refers to the recruitment of expiratory muscles for breathing. This occurs only when high levels of lung ventilation are required (exercise, hypercapnia); at rest, expiration is passive and relies purely on lung recoil.

RTN neurons can be transduced to express excitatory or inhibitory actuators by using viral vectors that use a powerful Phox2b-activated artificial promoter, PRSx8 (Abbott et al., 2009; Hwang et al., 2001) (Figure 1F_1). In conscious rats, optogenetic stimulation of RTN neurons increases the rate (f_R) and amplitude of breathing (tidal volume, V_T) in a manner that mimics effects of hypercapnia (Abbott et al., 2011). RTN stimulation also increases lung ventilation by transiently reducing airflow during early expiration and by triggering active expiration (Figure 1H) (Burke et al., 2015). Conversely, opto- or pharmacogenetic inhibition of RTN neurons reduces f_R and V_T and eliminates CO_2-induced active expiration (Basting et al., 2015; Marina et al., 2010). In conscious rats, opto-inhibition of RTN neurons reduces f_R and V_T in direct proportion to arterial blood pH below a threshold of 7.5; above this level, opto-inhibition is ineffective suggesting that RTN neurons are silent (Figures 1F_1–1F_4) (Basting et al., 2015).

The RTN region receives input from brain areas ranging from spinal cord to insular cortex (Craig, 1995; Lazarenko et al., 2009; Otake et al., 1992; Rosin et al., 2006; Song et al., 2012; Tan et al., 2010); however, ultrastructural confirmation of defined synaptic contacts with the chemoreceptors is largely lacking. Next, we describe inputs whose existence and function are supported by physiological data. Inputs from the spinal cord and the raphe are considered later.

RTN neurons regulate breathing in concert with the carotid bodies. The dorsal medullary neurons that receive carotid body afferent input (CB second-order neurons) are glutamatergic and target the same pontomedullary regions as RTN, where final integration between central and peripheral chemoreflexes presumably occurs (Guyenet, 2014; Song et al., 2012). However, CB second-order neurons also powerfully activate RTN (Takakura et al., 2006), and this connection has important functional implications. Usually, arterial PO_2 and arterial PCO_2 vary in opposite directions, so that central and peripheral chemoreceptors are either co-activated (during hypoventilation) or co-inhibited (with hyperventilation). Under such conditions, the excitatory input from the carotid bodies to RTN neurons contributes importantly to the breathing changes, and the carotid bodies and central chemoreceptors (RTN) act in concert to regulate breathing (Figures 1D and 1G) (Blain et al., 2010; Mulkey et al., 2004). By contrast, in hypoxia (e.g., at altitude), carotid body-mediated hyperventilation alkalizes the plasma, which inhibits RTN and renders it unresponsive to carotid body input (Figures 1F_3 and 1G) (Basting et al., 2015). Thus, under hypocapnic hypoxia, the RTN fails to support the breathing stimulation elicited by the carotid bodies. The potential consequences are insufficient alveolar ventilation and, at night, periodic breathing (Dempsey et al., 2012). Conversely, if the normally tonic input from the carotid body is reduced (e.g., by hyperoxia), RTN neurons provide a proportionally larger drive to breathe to minimize the change in CO_2 (Figure 1F) (Basting et al., 2015).

Glycinergic/GABAergic neurons located in the NTS mediate inhibitory effects of lung inflation on RTN neurons (Takakura et al., 2007), reducing inspiratory drive during lung overinflation. RTN neurons also receive inhibitory inputs during specific periods of the respiratory cycle (early inspiration, post-inspiration, late expiration) (Guyenet et al., 2005). These inputs likely arise from the ventral respiratory column and the pontine respiratory group (Song et al., 2012; Tan et al., 2010). Their role may be to reduce RTN neuron activity when the respiratory pattern generator is already highly active for reasons other than a blood gas imbalance (e.g., emotional or voluntary control of breathing).

In brief, RTN neurons are highly sensitive to changes of CO_2/H^+ in vivo, they activate breathing in proportion to CNS acidification in conscious animals, and their genetic elimination in mice reduces the central chemoreflex and increases apneas. RTN neurons regulate breathing in concert with the carotid bodies, and they are controlled by feedback from the respiratory pattern generator and lung afferents.

Pathways Mediating the Effects of RTN on Breathing

RTN neurons target only four brainstem regions, all of which are essential for breathing (Figure 1B): the ventral respiratory column (VRC), the Kölliker-Fuse nucleus, the lateral parabrachial nucleus (lPBN), and the nucleus of the solitary tract (NTS) (Bochorishvili et al., 2012; Smith et al., 2013). The VRC consists of four rostrocaudally stacked modules all of which receive RTN input (Figures 1B and 1I–1K). The rostral-most module is the Bötzinger region, followed by the preBötzinger complex. The latter contains a bilateral cluster of glutamatergic bursters that operate as rhythm generator for breathing (Bouvier et al., 2010; Feldman et al., 2013; Feldman and Kam, 2015). The rostral ventral respiratory group (rVRG) and the caudal VRG (cVRG) reside more caudally in the VRC and harbor inspiratory and expiratory premotor neurons, respectively (Smith et al., 2013). The pump (e.g., phrenic, lumbar) and airway motoneurons (hypoglossal, facial) have no

(F_2) Optogenetic inhibition of one ArchT-transduced RTN neuron (anesthetized rat).
(F_3) Effects of bilateral optogenetic inhibition of RTN neurons on respiratory frequency (fR) and air flow in a conscious rat exposed to four FiO_2.
(F_4) The breathing reduction elicited by inhibiting RTN is a linear function of arterial pH (pHa); RTN is silent above pHa 7.53. Open symbols: pHa changed via respiratory alkalosis (graded hypoxia); filled circles, pHa changed by administration of acetazolamide ($F_{1–4}$ reproduced from Basting et al., 2015). Error bars represent SEM; 4–9 rats/point.
(G) Contribution of the carotid bodies and RTN to respiratory homeostasis in normoxia versus hypoxia. Magenta denotes reduced activity (neurons, glia), shades of green depict increasing activity and font sizes symbolize changes in plasma or brain [H^+], PCO_2 or PO_2. From Basting et al., 2015.
(H) Optogenetic gain of function experiment. ChR2-mediated activation of RTN increases inspiration amplitude (inspiration downward), produces active expiration (E2 phase), and delays peak expiratory flow suggesting brief glottis closure after inspiration (modified from Burke et al., 2015). E1, early (passive) expiratory phase.
(I–K) Possible connections through which RTN increases breathing frequency, inspiratory amplitude, and active expiration (see text for details).

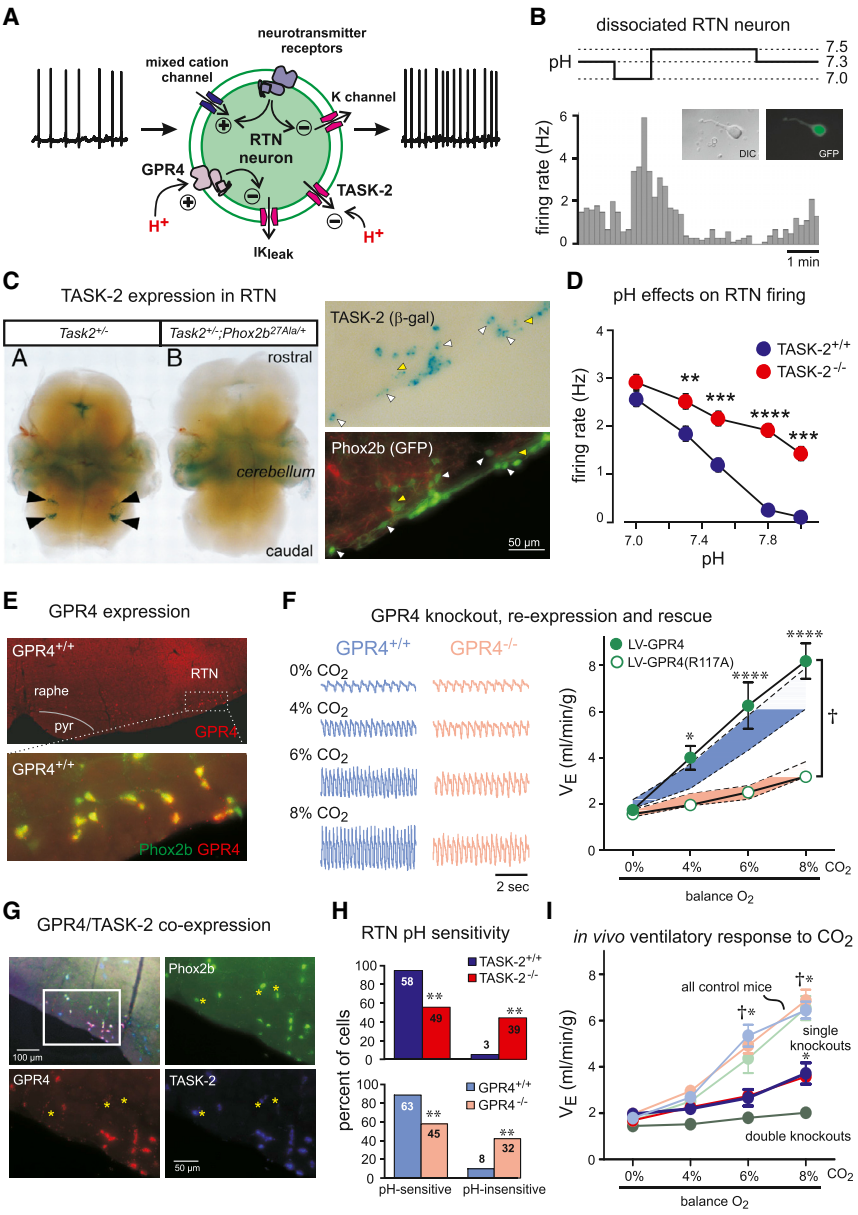

Figure 2. TASK-2 and GPR4 Are Proton Detectors in RTN Neurons Required for CO_2 Stimulation of Breathing

(A) Schematic of RTN neuron showing ionic mechanisms for intrinsic pH sensitivity and transmitter modulation.

(B) Firing rate histogram from GFP-expressing, dissociated RTN neuron.

(C) Left: staining for β-galactosidase (β-gal; from the TASK-2 locus) in embryo whole mounts from the indicated genotypes; arrowheads indicate RTN region. Right: β-gal staining for TASK-2 (upper) and GFP and TH (lower) in Phox2b::GFP;TASK-$2^{+/-}$ mice; white arrowheads indicate Phox2b-expressing RTN neurons that also express TASK-2.

(D) Averaged firing rates at different bath pH for RTN neurons from TASK-$2^{+/+}$ and TASK-$2^{-/-}$ mice. Error bars represent SEM; TASK-$2^{+/+}$, 61 neurons; TASK-$2^{-/-}$, 88 neurons.

(E) GPR4 and Phox2b expression detected by in situ hybridization in transverse mouse brainstem section.

(F) Left: respiratory flow recording from GPR4$^{+/+}$ and GPR4$^{-/-}$ mice with increased inspired CO_2 concentrations (balance O_2). Right: Lentiviral-mediated, PRSx8-driven re-expression in the RTN of GPR4, but not a non-functional mutant GPR4(R117A), fully rescued ventilatory response to CO_2 in GPR4-deleted mice. Shaded areas are 95% confidence intervals for GPR4$^{+/+}$ (blue) or GPR4$^{-/-}$ mice before lentiviral injection (pink). Error bars represent SEM; n = 7–8/group.

(G) Multiplex in situ hybridization illustrates differential, but overlapping, expression of GPR4 and TASK-2 in Phox2b-expressing RTN neurons; TASK-2-expressing Phox2b+ neurons without GPR4 are indicated (asterisks).

(H) Percent of pH-sensitive and pH-insensitive RTN neurons recorded from mice of the indicated genotypes.

(I) Ventilation during incremental CO_2 challenge for the indicated genotypes. †, all controls (TASK-$2^{+/+}$, light blue; GPR4$^{+/+}$, light pink; and TASK-$2^{+/+}$:GPR4$^{+/+}$, light green) greater than single (TASK-$2^{-/-}$, blue; and GPR4$^{-/-}$, red) or double knockouts (TASK-$2^{-/-}$:GPR4$^{-/-}$, green); *, both single knockouts greater than double knockouts. Error bars represent SEM; n = 4–50/group. (B) adapted from Wang et al., 2013b; (C, left) from Gestreau et al., 2010; (C, right) and (D) from Wang et al., 2013a; and (E)–(I) from Kumar et al., 2015.

direct input from RTN (Bochorishvili et al., 2012). Therefore, RTN neurons must increase breathing amplitude via inputs to either premotor neurons or to neurons further up the network. Finally, most RTN neurons innervate both the VRC and the dorsolateral pons, controlling several components of the respiratory pattern generator simultaneously (Mulkey et al., 2004).

RTN neurons could accelerate breathing frequency via monosynaptic projections to the rhythm-generating neurons of the pre-Bötzinger complex (Figure 1I) (Bochorishvili et al., 2012). They could also disinhibit these neurons as shown in Figure 1I (Bochorishvili et al., 2012; Potts et al., 2005) or via more complex pathways involving the dorsal pons (Mizusawa et al., 1995).

RTN effects on inspiratory amplitude may derive from direct projections to inspiratory premotor neurons located in the rVRG (Figure 1J), the Kölliker-Fuse nucleus (not illustrated) (Bochorishvili et al., 2012; Damasceno et al., 2014; Mizusawa et al., 1995; Smith et al., 2013; Yokota et al., 2007) and the lateral parabrachial nucleus (Yokota et al., 2015). Control of expiration by the RTN (Abbott et al., 2011; Marina et al., 2010) may involve projections to bulbospinal expiratory premotor neurons located in the cVRG (Bochorishvili et al., 2012; Gerrits and Holstege, 1996) and to the nearby parafacial oscillator for active expiration (Figure 1K) (Feldman et al., 2013; Huckstepp et al., 2015). Finally, RTN regulation of airway resistance (e.g., laryngeal adductors) likely occurs by way of the Kölliker-Fuse nucleus (Dutschmann and Herbert, 2006; Song et al., 2012).

In sum, RTN neurons regulate alveolar ventilation by controlling breathing rate, inspiratory amplitude, active expiration, and

airway patency. These effects are mediated via axonal projections to unidentified neurons located within four respiratory-related lower brainstem regions.

Proton Detection by RTN Neurons

Via carbonic anhydrase, molecular CO_2 is in equilibrium with protons, hydroxyl radicals, and bicarbonate. The effects of CO_2 on breathing are presumably mediated via changes in $[H^+]$ but additional mechanisms are being considered such as carbamylation reactions (e.g., to activate connexin-26) or via bicarbonate-regulated adenylyl cyclase (Huckstepp and Dale, 2011; Meigh et al., 2013). As reviewed below, RTN neurons respond to changes in local tissue PCO_2 at least partially in a cell autonomous manner (Figure 2A) via at least two molecular proton detectors: TASK-2 and GPR4 (Gestreau et al., 2010; Kumar et al., 2015; Wang et al., 2013a).

In brain slices, RTN neurons maintain a regular tonic discharge that is dynamically modulated by an intrinsic sensitivity to extracellular $[H^+]$. The actions of CO_2 on RTN neurons in vitro are also mediated via changes in $[H^+]$ (Mulkey et al., 2004). The pH sensitivity of RTN neurons persists in vitro during blockade of fast synaptic transmission (Lazarenko et al., 2010; Mulkey et al., 2004, 2007b) and, after acute RTN neuron isolation, it is indistinguishable from that observed in slice preparations (Wang et al., 2013b) (Figure 2B).

Initial characterization of pH sensitive membrane currents in RTN neurons identified a pH-sensitive background K^+ current (Mulkey et al., 2004), suggesting possible contributions from the K_{2P} family of background K channels. Despite evidence for widespread expression of acid-sensitive TASK-1 ($K_{2P}3$) and TASK-3 ($K_{2P}9$) channels in brainstem respiratory neurons, genetic deletion of those channels had no effect on RTN neuronal pH sensitivity or the ventilatory response to CO_2 (Bayliss et al., 2015; Mulkey et al., 2007b). On the other hand, strong evidence implicates TASK-2 ($K_{2P}5$), a member of the alkaline-activated subgroup of K_{2P} channels, both in RTN neuronal pH sensitivity and the central respiratory chemoreflex (Bayliss et al., 2015; Gestreau et al., 2010; Wang et al., 2013b). By using a gene-trap mouse line, TASK-2 expression was revealed in the RTN and very few other brainstem regions (Figure 2C) (Gestreau et al., 2010; Kumar et al., 2015). Notably, TASK-2 is undetectable in the RTN of *Phox2b*$^{27Ala/+}$ mice, in which the chemoreceptors do not develop and TASK-2 co-localizes with Phox2b and VGlut2 in most RTN neurons, as expected for expression within the pH-sensitive cell population (Figures 2C and 2G) (Gestreau et al., 2010; Wang et al., 2013a). Consistent with this, genetic elimination of TASK-2 yields a subgroup of RTN neurons (~44%) that are pH-insensitive (Figures 2D and 2H) and lack a pH-sensitive background K^+ current; in the remaining pH-sensitive RTN neurons the effect of pH on firing rate is blunted (Wang et al., 2013a). The aggregate firing rate is higher in TASK-2-deleted RTN neurons through the physiological pH range, as expected for elimination of a background K^+ channel (Figure 2D) (Bayliss et al., 2015; Wang et al., 2013a). Importantly, the ventilatory response to CO_2 is significantly reduced in mice lacking TASK-2 channels (Gestreau et al., 2010; Kumar et al., 2015).

A proton-activated G protein-coupled receptor, GPR4, also accounts for pH sensitivity in a subset of RTN neurons (Kumar et al., 2015; Ludwig et al., 2003). In the brainstem, GPR4 expression is very high in Phox2b-expressing RTN neurons (Figures 2E and 2G), low in raphe neurons and undetectable elsewhere (Kumar et al., 2015). A subgroup of pH-insensitive RTN neurons (~40%) is found in mice deleted for GPR4 (Figure 2H) or following exposure to a small molecule GPR4 antagonist, and RTN neuronal pH sensitivity is disrupted by interfering with intracellular G protein signaling (Kumar et al., 2015). In GPR4$^{-/-}$ mice, CO_2-evoked RTN neuronal activation (i.e., cFos activation) and CO_2-stimulated breathing are diminished (Figure 2F) (Kumar et al., 2015). Crucially, virally mediated re-expression of GPR4 selectively in RTN neurons of GPR4$^{-/-}$ mice restores CO_2-evoked neuronal activation in vivo (i.e., cFos expression) and rescues the ventilatory phenotype (Figure 2F) (Kumar et al., 2015).

GPR4 does not affect pH-sensitive TASK-2 currents in recombinant expression systems, and GPR4 and TASK-2 are expressed in distinct, but overlapping, subsets of Phox2b-expressing RTN neurons (Figure 2G) (Kumar et al., 2015). Consistent with independent cellular actions in RTN neurons, the ventilatory response to CO_2 is reduced by more than 85% in double GPR4$^{-/-}$:TASK-2$^{-/-}$ mice but by only ~60% in single knockout mice (Figure 2I) (Kumar et al., 2015). These results implicate both GPR4 and TASK-2 as molecular proton detectors for RTN neuronal pH sensitivity and for RTN-mediated central respiratory chemosensitivity.

The ongoing activity of other ion channels in RTN neurons, and their modulation by neurotransmitter systems, may also alter RTN cellular activity and transduction of chemoreceptor stimuli into respiratory output (Figure 2A). This may be true whether or not the cellular source of the transmitters, or the channels themselves, have any intrinsic pH sensitivity. In this respect, the effect of serotonin on RTN neurons and breathing is instructive. Hypercapnia enhances the respiratory activation induced by optogenetic stimulation of raphe obscurus neurons, even though that particular group of serotonergic neurons is not CO_2 responsive (Brust et al., 2014; Depuy et al., 2011). Serotonin excites RTN neurons, in part, via 5-HT_2-mediated inhibition of a K_V7 channel current and 5-HT_7-mediated activation of HCN channels (Figures 2A and 3A–3C) (Hawkins et al., 2015; Hawryluk et al., 2012), even though the pH sensitivity of RTN neurons is unchanged by serotonin (Figures 3A and 3B) or by pharmacologically modulating K_V7 and/or HCN channels (Hawryluk et al., 2012; Mulkey et al., 2007a). However, both 5-HT exposure and direct K_V7/HCN channel modulation in the RTN can shift the CO_2 threshold for respiration and enhance respiratory output at physiological pH or PCO_2 levels (Hawryluk et al., 2012; Mulkey et al., 2007a). Thus, changes in RTN neuron excitability elicited by serotonin, independent of pH sensing per se, can be manifest as altered threshold or gain of the ventilatory response to CO_2; this may also be true for other neurons that contribute, directly or indirectly, to the respiratory chemoreflex.

CO_2 Sensitivity of RTN Neurons: The Role of Astrocytes

RTN neurons display greater CO_2 sensitivity in vivo than in vitro (5 Hz/0.1 pH, mean dynamic range 0–10 Hz versus ~0.6 Hz/0.1 pH, 0–3 Hz) (Guyenet et al., 2005; Lazarenko et al., 2009;

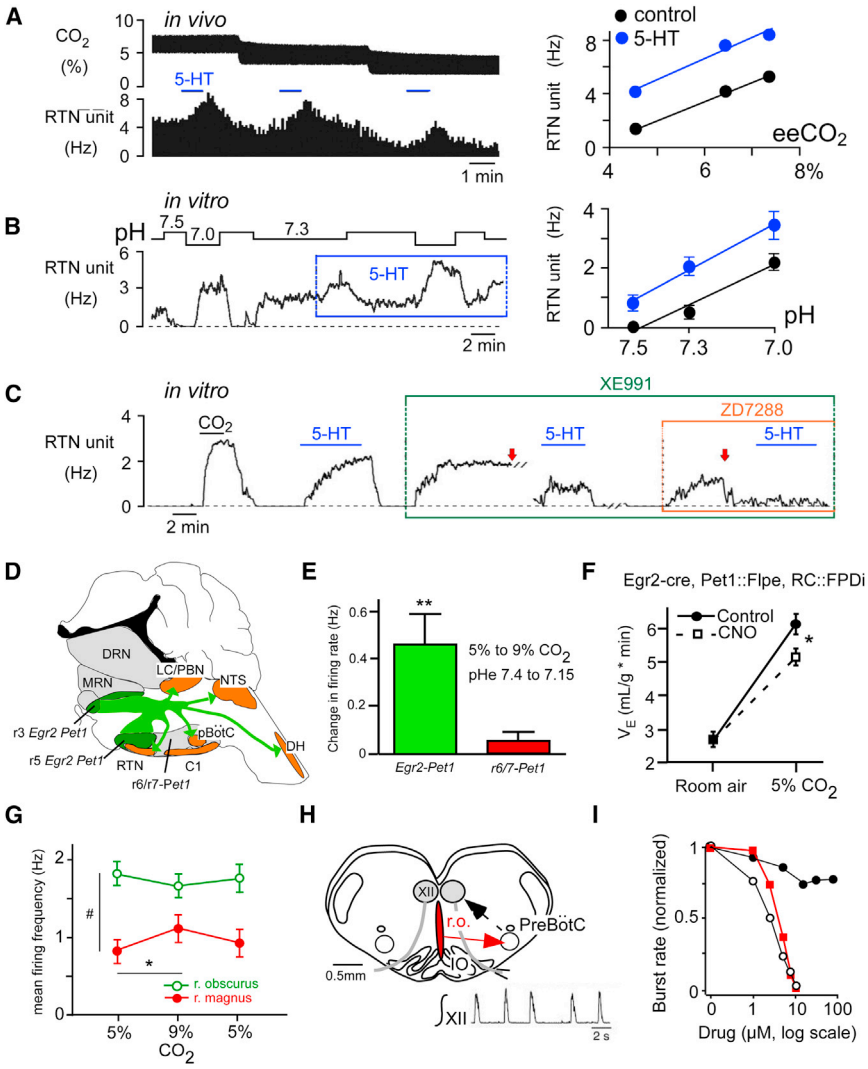

Figure 3. Lower Brainstem Serotonergic Neurons and Chemoreflexes

(A) CO_2 sensitivity of RTN neurons in vivo (ΔHz/ΔarterialPCO$_2$) is unchanged by iontophoretic application of serotonin (5-HT; raw data for a single neuron at left) (from Mulkey et al., 2007a). Error bars represent SEM; n = 5.

(B) pH sensitivity of RTN neurons in vitro (ΔHz/ΔpH) is unchanged by bath application of 5 μM 5-HT (raw data for one neuron at left) (from Mulkey et al., 2007a).

(C) Blocking K_V7 channels (with 10 μM XE991) and HCN channels (with 50 μM ZD7288) essentially eliminated 5-HT effects on firing rate in a CO_2-sensitive rat RTN neuron in vitro; arrows indicate current injection to reset baseline firing (from Hawkins et al., 2015).

(D) Serotonergic neurons of *egr-2* lineage innervate most lower brainstem and spinal cord regions implicated in respiratory control (C1, C1 adrenergic neurons; DRN, dorsal raphe; MRN, median raphe; LC, locus coeruleus; DH, dorsal horn; see Figure 1 for other abbreviations) (redrawn from Brust et al., 2014).

(E) Serotonergic neurons of *egr-2* lineage are significantly activated by hypercapnia in mouse brain slices unlike serotonergic neurons of rhombomere 6/7 origin (redrawn from Brust et al., 2014). Error bars represent SEM; *Egr-2 Pet1*, n = 8 neurons; *r6/7 Pet1*, n = 17 neurons.

(F) Pharmacogenetic inhibition of *Egr-2*-derived serotonergic neurons attenuates the hypercapnic ventilatory reflex in mice (redrawn from Brust et al., 2014). Clozapine-N-oxide (CNO) was administered to activate an inhibitory DREAAD expressed selectively in *Egr-2*-derived serotonergic neurons. Error bars represent SEM; n = 23.

(G) Mild activation of raphe magnus serotonergic neurons by hypercapnia in an arterially perfused rat (redrawn from Iceman et al., 2013). Error bars represent SEM; raphe obscurus, n = 9; raphe magnus, n = 7.

(H) Rat "breathing slice" preparation. Inset shows integrated respiratory-like activity of hypoglossal nerve rootlet.

(I) Dose-dependent inhibition of the respiratory burst rate by bath application of methysergide (broad-spectrum serotonin antagonist; open circles), a substance P receptor antagonist (SR140333; red squares) and RS102221, an inactive serotonin receptor antagonist (black circles). The antagonists blocked the excitatory effects of serotonin and substance P presumably released by raphe obscurus (ro) neurons (H and I redrawn from Ptak et al., 2009). IO, inferior olive; r.o., raphe obscurus; XII, hypoglossal motoneurons.

Mulkey et al., 2004). These differences may be technical and trivial (e.g., for slices or isolated cells: low temperature recordings, tissue immaturity, neuronal damage; for unit recordings in vivo: effects of anesthesia), but they might also reflect other important mechanisms at play in vivo. For example, the RTN response to CO_2 may be facilitated in vivo by neuromodulators (Hawryluk et al., 2012) or inputs from additional CO_2-activated CNS neurons. These quantitative differences may also reflect the role of astrocytes (Figure 4) (Erlichman and Leiter, 2010; Gourine et al., 2010; Wenker et al., 2010).

CO_2-induced ATP release and acid-depolarized astrocytes are present throughout the ventral surface of the medulla oblongata (Fukuda and Honda, 1975; Gourine et al., 2005; Kasymov et al., 2013; Wenker et al., 2010). Injection into RTN of fluorocitrate, which selectively depolarizes astrocytes by blocking their metabolism, produces local acidification and activates breathing (Erlichman et al., 1998). Most famously, ChR2-mediated depolarization of nearby astrocytes activates RTN neurons and increases breathing in anesthetized rats (Gourine et al., 2010). The mechanisms of astrocytic ATP release may include depolarization-induced exocytosis (Kasymov et al., 2013), or CO_2-evoked carbamylation and activation of ATP-permeant connexin-26 channels (Huckstepp et al., 2010; Meigh et al., 2013). Because effects of astrocytic opto-depolarization were blocked by MRS 2179, a P2Y1 purinergic receptor antagonist, it was proposed that astrocytes, not neurons, are the cellular proton/CO_2 sensors responsible for respiratory chemoreception, with ATP providing an obligate excitatory signal from astrocytes to neurons via P2Y1 receptors (Gourine et al., 2010). However, later work revealed that RTN neurons are intrinsically chemosensitive (Wang et al., 2013b), and that various P2 receptor antagonists, including MRS 2179, reduced the activation of RTN neurons by CO_2 modestly, at best (Mulkey et al., 2006; Onimaru et al., 2012; Wenker et al., 2012).

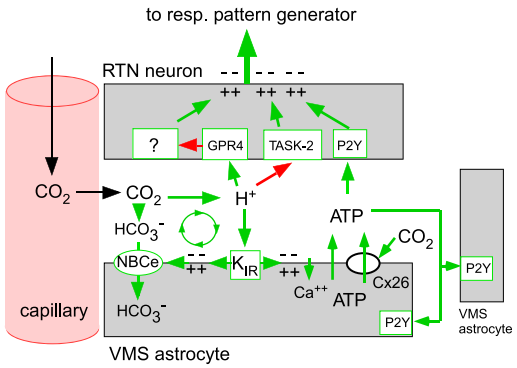

Figure 4. Hypothetical Contribution of Astrocytes to the CO_2 Sensitivity of RTN Neurons

The activation of RTN neurons by CO_2 requires the expression of two proton receptors (TASK-2 and GPR4) but this response may be facilitated or potentiated by surrounding astrocytes in several ways. Extracellular acidification depolarizes RTN astrocytes by closing an inwardly rectifying potassium channel (K_{IR}). This depolarization, which can also be mimicked optogenetically by introducing ChR2 into these astrocytes, elicits the release ATP and, possibly, other gliotransmitters (Gourine et al., 2010; Kasymov et al., 2013). ATP then contributes to the activation of RTN neurons via P_{2Y} receptors and recruits more astrocytes. Astrocyte depolarization may also activate an electrogenic sodium-bicarbonate transporter (NBCe) which moves bicarbonate into the cells, thereby further acidifying the extracellular space and enhancing the depolarization of RTN neurons (Erlichman and Leiter, 2010). Finally, CO_2 may also trigger ATP release through Cx-26 hemichannels (Huckstepp et al., 2010).

Can the initial suggestion of a primary sensory role for astrocytes be reconciled with new data showing that $[H^+]$ detectors in RTN neurons are required for CO_2-stimulated breathing? As usual, there are potential technical issues to consider: ChR2 is permeable to protons and acidifies the cytoplasm of glial cells causing the release of lactate and glutamate, which can depolarize surrounding neurons (Beppu et al., 2014; Tang et al., 2014). In addition, the enhanced GFAP promoter used to transduce astrocytes with ChR2 may not be totally selective for glia. A more interesting possibility is that the acid-depolarized glia enhances the apparent pH sensitivity of RTN neurons by augmenting local extracellular acidification relative to arteriolar pH. Astrocytes are capable of dynamic and bidirectional regulation of local blood flow (Gordon et al., 2011). If CO_2 (or ChR2)-mediated depolarization of astrocytes in vivo were to cause vasoconstriction, the consequence would be tissue CO_2 retention and breathing activation via local acidification. Yet another possibility is astrocytic depolarization-induced alkalization (DIA), which causes simultaneous extrusion of protons (Figure 4) (Erlichman et al., 2004). These hypotheses are appealing given that specific proton detectors, selectively expressed by RTN neurons but absent from glia, are required for the ventilatory response to CO_2.

In conclusion, pH-sensitive astrocytes may contribute to the CO_2 response of RTN neurons by releasing ATP, lactate or glutamate, or by exaggerating local changes in extracellular pH. However, the importance of these mechanisms to central respiratory chemoreception is not clearly established.

RTN Development

RTN originates from an Egr-2-dependent embryonic domain (rhombomeres 3/5) and co-expresses Atoh-1 and Phox2b during late embryogenesis (Dubreuil et al., 2009; Ramanantsoa et al., 2011). Deletion of any one of these three transcription factors prevents RTN from developing (Ruffault et al., 2015) or, in the case of Atoh-1, from establishing proper connections (Huang et al., 2012).

RTN or the RTN region has been described as an inspiratory rhythm generator, an oscillator for active expiration, or a central respiratory chemoreceptor (Guyenet, 2008; Marina et al., 2010; Pagliardini et al., 2011; Wittmeier et al., 2008). These interpretations should be considered in a proper developmental and physiological context.

Prenatally, RTN neurons, a.k.a. embryonic parafacial oscillator (e-pF), exhibit group pacemaker properties that rely on persistent Na current (I_{NaP}), hyperpolarization-activated cationic current (I_h), and gap junctions (Fortin and Thoby-Brisson, 2009; Ruffault et al., 2015; Thoby-Brisson et al., 2009). RTN is pH-modulated and activates the preBötzinger complex. In vitro the embryonic RTN and the preBötzinger complex operate as coupled oscillators (Fortin and Thoby-Brisson, 2009; Thoby-Brisson et al., 2009).

Around birth (postnatal day 0 to postnatal day 2), RTN neurons seem to retain many of their embryonic characteristics. Originally described as the ''parafacial respiratory group'' (pfRG), many neonatal RTN neurons (that are pH-sensitive and express Phox2b, VGlut2, and NK1 receptors) retain intrinsic burst-generating properties in vitro (Onimaru and Homma, 2003; Onimaru et al., 2008, 2014). Like the e-pF, neonatal RTN is pH-modulated and operates as an oscillator coupled to the preBötzinger complex (Onimaru and Homma, 2003).

In certain preparations of neonatal rats, the abdominal muscles or abdominal nerve rootlets display a double burst that resembles the discharge pattern of pfRG neurons (Janczewski and Feldman, 2006; Janczewski et al., 2002; Onimaru et al., 1987). In neonatal anesthetized midbrain transected rats (7–12 days), opiate agonists produce quantal slowing of the inspiratory air flow but have little effect on the respiratory-synchronous contractions of expiratory abdominal muscles (Feldman et al., 2013; Janczewski and Feldman, 2006). A second brain transection at mid-facial nucleus level eliminates the respiratory-like abdominal contractions, suggesting that the transected region (RTN/pfRG) contains an opiate-resistant expiratory oscillator (Feldman et al., 2013; Janczewski and Feldman, 2006). Consistent with this interpretation, respiratory-synchronous discharges of lumbar nerves can be enabled or silenced in anesthetized rats by activating or inhibiting neurons located near the facial motor nucleus (Huckstepp et al., 2015; Pagliardini et al., 2011). Neurons other than RTN have been implicated in these effects (Huckstepp et al., 2015) but selective activation of RTN neurons in adult conscious rats does elicit active expiration and active expiration is suppressed when these neurons are inhibited (Abbott et al., 2011; Marina et al., 2010). Thus, as summarized in Figure 1K, RTN neurons may either gate or enable a nearby parafacial network that drives active expiration.

In conclusion, the use of preparations varying in age from embryos to adults has contributed to the divergent views regarding non-chemosensory roles of RTN neurons. During the late embryonic and early postnatal periods, RTN neurons have intrinsic bursting properties and operate together with the preBötzinger

complex as coupled oscillators, at least in vitro. There is no evidence that such pacemaker properties persist in the adult RTN. By contrast, this nucleus clearly functions as a central chemoreceptor in adults and controls multiple aspects of breathing including frequency, depth of inspiration, active expiration, and airway patency.

RTN, Phox2b, and Congenital Central Hypoventilation Syndrome

As already mentioned, mice with a Phox2b mutation that causes a severe form of CCHS (*Phox2b*$^{27ala/+}$) die at birth of respiratory failure and are born without RTN neurons (Amiel et al., 2003; Dubreuil et al., 2008); however, these mice have normal numbers of other neurons that also depend on Phox2b for their development e.g., locus coeruleus, type-I glomus cells (the oxygen sensors of the carotid bodies), and serotonergic neurons (Dubreuil et al., 2008). Thus, for unknown reasons, RTN development is especially vulnerable to this *Phox2b* mutation. When the *Phox2b*^{27ala} mutation is restricted to neurons of r3/r5 lineage, RTN neurons and the ventilatory response to CO_2 are again largely absent at birth but these mice survive and their chemoreflex is partially (~35%) restored by 2 weeks after birth (Ramanantsoa et al., 2011). An even more selective genetic lesion of RTN produces a similar respiratory phenotype (Ruffault et al., 2015). These mice may survive because of an incomplete loss of RTN neurons and a compensatory increase in contributions from other central chemoreceptors or carotid bodies (Ramanantsoa et al., 2011).

An RTN-like structure has been identified in humans (Rudzinski and Kapur, 2010). However, the crucial evidence that these neurons are actually missing in patients with CCHS-causing *Phox2b* mutations is yet to be produced.

Serotonergic Neurons, Breathing, and CO_2 Homeostasis

Several lines of evidence suggest that subsets of serotonergic neurons may have central respiratory chemoreceptor properties. CO_2-induced ventilation in mice is reduced when serotonergic neuron development is impaired (Buchanan and Richerson, 2010; Hodges et al., 2008, 2009). Global acute pharmacogenetic inhibition of serotonergic neurons or selective inhibition of r3/5-derived serotonergic neurons (Figure 3F) attenuates CO_2-stimulated ventilation (Brust et al., 2014; Ray et al., 2011). In addition, optogenetic stimulation of medullary raphe serotonergic neurons activates breathing in conscious or anesthetized mice (Depuy et al., 2011). Thus, two facts are clearly established: activation of serotonergic raphe neurons stimulates ventilation and full effects of CO_2 on breathing require ongoing activity of raphe neurons.

In the mature brain, classically defined serotonergic raphe neuronal clusters (B1–B9) contain neurons derived from several embryonic domains or rhombomeres, with the pontine and medullary raphe nuclei providing most of serotonergic innervation to the lower brainstem and spinal cord (Bang et al., 2012; Brust et al., 2014; Jensen et al., 2008). In conscious cats, midbrain and lower brainstem raphe cells display a slow, regular discharge rate with a prominent state-dependence (i.e., highest during active waking, silent during REM) and about 20% of these neurons are activated by elevated CO_2 (Jacobs et al., 2002; Martín-Cora et al., 2000, 2005; Veasey et al., 1995). In rodents, serotonergic neurons located in raphe magnus are usually mildly activated by CO_2 in slices or in an arterially perfused preparation (<0.5 Hz, on average) (Brust et al., 2014; Iceman et al., 2013), whereas those in raphe obscurus are typically unresponsive (Figures 3E and 3G) (Depuy et al., 2011; Iceman et al., 2013) and parapyramidal serotonergic cells usually inhibited (Mulkey et al., 2004). A much higher proportion of serotonergic neurons from various raphe nuclei (73%–100%) are CO_2-responsive when recorded in slices or in culture (Severson et al., 2003; Wang et al., 2001). Thus, CO_2/H^+-activated serotonergic neurons have been repeatedly identified within a variety of classically defined raphe neuron subgroups, albeit in highly variable proportion depending on the preparation. These discrepancies may be related to the intermingling of CO_2-sensitive and insensitive serotonergic neurons within a given brain region. Indeed, when distinct serotonergic neuron subtypes are identified based both on anatomic location and intersection of select genetic markers, those Egr2- and Pet1-expressing lower brainstem serotonergic neurons located primarily in raphe magnus are nearly all CO_2 sensitive ex vivo, and inhibition of that genetically identified population reduces CO_2 effects on breathing in vivo (Brust et al., 2014). This particular group of genetically defined, CO_2-sensitive serotonergic neurons may therefore function as central respiratory chemoreceptors (Teran et al., 2014). However, it is important to note that serotonergic neurons can facilitate the respiratory chemoreflex in other ways. For example, the ventilatory deficits caused by inhibition or lesion of serotonergic neurons are completely reversed by intracerebral administration of serotonin (Hodges et al., 2008); this observation suggests that serotonin facilitates a respiratory reflex initiated by CO_2 sensors located elsewhere. Also, serotonergic neurons recently judged to be insensitive to CO_2, in vivo or ex vivo (e.g., raphe obscurus) are clearly able to activate breathing in reduced preparations or in conscious mice (Brust et al., 2014; Depuy et al., 2011; Ptak et al., 2009) (Figures 3H and 3I).

In summary, although several medullary raphe subdivisions seem capable of activating breathing, intrinsic pH sensitivity may be restricted to a subset of raphe magnus serotonergic neurons that serve as bona fide respiratory chemoreceptors (Brust et al., 2014; Teran et al., 2014). However, as recently achieved for RTN, a critical test of this hypothesis will require identification of the molecular bases for their pH sensitivity, and demonstration that selective elimination of that sensing mechanism (rather than wholesale inhibition or destruction of the neurons) attenuates the respiratory chemoreflex. The most obvious changes in the activity of serotonergic neurons in vivo are state-related (Jacobs and Azmitia, 1992) and serotonin loss-of-function experiments may partially reproduce the generally depressant effects of REM sleep on muscle tone, breathing, autonomic functions, and thermogenesis (Berthon-Jones and Sullivan, 1984; Horner et al., 2002; Lovering et al., 2003; Teran et al., 2014). Thus, lower brainstem serotonergic neurons likely contribute to arousal state-dependent modulation of multiple systems, including breathing.

Serotonergic Neurons and Sudden Infant Death Syndrome

A triple threat hypothesis posits that SIDS requires: (1) a genetic predisposition, (2) an immature and inherently unstable respiratory network, and (3) precipitating environmental factors (Becker, 1990; Kinney and Thach, 2009). Many sudden infant

death syndrome (SIDS) cases may be caused by a defect in asphyxia-induced arousal or auto-resuscitation (Darnall, 2013). Severe brain hypoxia can cause the glottis to constrict during inspiration as opposed to immediately after, with potentially dire consequences on airflow (Dutschmann and Paton, 2005). Auto-resuscitation is a powerful stimulation of breathing that is probably triggered by severe CNS hypoxia. Its main respiratory manifestation, gasping, is a brief and intense series of inspiratory efforts that, if unsuccessful in restoring normal breathing and oxygenation, precedes death. The failure to arouse may also prevent a life-saving shift in body position that would otherwise free obstructed airways (Garcia et al., 2013).

The brainstem cholinergic system, the RTN, peripheral chemoreceptors, and brainstem serotonergic neurons have been judged abnormal in some cases of SIDS (Duncan et al., 2010; Lavezzi et al., 2012; Peña et al., 2004; Porzionato et al., 2013); however, defects of the serotonergic system may be most critical. Transgenic mice lacking serotonin neurons have high mortality during development, severe neonatal apneas (Hodges et al., 2009), and fail to arouse when exposed to CO_2 (Buchanan and Richerson, 2010). In addition, lesioning serotonergic neurons in neonate rodents weakens hypoxia-induced gasping (Cummings et al., 2011). Gasping is attributed to increased I_{NaP} in lower brainstem respiratory neurons (Del Negro et al., 2002; Paton et al., 2006; Ramirez et al., 1998) with possible contribution of ATP release from astrocytes (Marina et al., 2013). An I_{NaP} -dependent gasp-like inspiratory pattern is also observed in hypoxic ''breathing'' slices, which is facilitated by serotonin (Peña et al., 2004; Tryba et al., 2006).

In summary, in rodents, the serotonergic system is critical to breathing during the neonatal period. Severe deficits of CNS serotonin neurons impair two mechanisms considered essential to survive central apneas or accidental airway obstruction during sleep: asphyxia-induced arousal, and hypoxia-induced gasping and resuscitation. Abnormalities of the lower brainstem serotonergic system and many other areas, including RTN, have been reported in SIDS. The proximate cause of SIDS, however, remains unknown.

Chemoreflexes and Sleep

The chemoreflexes are depressed during sleep whereas chemoreceptor stimulation produces arousal from sleep. These reciprocal interactions have important implications for sleep medicine (Javaheri and Dempsey, 2013).

During non-REM sleep, the pontomedullary respiratory pattern generator is presumed to be autorhythmic and its activity is highly dependent on inputs from central and peripheral chemoreceptors (Janczewski et al., 2013; Javaheri and Dempsey, 2013). Consistent with this, opto-inhibition of RTN reduces breathing considerably during non-REM sleep and this inhibition is much greater when the carotid bodies are silenced by hyperoxia (Burke et al., 2015). The low level of breathing present during non-REM sleep and its heavy dependence on chemoreceptors explains why minor fluctuations of PCO_2 can cause apneas or periodic breathing and CNS-damaging hypoxemia (Dempsey et al., 2012; Javaheri and Dempsey, 2013). Periodic breathing, commonly present during sleep at altitude (i.e., in hypobaric hypoxia) and in advanced heart failure, is attributed to an increase in peripheral chemoreflex gain and/or to a longer time constant of the central chemoreflex (Dempsey et al., 2012; Marcus et al., 2014). The apneas are probably caused by recurring episodes of CNS hypocapnia and the ensuing inactivity of central respiratory chemoreceptors such as RTN (Basting et al., 2015).

During REM sleep, the chemoreflexes are greatly attenuated because breathing frequency is no longer under the control of chemoreceptors (Burke et al., 2015 and refs. therein). Despite the presumed loss of the stimulatory effects of wake-on modulators (e.g., orexin, noradrenaline, and serotonin), the relative atonia of many respiratory muscles in REM (e.g., abdominals, airways) and a reduced tidal volume, overall ventilation is actually well maintained during REM sleep; this is due to an net increase in mean breathing frequency, with highly variable inspiratory burst intervals, of unknown origin (Orem et al., 2005) and no longer under RTN control (Figure 5) (Burke et al., 2015). This evidence suggests that the preBötzinger complex is no longer autorhythmic in REM sleep and that the frequency of inspiratory bursts is governed by inputs from other brain regions, conceivably the cortex (analogous to the voluntary control of breathing) or brainstem structures that contribute to other aspects of REM sleep (Fraigne and Orem, 2011). Serotonergic and pontine noradrenergic neurons are generally silent during REM sleep and those serotonergic neurons that remain active during REM sleep no longer respond to CO_2 (Veasey et al., 1995). Therefore, these aminergic neuron groups are unlikely to operate as central chemoreceptors during REM sleep. The only central chemoreceptors known to exert any influence on breathing during REM sleep are the RTN neurons, and they influence breathing amplitude but not frequency.

Chemoreceptor stimuli produce arousal from sleep (Ayas et al., 2000; Berry and Gleeson, 1997; Guyenet and Abbott, 2013). Either hypoxia or hypercapnia alone can produce arousal but, because severe hypercapnia is normally paired with hypoxemia during sleep (hypoventilation), arousal typically occurs under the combined effect of central and peripheral chemoreceptors. The lateral parabrachial nucleus contributes to the arousal produced by chemoreceptor stimulation in mice (Kaur et al., 2013). This region receives convergent input from RTN, from caudal NTS neurons that relay carotid body inputs, from serotonergic neurons and from the C1 adrenergic cells (Bochorishvili et al., 2012; Burke et al., 2014; Song et al., 2011). The C1 neurons are highly responsive to carotid body stimulation and brain hypoxemia (Guyenet, 2014; Koganezawa and Paton, 2014) and arousal from NREM sleep is reliably elicited by C1 cell activation (Burke et al., 2014).

In brief, RTN generates a considerable portion of the drive to breathe during non-REM sleep. During REM sleep, the contribution of RTN is reduced but still present, REM-off aminergic systems are generally silent, and breathing frequency is regulated by unknown mechanisms. Drugs that selectively activate RTN neurons could, in principle, be useful to treat central sleep apnea syndromes and periodic breathing that are prevalent during nREM sleep.

The Hyperpnea of Exercise

Breathing increases instantly at the beginning of exercise (phase 1 of hyperpnea), and then more slowly until a steady state is reached (phase 2, $t_{1/2}$ ~1 min in humans). For mild to moderate aerobic exercise, $PaCO_2$ is invariant throughout (or even drops a

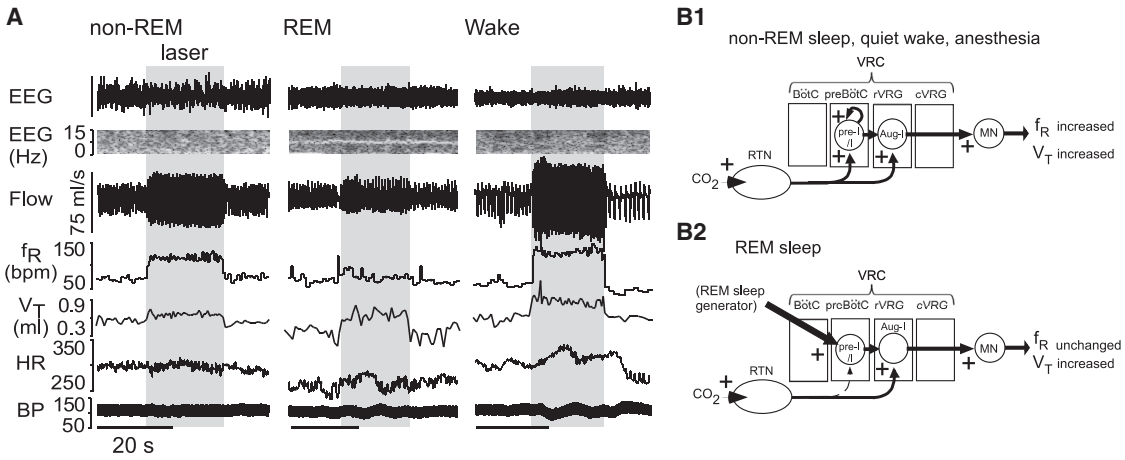

Figure 5. State-Dependent Control of Breathing by RTN

(A) Unilateral optogenetic activation of RTN (ChR2) increases breathing frequency during non-REM sleep and quiet waking but has no effect during REM sleep (identified by ~7 Hz theta rhythm in EEG). In contrast, inspiratory (tidal) volume (V_T) is increased regardless of the state of vigilance (reproduced from Burke et al., 2015).

(B) Speculative interpretation.

(B_1) During quiet waking, non-REM sleep, or anesthesia, the breathing rhythm is generated autonomously by the synchronized bursts of prel/I neurons located in the pre-Bötzinger complex (Janczewski et al., 2013; Koshiya and Smith, 1999; St-John et al., 2009). The pacemaker depolarization of these neurons is accelerated by RTN thereby increasing breathing frequency. The RTN input to the premotor neurons (rVRG) increases the burst amplitude, and thus V_T.

(B_2) During REM sleep, we speculate that the burst frequency of the pre-Bötzinger complex is controlled by inputs that originate outside the respiratory pattern generator (hypothetical REM sleep generator) and prevent RTN from modulating the respiratory frequency. The excitatory input from RTN to the inspiratory premotor neurons is still operating; hence, the control of inspiratory amplitude by RTN persists.

little in non-human mammals) and the gain of the hypercapnic ventilatory reflex is unchanged (Forster et al., 2012). Therefore, the hyperpnea of moderate exercise is not driven by an increase in $PaCO_2$ or proton concentration caused by the sudden increase in metabolism. This evidence has been taken to rule out the participation of "central chemoreceptors" in exercise hyperpnea, but such an interpretation should be qualified. First, the chemoreflex can attenuate the ventilatory overshoot that occurs at the initiation of exercise (Figure 6 of Forster et al., 2012). Second, RTN and lower brainstem serotonergic neurons are activated during dynamic exercise (Barna et al., 2012; Veasey et al., 1995). Thus, central chemoreceptor neurons may well contribute to exercise hyperpnea but by mechanisms that are independent of a change in brain PCO_2. Finally, during intense anaerobic exercise, when blood lactate accumulation occurs, breathing increases even further. The fall in $PaCO_2$ associated with this hyperventilation moderates lactic acid-induced acidosis, with additional benefits for blood oxygenation and thermoregulation (Forster et al., 2012 for review). The chemoreceptors, in particular the carotid bodies, contribute to this hyperventilation.

Exercise hyperpnea and the concomitant increase in sympathetic tone (exercise pressor reflex) presumably rely, in part, on activation of group III (thinly myelinated) and group IV (unmyelinated) muscle afferents (Forster et al., 2012; Kaufman, 2012). These afferents are activated by mechanical distortion of their receptive field, metabolic by-products of muscle contraction (H^+, K^+, and lactate), local inflammation, a rise in tissue temperature, and factors that cause muscle pain (Haouzi et al., 1995; Jankowski et al., 2013; Kaufman, 2012). Some of these fibers also respond to venous distension, a variable that could conceivably encode the overall metabolic activity of the muscles via the proxy of muscle blood flow (Haouzi et al., 1995). Groups III and IV muscle afferents innervate and activate lamina I of the dorsal horn (Craig and Mense, 1983; Jankowski et al., 2013; Wilson et al., 2002). In turn, lamina I neurons directly innervate the intermediolateral cell column and several lower brainstem regions involved in breathing and blood pressure control (Craig, 2002, 2013). Therefore, lamina I neurons are the likely initial relay for cardiorespiratory effects produced by unmyelinated muscle afferents.

The degree to which muscle afferents contribute to the hyperpnea of exercise is somewhat controversial. The sufficiency criterion has been satisfied by showing that activation of muscle afferents increases breathing and blood pressure, albeit with the important caveat that these responses could be related to deep muscle pain rather than aerobic exercise. The necessity criterion would require showing that selectively silencing small caliber muscle afferents reduces exercise hyperpnea without affecting muscle work or the chemoreflexes. Toward this end, Amann et al. found that a very low dose of fentanyl given intrathecally to attenuate synaptic transmission between muscle afferents and lamina I neurons blunts exercise hyperpnea (Amann et al., 2010; Dempsey, 2012).

The central command theory posits that motor pathways for locomotion and respiration are driven in parallel by a central feed-forward mechanism (reviewed in Forster et al., 2012; Paterson, 2014). A seminal observation was that electrical and chemical stimulation of the caudal hypothalamus (subthalamic locomotor region) produces parallel and proportional activation of locomotion and breathing in decorticated cats (Eldridge et al., 1981). The interpretation of this experiment depends on

the assumption, still unverified, that the increase in locomotion and breathing were not caused by the simultaneous stimulation of two functionally independent pathways. Similar interpretative problems exist concerning the role of the other "locomotor centers": the spinal cord (Le Gal et al., 2014), the periaqueductal gray matter (Paterson, 2014) and the mesencephalic locomotor region (MLR) (Gariépy et al., 2012; Karachi et al., 2010; Le Ray et al., 2011).

Conclusions

The neural control of CO_2 homeostasis relies on three processes: the chemoreflexes, central command, and somatic afferent feedback. The past decade has witnessed rapid progress in understanding the cellular, molecular, and integrative mechanisms underlying the chemoreflex regulation of breathing. Comparable insights into the neural substrates and processes underlying the central command and muscle feedback that drive exercise hyperpnea are lagging.

The RTN is the most thoroughly characterized cluster of central respiratory chemoreceptor neurons. Genetic elimination of these neurons reduces the central chemoreflex to a very large extent. Two proton receptors (TASK-2 and GPR4), with sparse representation elsewhere in the brain are required for RTN neurons to detect changes in brain pH; in the absence of these proton detectors, the central respiratory chemoreflex is nearly abolished. Accordingly, the stimulatory effect of brain PCO_2 on breathing is ultimately mediated by changes in brain $[H^+]$ that are detected, directly and predominantly, by RTN neurons. This conclusion is at variance with the view that the central respiratory chemoreflex results from actions of H^+ or CO_2 distributed throughout the respiratory pattern generator and the rest of the brain but it is consistent with earlier ideas hypothesizing specific chemoreceptors in the rostral medulla (Loeschcke, 1982).

Although proton receptors (TASK-2 and GPR4) are required for RTN neurons to respond to elevated brain PCO_2, the CO_2 sensitivity of these neurons may be boosted by specialized astrocytes that respond to pH and/or to molecular CO_2 by releasing ATP locally, enhancing extracellular acidification relative to intravascular pH changes, and modifying local blood flow. The relative importance of these mechanisms requires further evaluation.

RTN neurons regulate alveolar ventilation by adjusting the breathing rate, inspiratory and expiratory muscle activity, and airway resistance. These effects occur via excitatory projections to multiple segments of the lower brainstem respiratory pattern generator but the targeted respiratory neurons have yet to be identified. The effect of RTN on breathing is state dependent; it is most prominent when the brainstem respiratory network is auto-rhythmic (e.g., during non-REM sleep) and breathing frequency is presumably defined by the group pacemaker properties of the preBötzinger complex. During REM sleep, RTN regulates tidal volume but not breathing frequency.

RTN development depends on expression of at least three transcription factors: Atoh-1, Egr-2 and Phox2b and is particularly vulnerable to a Phox2b mutation that causes CCHS in humans (*Phox2b*$^{27ala/+}$). This mutation recapitulates in mice the cardinal respiratory signs of the human disease. The congenital absence of RTN neurons could therefore underlie the respiratory deficits observed in CCHS but, in the absence of histopathological evidence from patients, this interpretation remains tentative.

RTN neurons and the carotid bodies normally work in concert to stimulate or reduce breathing in response to hypo- or hyperventilation. During hypoxia, the ventilatory stimulation elicited by carotid body hyperactivity is opposed by a reduction in RTN neuronal activity caused by the concomitant alkalosis. This phenomenon limits the increase in breathing elicited by hypobaric hypoxia and likely contributes to altitude sickness. Conversely, when respiratory drive from the carotid bodies is reduced or eliminated (e.g., hyperoxia), the contribution of RTN neurons to breathing increases, minimizing the respiratory deficit.

Serotonergic, orexinergic and noradrenergic neurons increase breathing and can facilitate the chemoreflex by multiple mechanisms, including RTN stimulation. A subset of serotonergic neurons located in raphe magnus likely has central chemoreceptor properties but their response to CO_2 in vivo is small and evidence that actions on the respiratory chemoreflex reflect a specific effect of pH in those cells, via an intrinsic molecular proton detector, has not yet been obtained. Major perturbations in serotonergic systems exacerbate effects of asphyxia in rodents by attenuating CO_2-induced arousal and hypoxia-induced autoresuscitation, and developmental defects in serotonergic transmission may contribute to SIDS.

The stability of PCO_2 during exercise is based on coincident activation of the respiratory pattern generator by circuits involved in locomotion (central command) and by small caliber muscle afferents that relay via lamina I of the dorsal horn. Ultimately, a more detailed phenotypic and functional characterization of these afferents and their central connections will require genetic approaches similar to those recently implemented for the study of cutaneous and vagal afferents (Abraira and Ginty, 2013; Chang et al., 2015). The projections from lamina I to the brainstem convey multiple modalities of interoceptive and exteroceptive information, and the challenge remains to identify which particular neurons relay inputs from muscle afferents that are relevant to exercise hyperpnea.

Central command is the least understood of the three mechanisms involved in CO_2 homeostasis. The role of the various candidate locomotor centers to exercise hyperpnea should be thoroughly reinvestigated, with a goal of identifying specific roles and precise connectivity of chemically defined neuronal populations present within the broadly defined locomotor regions. This recalcitrant research area is ready to yield to the ever-expanding toolkit of contemporary integrative neuroscience.

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