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Serotonin Neurons and Central Respiratory Chemoreception: Where are we now?

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Abstract

Serotonin (5-hydroxytryptamine; 5-HT) neurons are widely considered to play an important role in central respiratory chemoreception. Although many studies in the past decades have supported this hypothesis, there had been concerns about its validity until recently. One recurring claim had been that 5-HT neurons are not consistently sensitive to hypercapnia in vivo. Another belief was that 5-HT neurons do not stimulate breathing; instead, they inhibit or modulate respiratory output. It was also believed by some that 5-HT neuron chemosensitivity is dependent on TASK channels, but mice with genetic deletion of TASK-1 & TASK-3 have a normal hypercapnic ventilatory response (HCVR). This review explains why these principal arguments against the hypothesis are not supported by existing data. Despite repeated challenges, a large body of evidence now supports the conclusion that at least a subset of 5-HT neurons are central chemoreceptors.

Keywords

5-HT neurons; chemoreceptors; control of breathing; hypercapnia; acidosis; raphé

Introduction

The primary purpose of breathing is to maintain normal O_2 and CO_2 levels in the blood. Under resting conditions and at sea level, ventilation is primarily regulated by sensory feedback from central respiratory chemoreceptors (CRCs) as well as by peripheral chemoreceptors (PCRs) to a lesser extent. CRCs, located in the brainstem, are sensitive to parenchymal pH, which is strongly influenced by blood PCO_2. PCRs, which will not be discussed in detail in this review, are located in the carotid and aortic bodies and under normal conditions are influenced primarily by blood PO_2 and less so by blood PCO_2. For more than four decades, central chemoreception has been the subject of extensive research, and much attention has been paid to the ventrolateral medulla (VLM) as the site of CRCs.

However, the precise location within the VLM and phenotype of the neurons (or glia) that are CRCs has been disputed. In addition, neurons in other regions of the brainstem have been proposed as CRCs (Richerson 2004, Corcoran et al. 2009, Guyenet et al. 2010). For example, substantial evidence has supported the conclusion that 5-HT neurons are CRCs, only some of which are in the VLM.

In order for any cells to be considered CRCs, they need to display chemosensitivity to physiologically relevant changes in pH/PCO_2, and their response must be intrinsic. They also must be connected to the respiratory network in a way that increases respiratory output in response to hypercapnia. Medullary 5-HT neurons possess these properties (Richerson 1995, Wang et al. 2001, Richerson 2004, Richerson et al. 2005, Corcoran et al. 2009). A number of recent studies have strengthened the support for a critical role of 5-HT neurons in central respiratory chemoreception, yet until recently there were still some that remained skeptical about this possibility. Due to rapid changes in this field and new evidence in favor of the 5-HT CRC hypothesis, now is an appropriate time to provide a comprehensive account of research that has occurred in recent years. This review identifies each of the major objections made against the 5-HT central chemoreceptor hypothesis, describes the data that led to these objections, and addresses why the original interpretation of those data have not held up to deeper scrutiny. In the process, we describe evidence that provides support that 5-HT neurons are central chemoreceptors.

Unraveling the Apparent Contradictions

A subset of 5-HT neurons increases their firing rate *in vivo* in response to hypercapnia

It has been claimed that 5-HT neurons are not central chemoreceptors based on data showing that 5-HT neurons do not respond to CO_2 *in vivo* (Mulkey et al. 2004, Guyenet et al. 2008, DePuy et al. 2011). However, there have been two studies using extracellular recordings *in vivo* that have shown 5-HT neurons do, in fact, respond to hypercapnia with an appreciable increase in firing rate (Veasey et al. 1995, Veasey et al. 1997, Richerson et al. 2005). In addition, multiple studies have shown that hypercapnia causes c-*fos* activation in the raphe, with some demonstrating that a subset of activated cells are immunoreactive for markers of serotonin neurons [Reviewed in Richerson et al, 2005].

In order to understand why there is inconsistency in the literature on this particular point, it is necessary to identify differences in experimental conditions. 5-HT neurons in the raphé obscurus (DePuy et al. 2011) and VLM surface (Mulkey et al. 2004) of anaesthetized rats *in vivo* did not increase their firing rate in response to inhalation of 10% CO_2 (a non-physiologically high level). It was unclear why 5-HT neurons were not chemosensitive during these *in vivo* experiments using anesthesia (DePuy et al. 2011) until it was realized that the HCVR was markedly smaller than that seen in other laboratories that did not use anesthesia. Rats studied by DePuy et al (2011) only increased their ventilation by 35% in response to 8% CO_2 (Fig. 1a). In contrast, multiple other laboratories have found that rodents have much greater sensitivity, typically responding to 7% CO_2 with an increase in ventilation to 250% of control (Fig. 1b) (Taylor et al. 2005, Davis et al. 2006, Hodges et al. 2008). We hypothesized that the blunted HCVR was due to the use of isoflurane or halothane anesthesia. Consistent with this we have obtained preliminary data showing that

1% isoflurane caused profound depression of the HCVR by 81% (n=16; CO_2 7%) (Massey et al. 2012). Halogenated anesthetics have also been shown to decrease the HCVR in other species (Fig. 1c) (Hirshman et al. 1977, Dahan and Teppema 2003).

The effect of halogenated anesthetics on the HCVR appears to be due in part to an effect on 5-HT neurons. The blunting of chemoreception was accompanied by complete loss of the increase in firing in response to acidosis of 5-HT neurons in culture and in an *in situ* perfused brain preparation (Johansen et al. 2012, Massey et al. 2012). Inhalational halogenated anesthetics strongly potentiate two-pore domain K^+ leak (TASK) channel currents causing membrane hyperpolarization (Patel et al. 1999, Sirois et al. 2000). Although TASK channels are widely expressed in many CNS neurons, 5-HT neurons have one of the highest levels of TASK channel expression (Talley et al. 2001). Halothane and isoflurane would therefore be expected to inhibit 5-HT neurons, which has been shown to decrease respiratory chemoreception (Ray et al., 2011).

The possibility that inhalational anesthetics introduced an artifact into *in vivo* recordings (Mulkey et al. 2004, DePuy et al. 2011) is supported by recordings from unanesthetized cats. A subset of 5-HT neurons in the medullary raphé (Veasey et al. 1995) and the dorsal raphé (Veasey et al. 1997) is sensitive to hypercapnia *in vivo*. There are numerous additional studies performed in unanesthetized animals in which 5-HT neurons are stimulated by hypercapnia *in vivo*. Microdialysis in the hypoglossal (XII) nucleus of unanesthetized mice demonstrated a 2.4–2.6-fold increase in extracellular 5-HT levels during inhalation of 7% CO2 (Kanamaru and Homma 2007). C-*fos* staining of some 5-HT neurons increases in the medullary raphé of cats (Larnicol et al. 1994), rats (Sato et al. 1992, Pete et al. 2002, Johnson et al. 2005), and mice (Haxhiu et al. 2001) when exposed to hypercapnia. Recent studies have examined *Lmx1b*$^{f/f/p}$ mice, in which *Lmx1b* (the gene for a transcription factor necessary in the differentiation of 5-HT neurons) is excised from genomic DNA selectively in *Pet-1* expressing cells (i.e. 5-HT neurons). This results in specific deletion during embryonic development of >99% of 5-HT neurons in the CNS (Zhao et al. 2006). As adults, *Lmx1b*$^{f/f/p}$ mice exhibit an HCVR that is 50% smaller than that of wild type littermates (Fig. 1b) (Hodges et al. 2008). A 50% decrease in chemoreception was also seen when 5-HT neurons were silenced by activation of genetically encoded DREADD receptors selectively expressed in 5-HT neurons (Ray et al. 2011). It is not known which cells contribute to the remaining 50% of the HCVR in these transgenic lines. However, 5-HT neurons make a major contribution to the HCVR, possibly more than 50% of the total response.

It remains to be determined what percentage of 5-HT neurons are chemoreceptors. In cell culture, between 70% & 90% of 5-HT neurons respond to a decrease in pH from 7.4 to 7.2 (Wang et al. 2001). In slices the percentage of 5-HT neurons that are chemosensitive to this range of pH is smaller, but it is not clear if there are more neurons that have no response, if slices buffer the pH changes to mask some responses, or there are some neurons that have very large responses and others that have smaller responses. It is possible that the former project to respiratory neurons and the latter project to other brain regions involved in functions that are less sensitive to pH/CO_2.

5-HT neurons stimulate breathing; they do not inhibit it

In order to be considered a chemoreceptor, a neuron is required to have more properties than just chemosensitivity; it must provide excitatory drive to the respiratory network. Only recently has it become widely accepted that 5-HT neurons stimulate respiratory output. Previously, investigators described the respiratory effects of 5-HT as inhibitory, modulatory, "stabilizing" or primarily involved in plasticity (Hodges and Richerson 2008a). However, a number of recent experiments have convincingly demonstrated that 5-HT neurons stimulate respiratory output. Here we describe possible explanations for why it had previously been unclear how 5-HT affects breathing.

Stimulation—There is now ample evidence that 5-HT neurons stimulate breathing. Early studies of serotonergic raphé neurons *in vivo* observed a direct correlation between firing rate and the increase in minute ventilation induced by hypercapnia (Fig 2a) (Veasey et al. 1995). An increase in fractional inspired CO_2 of as little as 3% led to a statistically significant increase in both 5-HT neuron firing rate and ventilation (Fig 2a). Hodges et al. (2008) also observed increased ventilation after providing exogenous 5-HT given intracerebroventricularly (ICV). Similarly, exogenous application of 1-[2,3-dimethoxy-4-iodophenyl]-2-aminopropane (DOI), a 5-$HT_{2A/2C}$ agonist, increases ventilation in rats (Cayetanot et al. 2002) and neonatal mice *in vivo* (Hodges et al. 2009) (Fig 2b). Conversely, iontophoretic application of 5-HT_{2A} antagonists such as ketanserin in decerebrate dogs *in vivo* and unilateral injection of MDL 100,907 into the XII nucleus of rats *in vivo* decreases respiratory output of hypoglossal motor neurons (Brandes et al. 2006). These antagonists also block the effects of 5-HT microinjected into the ventral aspect of the XII nucleus of rats (Fenik and Veasey 2003) thus supporting the excitatory effect of 5-HT on respiratory motor neurons.

BIMU8, a 5-HT_{4A} receptor agonist, significantly increases respiratory motor output (Manzke et al. 2003). This stimulatory effect is blocked by the 5-HT_{4A} receptor-specific antagonist GR 113808. In addition, BIMU8 is effective in overcoming fentanyl-induced respiratory depression and apnea (Manzke et al. 2003). Systemic application of the 5-HT_{1A} receptor agonist 8-hydroxy-[N-n-dipropyl-N-(3-iodo-2-propenyl)amino] tetralin (8-OH-DPAT) also restores eupneic respiration after fentanyl-induced apnea (Sahibzada et al. 2000). This latter observation is counterintuitive because 5-HT_{1A} receptors usually inhibit neurons through G_i second messenger pathways. Recent data from rhythmogenic neonatal mouse medullary slices indicate that this is due to disinhibition of the respiratory network via 5-HT_{1A} receptor activation on GABAergic or glycinergic interneurons (Corcoran et al. 2013).

Two sets of experiments were recently performed that demonstrate clearly that increased firing of 5-HT neurons causes increased respiratory output. First, an excitatory projection from 5–HT neurons to the respiratory network was revealed by Ptak et al (2009) in rhythmogenic slices from neonatal mice. Patch clamp recordings were made from raphé obscurus 5-HT neurons. It was found that focal application of the glutamate receptor agonist 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid (AMPA) into the raphe obscurus induces a graded increase in firing rate of 5-HT neurons which leads to a

proportional increase in frequency of hypoglossal nerve rootlet bursting (Fig. 3). This stimulation of respiratory motor output is blocked by 5-HT_{2A} receptor antagonists. Some neurons that are tryptophan hydroxylase (TpOH) immunoreactive have axons that project to the pre-Bötzinger complex (pre-BötC), others have axons that project to the XII motor nucleus, and still others have axons that send branches to both of these nuclei (Fig. 4). There are also projections of 5-HT neurons to other respiratory nuclei, including the nucleus of the solitary tract, nucleus ambiguus, RTN and phrenic motor nucleus (Jacobs and Fornal 1997, Lovick 1997, Mason 2001). Together these projections stimulate the respiratory network at multiple sites, via multiple receptors and using multiple mechanisms (Dekin et al. 1985, Richerson 2004, Ptak et al. 2009). Interestingly 5-HT neurons are also embedded within the respiratory network as they receive bursts of excitatory input during inspiration, indicating that there are synapses from the respiratory CPG onto 5-HT neurons (Ptak et al. 2009).

The second study employed optogenetics to selectively stimulate 5-HT neurons in the medullary raphé *in vivo*. This induces a large increase in frequency and amplitude of phrenic nerve output. This increase in phrenic nerve activity is blocked by methysergide, a non-selective antagonist of 5-HT receptors (Fig. 5) (DePuy et al. 2011). The use of optogenetics was important, because it allowed stimulation to be isolated specifically to 5-HT neurons. Previous experiments stimulating the raphe in adults *in vivo* were not able to isolate the effect of 5-HT neurons alone, and often got mixed effects that were likely to be due to stimulation of non-5-HT neurons.

Inhibition—Early studies led to the conclusion that 5-HT neurons normally inhibit breathing. These studies included the use of systemic inhibitors of TpOH, such as p-chlorophenylalanine (PCPA), 6-fluorotryptophan (6-FT), and para-chloroamphetamine (PCA). Each of these drugs reduces 5-HT levels in the brain and systemically, and also induces hyperventilation (Olson et al. 1979, Mitchell et al. 1983). However, one of these studies, as well as others performed around the same time, also reported that specific lesions of 5-HT neurons with the toxin 5,7-dihydroxytryptamine (5,7-DHT) cause significant hypoventilation (Olson et al. 1979, Mueller et al. 1984). A possible explanation for these contradictory results could be that 5,7-DHT does not cross the blood-brain barrier, unlike PCA, 6-FT, and PCPA, and thus must be administered ICV (Olson et al. 1979). Respiratory control is regulated by both central and peripheral neuronal inputs and the pharmacological agents that caused hyperventilation were given intraperitoneally. Beyond depletion of central 5-HT, PCPA can also alter or deplete peripheral 5-HT and other neurotransmitters (Olson et al. 1979, Reader and Gauthier 1984). Another explanation is that when PCPA is given at a dose sufficient to deplete central 5-HT by 90%, there is no change in the post-synaptic response to stimulation of ascending 5-HT fibers to the hippocampus, indicating that there is considerable compensatory reserve to prevent failure of neurotransmission at 5-HT synapses (Chaput et al. 1990). Finally, 5,7-DHT is neurotoxic, and can cause death of 5-HT neurons. The result is not just reduced release of 5-HT, as would occur with the other agents, but also reduced release of neuropeptides that are co-localized in 5-HT neurons, such as thyrotropin-releasing hormone (TRH) and substance P (SP). Conversely, PCPA, PCA and 6-FT deplete 5-HT, but the neurons remain intact and would continue to release TRH and SP. The decrease in extracellular 5-HT levels would lead to loss of 5-HT_{1A} receptor-

mediated autoinhibition, which could increase release of TRH and SP, both of which have powerful stimulatory effects on breathing (Yamamoto et al. 1981, Hedner et al. 1983, Nink et al. 1991). The end result of 5-HT depletion might be greater stimulation of the respiratory network rather than less.

There has been relatively little other direct evidence that 5-HT neurons inhibit respiratory output. There are regions of the midline medulla that when stimulated electrically induce inhibition of breathing (Lalley et al. 1997). Some of these responses are mediated by 5-HT release as they are blocked by methysergide. However, what is not known is whether the "inhibitory 5-HT neurons" project directly to the respiratory network. Alternatively, they may project to and inhibit another pool of 5-HT neurons, and this other pool might normally stimulate the respiratory network. Interconnections between different subsets of 5-HT neurons are not well understood.

Some neurons of the pre-BötC are inhibited by 5-HT_{1A} receptors (Lalley et al. 1997, Richter et al. 2003). The identity of these neurons is unknown, but if they were inhibitory interneurons then inhibition by 5-HT would lead to disinhibition of respiratory output. Direct projections have never been demonstrated from 5-HT neurons that inhibit output from the respiratory network.

Modulation—5-HT neurons have also been proposed to play a neuromodulatory role in control of breathing (Hodges and Richerson 2008a). However, it has not always been clear how neuromodulation is defined in this context, since this term has had many different definitions. According to Kaczmarek and Levitan (1987) it is "the ability of neurons to alter their electrical properties in response to intracellular biochemical changes resulting from synaptic or hormonal stimulation." It can often refer to changes in neuronal excitability induced through second messenger pathways upon activation of G protein-coupled receptors (GPCRs) (Hodges and Richerson 2010, Bocchiaro and Feldman 2004). In that sense, most actions of 5-HT are neuromodulatory, because all but one of the 5-HT receptor subtypes (5-HT_3) are GPCRs (Bockaert et al. 2006). Therefore, the effects of 5-HT on all of the GPCRs are neuromodulatory. However, they can still be stimulatory. As an example, 5-HT_{2A} receptors are GPCRs and therefore neuromodulatory. However, they stimulate respiratory output in slices (Pena and Ramirez 2002; Ptak et al. 2009) and *in vivo* (Hodges and Richerson 2008a, Doi and Ramirez 2010). 5-HT_{2A} receptor activation in neurons of the pre-BötC enhances a leak Na^+ current, which in turn promotes bursting pacemaker activity and strengthens respiratory motor output (Ptak et al. 2009).

Plasticity—Serotonin is necessary for some forms of neural plasticity observed in the respiratory network. A well-studied example is long-term facilitation (LTF), which is characterized by a prolonged increase in respiratory motor output after specific patterns of intermittent hypoxic challenges (Mitchell et al. 2001). LTF has most frequently been observed in anesthetized rats as increased phrenic nerve burst amplitude and a long-lasting increase in frequency (Karen B. Bach 1996, Baker and Mitchell 2000, Fuller et al. 2000, Mitchell et al. 2001). Methysergide (Baker-Herman and Mitchell 2002) and ketanserin (Kinkead and Mitchell 1999) block LTF. This effect of 5-HT could be viewed as a more complex form of respiratory stimulation on a longer time domain that is superimposed on

the immediate and short-term forms described above. The end result is an increase in respiratory motor output, so it could be viewed as a complementary form of stimulation mediated by chemoreceptors.

Chemosensitivity of adult 5-HT neurons is not due to TASK channels

It has previously been stated that 5-HT neurons are not central chemoreceptors, because of data from TASK knockout mice (Mulkey et al. 2007b). Dorsal raphé 5-HT neurons in brain slices from WT mice increase firing rate two-fold when pH decreases from 7.5 to 6.9. This *in vitro* chemosensitivity is abolished if either TASK-1 or TASK-3 channels are genetically deleted alone or together. However, the *in vivo* HCVR is normal in mice with genetic deletion of either or both TASK channels. The authors concluded that TASK-1/3 channels are necessary for chemosensitivity of 5-HT neurons, but that respiratory chemoreception is not dependent on 5-HT neurons in adult rodents. However, one must be cautious in accepting those conclusions for several reasons. First, the 5-HT neurons that have been proposed to be respiratory chemoreceptors are in the medulla, not the dorsal raphe of the midbrain, although those in the midbrain may be important for arousal and anxiety/panic to hypercapnia (Buchanan and Richerson 2009, Buchanan and Richerson 2010). Second, the data obtained to make these conclusions were all from neurons in brain slices from P7-P12 mice, which is too young for a mature response. At this age 5-HT neurons require a very large decrease in pH to induce only a small increase in firing rate. Chemosensitivity of 5-HT neurons of the degree that is likely to be relevant to adult chemoreception does not begin to develop until P12 (Fig 6a) and is not mature until after P21 (Wang and Richerson 1999, Wu et al. 2008). This age dependence of 5-HT neuron chemosensitivity is seen *in vitro* in slices and culture and parallels the age-dependent increase in the HCVR in rodents *in vivo* (Serra et al. 2001, Davis et al. 2006). Similarly, inhibition of medullary raphé 5-HT neurons by microdialysis of 8-OH-DPAT does not affect the HCVR in piglets younger than P10, but does decrease it in older animals (Messier et al. 2004). Penatti et al. (2006) did not observe any change in the HCVR in conscious piglets at P4–P12 after lesioning medullary raphé 5-HT neurons using a SERT antibody-saporin conjugate. Additionally, 5,7-DHT increases the HCVR in neonatal rats (Cummings and Frappell 2008), whereas it causes hypoventilation in adult rats (Olson et al. 1979). In contrast, neurons of both the locus coeruleus and nucleus tractus solitarius (NTS) are chemosensitive early in development *in vitro* (Stunden et al. 2001, Conrad et al. 2009, Nichols et al. 2009). These data indicate that the HCVR in neonates is very small and depends primarily on non-5-HT neurons. During adulthood the HCVR becomes much larger and 5-HT neurons play a more significant role, contributing at least 50% to central chemoreception (Hodges and Richerson 2008b, Ray et al. 2011).

Another reason the conclusions from Mulkey et al (2007b) are not relevant to respiratory chemoreception is that the pH changes used to elicit chemosensitivity were not physiologically relevant. 5-HT neurons did not respond to a decrease in pH from 7.5 to 7.3, but instead required a decrease in pH from 7.5 to 6.9 in order to induce a response. Such a large pH change is not consistent with the well-established observation that 5-HT neurons respond to a pH decrease from 7.4 to 7.2 with an average increase in firing rate to 300% of baseline (Wang et al. 1998, Wang et al. 2001, Wang et al. 2002). It is unlikely a living mammal would have a blood pH of 6.9 unless it is *in extremis*. In the case of a pure

respiratory acidosis, the PCO_2 would have to rise above 100 mmHg in order for the pH to drop below 7.1 (Katsura et al. 1992a, Katsura et al. 1992b), which greatly surpasses the range of 50-70 mmHg that might occur in a critically ill patient with chronic obstructive pulmonary disease, and would likely only occur when a patient goes into respiratory failure, cardiopulmonary arrest or other medical emergency (Koo et al. 1975). When studying putative chemoreceptors, it is imperative to keep *in vitro* experimental conditions within a physiological range. Any cellular response to changes in PCO_2/pH beyond 80 mmHg/ 7.0 are unlikely to be relevant to normal respiratory control, since a PCO_2 above that range is not seen in healthy individuals, and is only seen in patients with severe illness such as cardiopulmonary arrest, diabetic ketoacidosis or other serious illness. Even in those cases CSF pH is maintained within a much tighter range, typically between 7.4 and 7.25, than arterial pH (Fencl, 1971). Another reason to not study such an extreme level of pH is that the HCVR plateaus above a very high level of $PaCO_2$ (Lambertsen 1980).

There are other reasons why it is unlikely that TASK channels mediate the response of adult 5-HT neurons to pH. For example, TASK channels have a relatively small response to changes in pH between 7.6 and 7.2 (Fig. 6b) (Washburn et al. 2002), whereas 5-HT neurons have a very large firing rate response over that pH range. TASK channels are sensitive to extracellular pH (Rajan et al. 2000, Morton et al. 2003), whereas 5-HT neuron firing rate is sensitive to intracellular pH (Wang et al. 2002). In addition, TASK channels are leak channels, and are not gated by Ca^{2+} or depolarization, whereas 5-HT neurons have a pH sensitive tail current that is activated by both depolarization and Ca^{2+} (Richerson et al. 2009). TASK channels are selective for K^+ whereas the pH sensitive channel in 5-HT neurons is permeable to both K^+ and Na^+ (Richerson et al. 2009). These data point to a novel pH sensitive calcium-activated non-selective cation (CAN) current (Richerson 2004, Massey et al. 2013) as mediating the response of mature 5-HT neurons to changes in pH between 7.4 and 7.2. There is no evidence that TASK channels mediate the large changes in firing rate that take place in mature raphé neurons in response to small changes in pH near 7.4 (Corcoran et al. 2009).

Although TASK channels are sensitive to pH (Rajan et al. 2000, Morton et al. 2003), there is other evidence that they are not crucial for central respiratory chemoreception. For example, TASK channels are broadly expressed in the CNS (Talley et al. 2001) and yet not all neurons and brain functions are chemosensitive to hypercapnia (Richerson 1998, Richerson et al. 2001). Hippocampal principal neurons and essentially all motor neurons express high levels of TASK channels (Talley et al. 2001, Taverna et al. 2005); however, hypercapnic acidosis does not stimulate these neurons, but rather has no effect or inhibits them (Richerson 1998, Wang and Richerson 2000, Somjen and Tombaugh 1998).

For all of the above reasons, the fact that a normal HCVR was seen in TASK knockout mice does not refute the hypothesis that 5-HT neurons are respiratory chemoreceptors.

Chemosensitivity of 5-HT neurons is reduced during sleep

It has previously been suggested that 5-HT neurons are not chemoreceptors because they decrease their firing rate and become less responsive to hypercapnia during sleep. A study performed on unanesthetized cats found that a subset of neurons in the medullary raphe

(identified as serotonergic by validated criteria) are activated by hypercapnia during wakefulness (Veasey et al. 1995), but the response to CO_2 disappeared during sleep. This was interpreted by others as showing that activation by hypercapnia was due to arousal rather than to intrinsic chemosensitivity (Guyenet et al. 2005). However, we disagree with this conclusion. It has long been known that there is a decrease in the HCVR during sleep, but the mechanism has never been defined. 5-HT neurons *in vivo* usually fire fastest during wakefulness, slower during non-rapid eye movement sleep (NREM), and are nearly silent during rapid eye movement sleep (REM) (McGinty and Harper 1976, Trulson and Jacobs 1979). The arousal state-dependent discharge pattern of 5-HT neurons is congruous with their ability to regulate respiration as central chemoreceptors. Arousal in response to hypercapnia is dependent on 5-HT neurons (Buchanan and Richerson 2010), presumably acting as CO_2 sensors within the midbrain. Previously it has been proposed that state-dependent control of upper airway tone is partly mediated by state-dependent changes in 5-HT neuron firing (Kubin et al. 1992, Buchanan 2008). There is also a progressive reduction in the slope of the HCVR in deeper stages of sleep (Fig. 7a) (Bulow 1963). The mechanisms of this effect are not known, but the firing rate of 5-HT neurons decreases during sleep, presumably due to inhibition from sleep generating centers such as the ventrolateral preoptic nucleus in the hypothalamus (Saper et al. 2005). Since generation of bursting activity in respiratory centers is enhanced by 5-HT and TRH input (Dekin et al. 1985, Pena and Ramirez 2002, Ptak et al. 2009), the decrease in firing of 5-HT neurons might contribute to the decrease in baseline ventilation that occurs during sleep. There is also a progressive decrease in chemosensitivity of 5-HT neurons during sleep (Fig. 7b), which may represent the cellular mechanism that underlies the decrease in the HCVR (Fig. 7a).

Recordings from unanesthetized, behaving cats identified neurons that responded to hypercapnia during wakefulness, but were less responsive or unresponsive during sleep (Veasey et al. 1995). This led to the conclusion that these neurons were simply responding to an increase in arousal induced by CO_2 (DePuy et al. 2011). Exposure to CO_2 during sleep induces arousal (Buchanan and Richerson 2010), which is an important protective reflex. We have proposed that this is mediated by chemosensitivity of midbrain 5-HT neurons (Richerson 2004). If true, the effect of CO_2 on arousal would occur at the same time as that on ventilation. As CO_2 increases, 5-HT neurons fire at a faster rate, inducing both arousal and increased ventilation. The arousal would also cause an increase in slope of the HCVR. If a 5-HT neuron increases its firing rate when an animal wakes up, the increase in neuronal firing would be ascribed to arousal. If all 5-HT neurons were excluded from analysis just because there was coexisting arousal, then many chemosensitive 5-HT neurons would go undetected.

The role of 5-HT in control of sleep and wakefulness has long been controversial. Early experiments led to the persistent belief that 5-HT causes sleep (Jouvet 1999). This conclusion was based on experiments using PCPA to deplete 5-HT levels. However, as with experiments on respiratory control using PCPA (see above), this approach led to complicated effects that opposed those obtained using other approaches. Although this view of 5-HT still continues to be perpetuated in the literature, it has been acknowledged to be incorrect (Jouvet 1999). The first experiments revealing that PCPA gave invalid results used

single unit recordings and found that 5-HT neurons fire fastest during wakefulness, as described above (McGinty and Harper 1976, Trulson and Jacobs 1979). This is the opposite one would expect if these neurons caused sleep. It is now accepted by the majority of sleep neuroscientists that 5-HT neurons are part of the ascending arousal system (Steriade et al. 1993, Monckton and McCormick 2002, Saper et al. 2005) and activation of these neurons causes arousal instead of sleep.

What is now emerging is a picture where 5-HT neurons induce arousal, but rather than doing so in an ill-defined way, they do so specifically in response to hypercapnia. It has been hypothesized that midbrain 5-HT neurons initiate the arousal response to hypercapnia (Washburn et al. 2002, Severson et al. 2003, Richerson 2004, Buchanan 2008). Functional MRI studies show that hypercapnia induced by brief apneic episodes leads to an increase in activity within the thalamus and cerebral cortex (Kannurpatti et al. 2003). 5-HT release within the thalamus changes thalamocortical rhythms from a bursting pattern to a tonic pattern (Monckton and McCormick 2002). This correlates with a change from high-voltage, low-frequency, synchronized activity in the cortical EEG compatible with slow wave sleep to low-voltage, high-frequency, desynchronized activity consistent with wakefulness (Steriade et al. 1993). Furthermore, arousal to 10% CO_2 is completely absent in *Lmx1b*$^{f/f/p}$ mice (Fig 8) (Buchanan and Richerson 2010), indicating that 5-HT neurons are required for the potentially life-saving response that should normally occur when hypercapnia occurs during sleep.

The relationship between 5-HT neurons and central arteries is important for accuracy, not speed, of blood PCO_2 measurement

Most 5-HT neurons in both the medullary and midbrain raphé are adjacent to large cerebral arteries (Severson et al. 2003, Bradley et al. 2002). This is an ideal position for 5-HT neurons to accurately monitor changes in arterial PCO_2. However, the functional significance of this anatomical specialization has been questioned. Data from dogs *in vivo* shows a prolonged delay in the ventilatory response to central chemoreceptor stimulation (Smith et al. 2006). The authors concluded that this favored a site for CO_2 reception distant from blood. However, the delay for a change in ventilatory output depends not only on how long it takes for a change in arterial PCO_2 to induce a change in 5-HT neuron firing rate, but also how long it takes for neurotransmitters released from 5-HT neurons to induce changes in activity of the downstream respiratory network. The purpose of respiratory chemoreceptors is to monitor how efficiently the lungs are oxygenating the blood. For this reason, they need to be positioned where they have the ability to faithfully measure arterial PCO_2, the physiological parameter that best reflects alveolar ventilation. This parallels the functional purpose of peripheral chemoreceptors, which are closely associated with the carotid arteries and the aorta - locations where they can monitor arterial blood as soon as it exits the left ventricle of the heart. 5-HT neurons in the medullary raphé are immediately adjacent to the basilar artery and its large midline branches (Fig. 9). This region is highly perfused by large arteries and is relatively devoid of veins, which would lead to a local tissue PCO_2 that more accurately reflects changes in lung ventilation and arterial blood PCO_2 compared to any other site within the brain such as locations near distal venous blood, parenchymal tissue or bulk CSF.

Close proximity to large arteries could also permit detection of changes in lung gas exchange rapidly after they occur (Smith et al. 2006). However, a rapid response would not necessarily be an advantage. For example, when the speed of negative feedback is not matched properly for a particular control system, oscillations in output can occur. The respiratory control system can be prone to oscillations, as seen in Cheyne-Stokes breathing; this can occur when the rate of feedback is poorly matched to the output of the system.

There is also no advantage for central CO_2 chemoreceptors to be fast. First, changes in arterial blood PCO_2 occur relatively slowly. The respiratory control system also has a number of properties that introduce a delay downstream of CO_2/pH sensation. For example, there is a slow cellular response of respiratory neurons to activation of the receptors for the neurotransmitters released by 5-HT neurons (5-HT, TRH and SP) almost all of which (other than 5-HT_3) are G-protein coupled receptors. Optogenetic experiments determined the time course for the response of the respiratory system to selective stimulation of 5-HT neurons (Fig 10) (DePuy et al. 2011). A rapid increase in firing of 5-HT neurons caused a half-maximum response of the amplitude of diaphragm EMG to occur in 15.8 s. The increase in respiratory frequency was even slower, continuing for up to 10 seconds after the end of a 20-second burst of 5-HT neuron firing. These delays are consistent with the expected response time following activation of GPCRs. For example, 5-HT_{2A} receptors, which are important for stimulation of respiratory output by 5-HT neurons, activate the IP_3 second-messenger system and subsequently increase intracellular Ca^{2+} levels (Bhattacharyya et al. 2002), processes which both introduce a delay. Thus, downstream motor activity is significantly delayed after 5-HT neurons are stimulated by CO_2, so there is no advantage to having a fast response of 5-HT neurons to arterial CO_2 changes. Instead, the proximity to arteries is likely to exist so that the measurement of PCO_2 can be as accurate as possible.

Other brainstem regions contain neurons that may also be chemoreceptors

There are multiple other regions of the brainstem that contain neurons that may also be chemoreceptors, including the RTN (Mulkey et al. 2004) nucleus tractus solitaries (NTS) (Dean et al. 1990), locus coeruleus (LC) (Pineda & Aghajanian 1997) and hypothalamus (Dillon & Waldrop 1992). Of these regions, the RTN has received the most attention. It contains neurons that express *Phox2b* and are chemosensitive *in vitro* and *in vivo* (Mulkey et al. 2004, Mulkey et al. 2007a, DePuy et al. 2011). These neurons have also recently been found to be chemosensitive after acute dissociation, albeit with a smaller response to pH (Wang et al. 2013).

The RTN projects throughout the respiratory network (Smith et al. 1989) including the Pre-BötC. Interestingly, the RTN receives synaptic input from many other putative chemoreceptors, including 5-HT neurons, peripheral chemoreceptors, and the NTS (Takakura et al. 2006). Therefore, it is possible that the response of RTN neurons to acidosis *in vivo* is enhanced by synaptic input from these other chemoreceptors. For example, when acidosis causes an increase in firing rate of 5-HT neurons, RTN neurons would respond to the increase in extracellular 5-HT (Mulkey et al. 2007a) at the same time they respond to the acidosis, with both responses being excitatory. It would be hard to differentiate how much of the response of RTN neurons was due to which mechanism. Thus, in addition to being

chemosensitive themself, RTN neurons relay information to the respiratory network from multiple other chemoreceptors or are integrators of inputs from multiple sources of chemoreceptor afferents (Nattie et al. 2004, Forster et al. 2010). The very large response of RTN neurons to inhalation of CO_2 *in vivo* (Mulkey et al, 2004) may be due in part to this summation of inputs from many different chemoreceptors.

On top of the complexity that has already been described, 5-HT may also amplify the effects of some chemoreceptors. 5-HT delivered into the lateral ventricles of *Lmx1b*$^{f/f/p}$ mice, can restore the blunted HCVR response back to normal (Hodges et al. 2008). The mechanism is unknown, but could be due to enhancement of synaptic input from peripheral chemoreceptors within the NTS or other sites, or amplification of chemosensitivity of non-5-HT neurons such as chemoreceptors in the RTN. In the latter case the response of pH sensitive ion channels in RTN neurons, which may include TASK-2 (Wang et al. 2013), might be enhanced by activation of 5-HT receptors.

Figure 11 shows a model that includes the interaction between 5-HT neurons and RTN neurons, connections to other elements of the respiratory network, and ATP release from neighboring glia, which has been proposed to be important for the response of RTN neurons as well (Gourine et al. 2010).

Summary—The field of central respiratory chemoreception has rapidly evolved over the past few years. 5-HT neurons have now met all of the criteria needed for designation as CRCs, and each of the major arguments against their role as CRCs has been found to lack support. Neurons of the RTN and other sites also contain CRC candidates. In addition, the RTN may be a site for integration of signals from CRCs in other locations. In some regions glia may play a critical role as either sensors or modulators of the response to pH. There is considerable work remaining to be done in order to define the importance of each of the putative CRC sites. In doing so it will be necessary to recognize the strengths and weaknesses of every experimental approach that we as a field use, and interpret data cautiously unless the data are concordant across multiple preparations. These considerations will be important as we refine our understanding of these cell that are so critically important for life.

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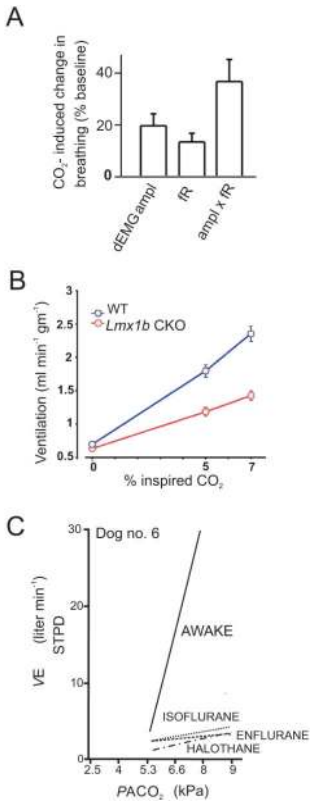

Figure 1. Halogenated anesthetics markedly depress the HCVR

a) *In vivo* recordings from 5-HT neurons in isoflurane anesthetized rats show only a 35% increase in ventilation in response to 8% CO_2. Reproduced with permission (DePuy et al. 2011). **b)** Whole-animal plethysmography recordings from unanesthetized WT mice show a greater than 250% increase in ventilation in response to 7% CO_2. In mice that lack 5-HT neurons there is a greater than 100% increase in ventilation in response to hypercapnia. Reproduced with permission (Hodges et al. 2008). **c)** Ventilation in an awake dog increases in response to hypercapnia. However, halogenated anesthetics depress the HCVR in the same dog. Adapted with permission (Hirshman et al. 1977).

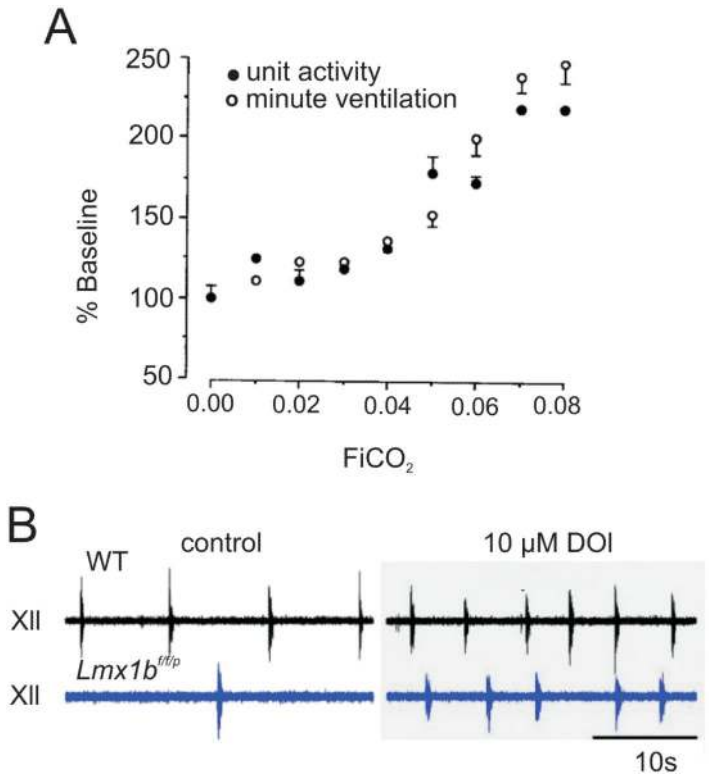

Figure 2. Medullary 5-HT neurons increase their firing in response to hypercapnia *in vivo*

a) Relationship between activity of a single medullary 5-HT neuron and minute ventilation as inspired CO_2 increases; there is a parallel increase in unit activity and minute ventilation in response to hypercapnia. Reproduced with permission (Veasey et al. 1995). **b).** Recordings from XII nerve roots show decreased respiratory activity in *Lmx1b*$^{f/f/p}$ mice (blue) compared to WT mice (black) under control conditions (left traces). DOI bath application (10 μM) increased respiratory activity in *Lmx1b*$^{f/f/p}$ mice to a comparable level as WT. Reproduced with permission (Hodges et al. 2009).

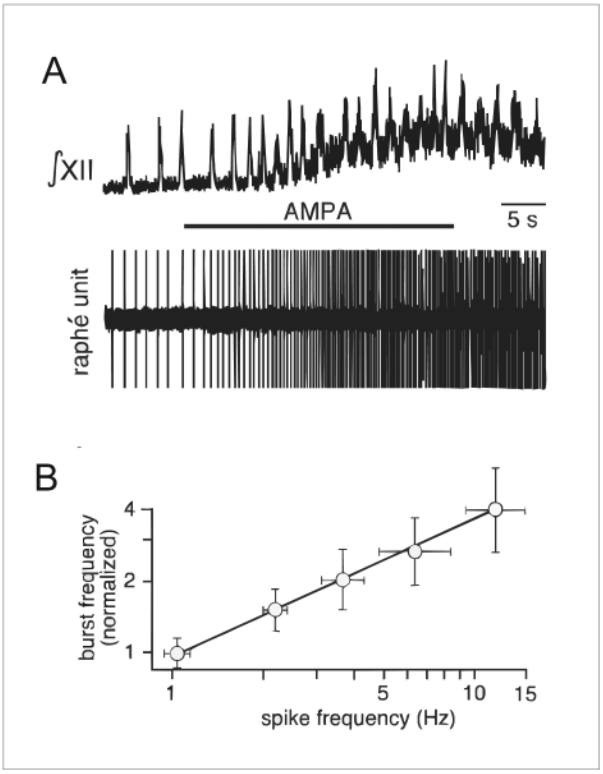

Figure 3. 5-HT neuron activity proportionally stimulates hypoglossal nerve rootlet bursting

a) XII output (top) recorded concurrently with extracellular potential of a 5-HT neuron (bottom trace). AMPA (5 μM) microinfusion into the raphé obscurus increased both firing rate of the 5-HT neuron and XII activity. **b)** XII nerve bursting activity increased proportionally with increased spike frequency of raphe unit. Reproduced with permission (Ptak et al. 2009).

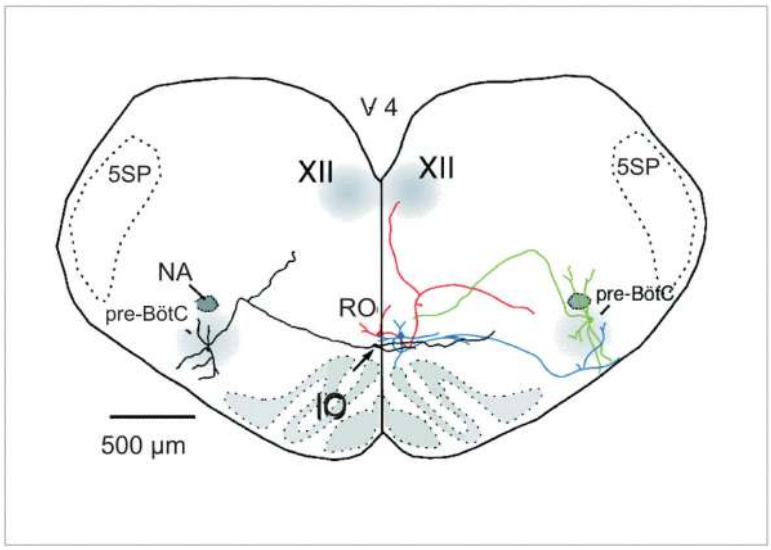

Figure 4. 5-HT neurons project to neurons in the major respiratory nuclei

Reconstruction of two biocytin-filled raphé obscurus 5-HT neurons (red, blue) and two pre-Bötzinger complex neurons (green, black) illustrating dendrites and axonal projections. Reproduced with permission from (Ptak et al. 2009).

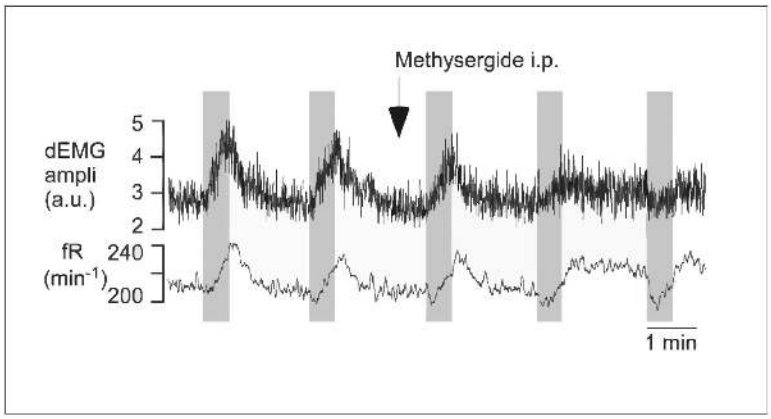

Figure 5. 5-HT neurons stimulate respiratory output

Raphé obscurus 5-HT neurons from rats expressing channelrhodopsin (ChR) stimulate breathing when activated by photostimulation. Intraperitoneal methysergide administration blocks this response. Reproduced with permission (DePuy et al. 2011).

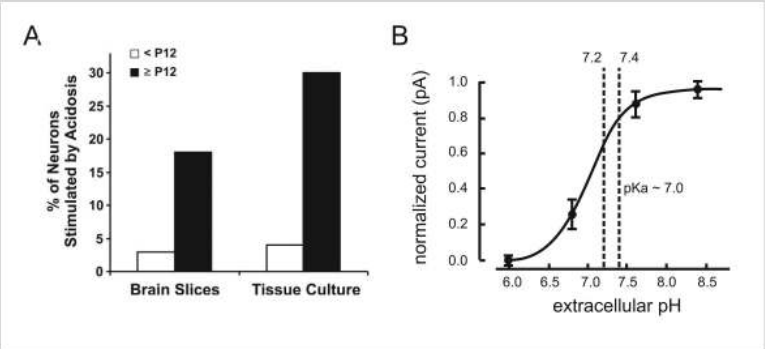

Figure 6. Chemosensitivity of 5-HT neurons is age-related, but not TASK-dependent

a) 5-HT neuron chemosensitivity does not mature until after P12. Recordings from Sprague-Dawley rat raphé neurons recorded in slices and culture are not chemosensitive until after P12. Reproduced with permission (Corcoran et al. 2009). **b)** A change in TASK channel conductance does not occur over the physiological pH range. Voltage-clamp recordings were made from dorsal raphé 5-HT neurons. Currents measured under pH variations from 6.0 to 8.4 in halothane were normalized to the maximum and minimum current. These were fitted to a logistic equation that predicted a pK of ~7.0. Dashed lines indicate the pH range over which 5-HT neurons are typically tested. Altered with permission (Washburn et al. 2002).

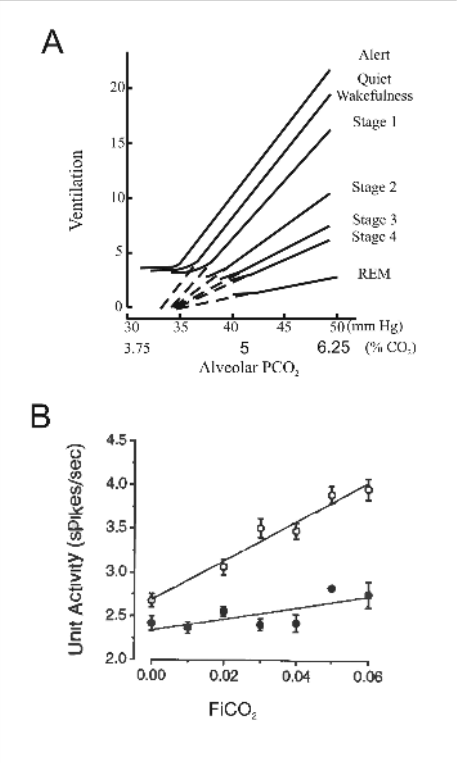

Figure 7. Respiratory CO_2 chemoreception decreases during sleep

a) The HCVR is robust in humans who are alert or in quiet wakefulness. It progressively decreases as subjects move from wakefulness to deeper sleep. REM sleep, when 5-HT neurons are silent, shows the lowest HCVR. Adapted with permission (Bulow 1963). **b)** State-dependent responses in neuronal activity for a 5-HT neuron responsive to a hypercapnia challenge during quiet waking (QW) and slow-wave sleep (SWS). Open circles, QW data; closed circles, SWS data. Reproduced with permission (Veasey et al. 1995).

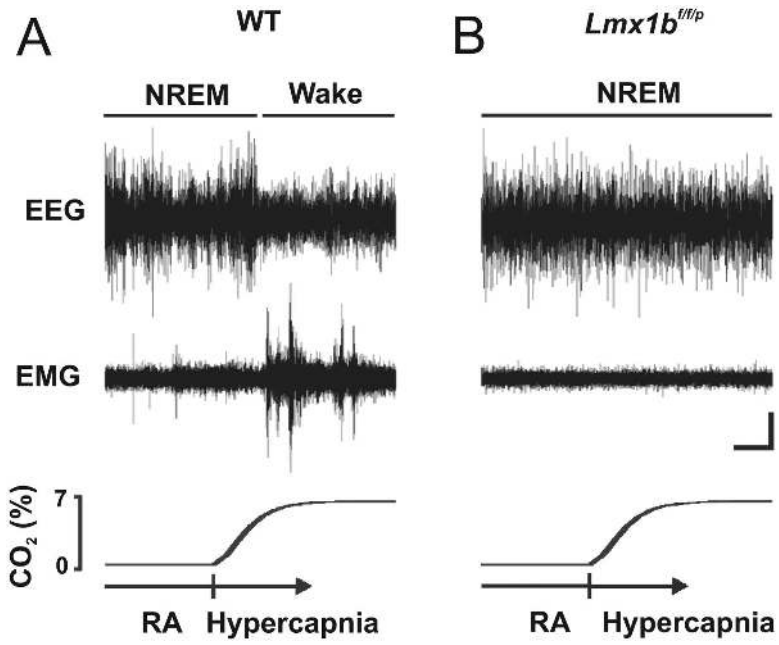

Figure 8. Genetic deletion of 5-HT neurons prevents arousal to hypercapnia

Four-minute EEG (top), EMG (middle), and PCO_2 (bottom) recordings showing an arousal response to 7% CO_2 in WT mice, but its absence in *Lmx1b*$^{f/f/p}$ mice. Reproduced with permission (Buchanan and Richerson 2010).

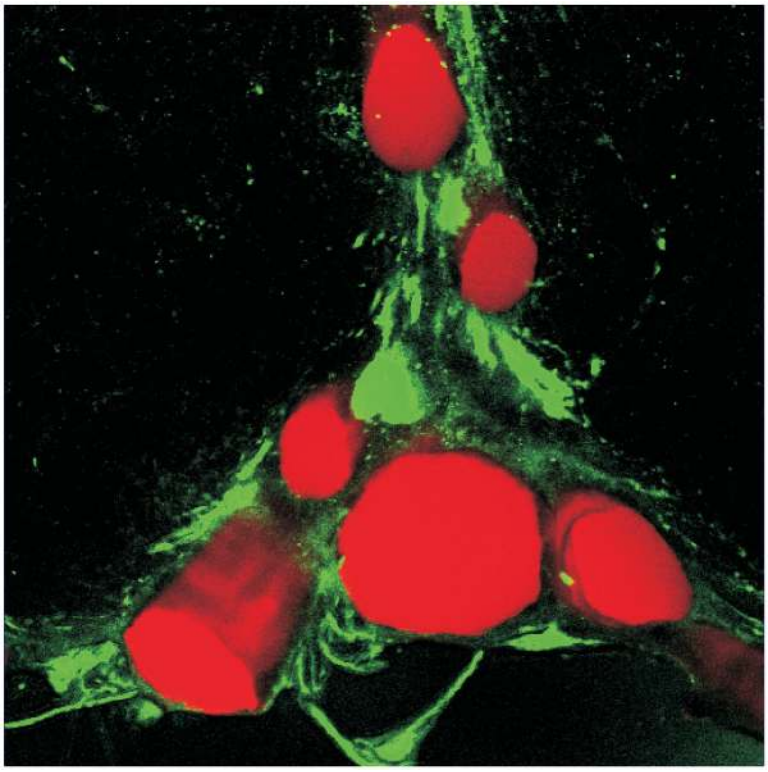

Figure 9. 5-HT neurons are located adjacent to large arteries in the medulla

5-HT neurons (green) are found in close approximation to the basilar and its associated arteries (red) in the medulla. Reproduced with permission (Fiske 2002).

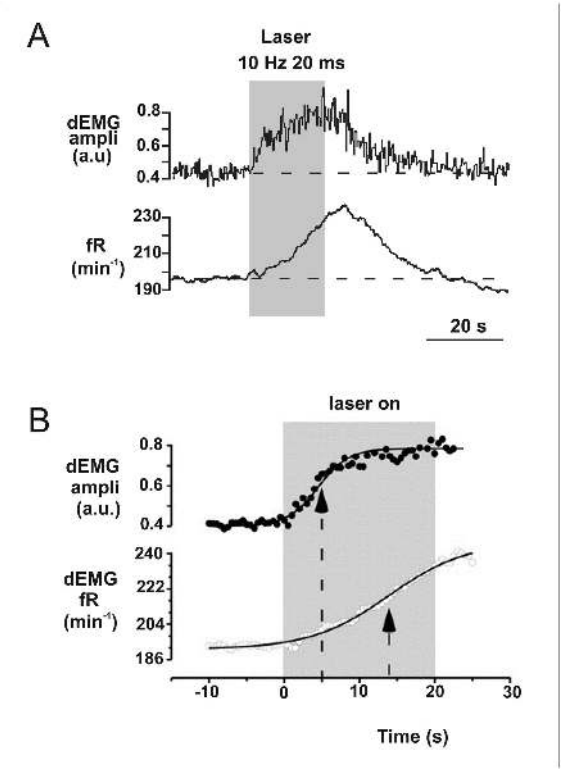

Figure 10. Respiratory response induced by 5-HT stimulation is delayed

a) Raphé obscurus 5-HT neurons from rats expressing channelrhodopsin (ChR) stimulate breathing when activated by photostimulation. Reproduced with permission from (DePuy et al. 2011). **b)** Amplitude and frequency response at onset of 5-HT photostimulation; data points are fitted to a Boltzmann sigmoid equation to determine half-maximal response. These data reveal that respiratory output is significantly delayed following 5-HT neuron stimulation. Reproduced with permission (DePuy et al. 2011).

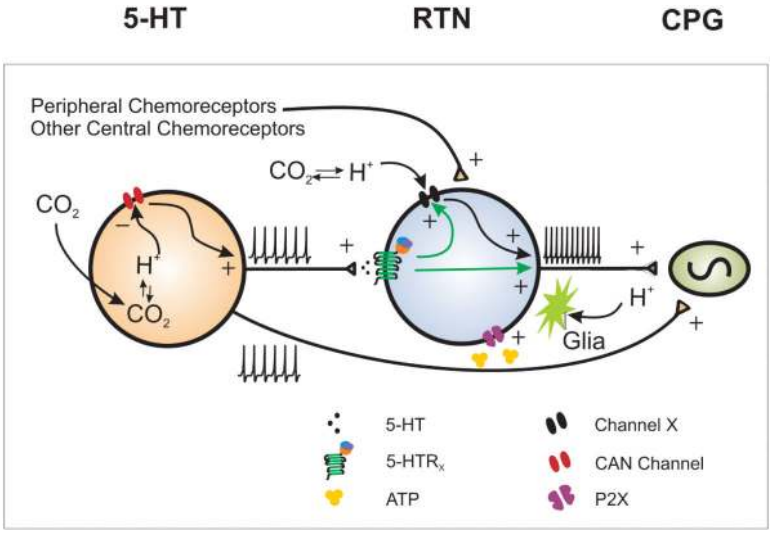

Figure 11. 5-HT neuron and RTN chemosensitivity

A model in which 5-HT neurons and RTN neurons are both chemosensitive to pH changes. In addition, 5-HT neurons enable or enhance RTN neuron chemosensitivity. 5-HT neurons are chemosensitive and stimulate RTN neurons. RTN neurons are chemosensitive and are also activated by chemosensitive glia and by projections from peripheral and other central chemoreceptors. There is evidence for each of these possibilities, as described in the text. Some are still hypotheses, and for others it is not known whether they occur under normal conditions *in vivo*.