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Medullary serotonin neurons and central CO₂ chemoreception

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Abstract

Serotonergic (5-HT) neurons are putative central respiratory chemoreceptors, aiding in the brain's ability to detect arterial changes in P_{CO₂} and implement appropriate ventilatory responses to maintain blood homeostasis. These neurons are in close proximity to large medullary arteries and are intrinsically chemosensitive *in vitro*, characteristics expected for chemoreceptors. 5-HT neurons of the medullary raphé are stimulated by hypercapnia *in vivo*, and their disruption results in a blunted hypercapnic ventilatory response. More recently, data collected from transgenic and knock-out mice have provided further insight into the role of 5-HT in chemosensitivity. This review summarizes current evidence in support of the hypothesis that 5-HT neurons are central chemoreceptors, and addresses arguments made against this role. We also briefly explore the relationship between the medullary raphé and another chemoreceptive site, the retrotrapezoid nucleus, and discuss how they may interact during hypercapnia to produce a robust ventilatory response.

1. Introduction

It has been several years since a comprehensive review on the role of serotonin (5-hydroxytryptamine, 5-HT) neurons in central chemoreception has been published (Richerson, 2004; Richerson et al., 2005). In that period, a number of studies have been carried out that contribute to the body of evidence supporting the hypothesis that 5-HT neurons are central respiratory chemoreceptors. Novel techniques have recently been employed, including the use of transgenic mouse models to study the relative importance of 5-HT neurons in the hypercapnic ventilatory response (HCVR). These data are consistent with the hypothesis that there are multiple sites that contain central chemoreceptors, and the set that is dominant may differ depending on the specific conditions. In addition, new evidence points to an important interaction between the medullary raphé and another putative chemoreceptor region, the retrotrapezoid nucleus (RTN). The current review aims to couple recent data with past results in an effort to synthesize the substantial and compelling evidence that 5-HT neurons are central

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respiratory chemoreceptors. We also discuss and dispute two arguments made recently that challenge the role of 5-HT neurons as central respiratory chemoreceptors.

2. Evidence that serotonergic neurons are respiratory chemosensors

There is a subset of 5-HT neurons located in the rostral ventrolateral medulla (VLM) in the region classically defined as the rostral chemosensitive zone (Mitchell et al., 1963). 5-HT neurons are also located in the caudal chemosensitive zone in the caudal VLM (Loeschcke, 1982). However, the majority of 5-HT neurons on the ventral surface are found in the midline – a region that was not examined in early *in vivo* experiments localizing the central chemoreceptors because of the risk of bleeding from the basilar artery, and there are even more 5-HT neurons in the midline deep to the surface which were inaccessible to ventral surface manipulations. It is not known whether 5-HT neurons within all of these regions share similar properties and functions, but those in the VLM are appropriately located to have mediated the responses induced by changes in pH on the VLM surface.

Within the medullary raphé, 5-HT neurons make up approximately 25% of neurons, and all of the raphé neurons stimulated by hypercapnic acidosis *in vitro* are serotonergic (Wang et al., 2001; Richerson, 2004). Although the firing rates of most raphé neurons *in vivo* are not phase-locked to respiratory output, it has been shown that the raphé participates in respiratory control. There are heavy projections from 5-HT neurons within the medullary raphé nuclei to the major respiratory nuclei, including hypoglossal and phrenic motor neurons (Holtman, Jr. et al., 1984a, 1984b; Connelly et al., 1989; Smith et al., 1989; Thor and Helke, 1989; Henry and Manaker, 1998; Aungst et al., 2008). Exogenous 5-HT, TRH and SP each stimulate respiratory motor output both *in vivo* and *in vitro* (Lalley, 1986; Morin et al., 1991; Monteau et al., 1994; Lalley et al., 1995; Hilaire et al., 1997; Cream et al., 1997, 1999; Pena and Ramirez, 2002, 2004; Manzke et al., 2003; Richerson, 2004; Brandes et al., 2006). Stimulation of raphé neurons causes an increase in respiratory output due to release of these transmitters (Lalley et al., 1986; Morin et al., 1990; Aungst et al., 2008), as recently reviewed in detail (Richerson, 2004; Hodges and Richerson, 2008b). 5-HT neurons, particularly those in the medullary raphé, also have many properties that would be expected for respiratory chemoreceptors. Collectively, the following evidence strongly suggests that some 5-HT neurons are chemoreceptors.

2.1. 5-HT neurons are closely associated with large arteries

The purpose of respiratory chemoreceptors, central and peripheral, is to monitor the effectiveness of lung ventilation. Therefore, the ideal location for them is a place where they could faithfully measure arterial P_{CO_2} . This is probably why peripheral chemoreceptors are near the aorta and carotid arteries where they would be exposed to arterial blood immediately after it leaves the left ventricle of the heart. 5-HT neurons in the midline and in the rostral and caudal chemosensitive zones are closely associated with the basilar artery and its largest branches (Bradley et al., 2002) (Fig. 1A). This proximity to large blood vessels would allow them to faithfully sense arterial P_{CO_2} , which would more closely reflect lung ventilation than would the P_{CO_2} of bulk cerebrospinal fluid or of tissue near small capillaries. This location may also allow changes in P_{CO_2} to be sensed rapidly, though a rapid response would not necessarily be an advantage since it could cause instability of the system. The rate of change in ventilation would depend on how rapidly changes in firing rates of 5-HT neurons are transduced into changes in respiratory output. There are a variety of central mechanisms that could introduce a delay downstream of pH sensation, such as a slow cellular response of respiratory neurons to activation of the G-protein coupled receptors that mediate the effects of neurotransmitters released by 5-HT neurons. Thus, a slow response of the respiratory system to activation of central chemoreceptors does not necessarily indicate that chemoreceptors do not sense CO_2 in a peri-arterial location (Smith et al., 2006).

2.2. 5-HT neurons are intrinsically chemosensitive *in vitro*

5-HT neurons increase their firing rate in response to increasing CO₂ in rat and mouse brain slices after blockade of fast glutamate and GABA receptors (Bradley et al., 2002; Wu et al., 2008). This response is not prevented by physically isolating rat and mouse raphé neurons in culture (Fig. 1B) (Wang et al., 1998, 2001; Wu et al., 2008), proving that chemosensitivity within the raphé is not due to input from another brainstem region. Within these cultures, 5-HT neurons are the only neurons that are stimulated by acidosis, making it unlikely that 5-HT neurons are synaptically driven by other neurons. Consistent with this conclusion, the chemosensitivity of raphé neurons in slices is not blocked by high Mg²⁺ low Ca²⁺ solution (Richerson, 1995). However, the only way to confirm intrinsic chemosensitivity is to physically isolate a neuron using acute dissociation, guaranteeing that there are no extrinsic influences. We have recently done this for 5-HT neurons from ePet-EYFP expressing mice (Scott et al., 2005), and found that they continue to be robustly chemosensitive (Wu et al., 2008) (Fig. 2).

Stimulation of 5-HT neurons by acidosis is not a non-specific effect, since most neurons from brain regions not involved in respiratory control are unaffected or inhibited by acidosis (Richerson, 1998; Wang and Richerson, 2000). The pH response of 5-HT neurons at room temperature is larger than that of other chemoreceptor candidates studied to date (Putnam et al., 2004) although strict comparisons are difficult since different conditions have been used by different labs. When the response of these neurons is normalized to a decrease in pH from 7.4 to 7.2 by using the Chemosensitivity Index (Wang et al., 1998), 5-HT neurons in culture increase their firing rate to approximately 300% of control on average at room temperature (Wang et al., 1998, 2001, 2002), and increase to 413% at 30°C (Wu et al., 2008). In slices, the response is similar in magnitude to that of neurons in culture of the same age, although for technical reasons recordings have only been made in slices from young animals, and the response is smaller in immature 5-HT neurons (see below).

2.3. 5-HT neurons are stimulated by hypercapnia in unanesthetized animals *in vivo*

There is evidence from a number of studies that some 5-HT neurons increase their firing rate *in vivo* in response to hypercapnia. In all of these studies, experiments were performed in unanesthetized animals. For example, two studies used chronic recordings from 5-HT neurons within the raphé in unanesthetized cats, one from the raphé obscurus and one from the dorsal raphé (Veasey et al., 1995, 1997). In both studies, a subset (22%) of electrophysiologically identified 5-HT neurons increased their firing rate in response to hypercapnia. On average there was a 60% increase in firing rate in response to inhalation of 8% CO₂, but there were large differences in sensitivity with the response being as large as 120% in response to 7% CO₂. Some neurons display non-linear CO₂ responses, with a large increase in firing rate above the response threshold (Fig. 3A). Thus, if a lower level of CO₂ had been used the response of these neurons would not have been detected. Consistent with a previous hypothesis (Richerson et al., 2005), it is possible that those 5-HT neurons with the largest response to CO₂ project to a neural system that is particularly sensitive to CO₂, such as the respiratory system or the thalamocortical network involved in arousal. Indeed, there is a direct correlation between firing rate of some 5-HT neurons and the increase in ventilation induced by hypercapnia (Fig. 3B). Other 5-HT neurons that are less sensitive to CO₂ may project to neural systems that also show sensitivity to CO₂ but with a higher threshold, such as the parts of the limbic system involved in anxiety/panic (Klein, 1993). Some of the 5-HT neurons that did not respond in the Veasey et al. (1995, 1997) studies may not be chemosensitive, consistent with the finding that not all 5-HT neurons are chemosensitive *in vitro*. However, others may have been chemosensitive but had a threshold that was higher than the maximum level tested. These studies controlled for changes in sleep state to avoid false positive responses that were due to arousal. Therefore, it is possible that some chemosensitive neurons were not detected in these studies because recordings were excluded if the arousal state was affected by hypercapnia, and arousal to

CO₂ may have occurred coincident with activation of a subset of 5-HT neurons. This is what would be expected if 5-HT neurons contribute to the arousal that is mediated by hypercapnia (Buchanan et al., 2007, 2008; Washburn et al., 2002; Severson et al., 2003).

There are numerous studies from unanesthetized animals that have demonstrated c-fos expression in response to hypercapnia in a subset of neurons within the midline medulla, including those that are immunoreactive for tryptophan hydroxylase (TpOH) (Larnicol et al., 1994; Haxhiu et al., 1996, 2001; Teppema et al., 1997; Okada et al., 2002; Pete et al., 2002; Johnson et al., 2005; Niblock et al., 2008). The conclusion that 5-HT neurons increase their firing rate in response to hypercapnia *in vivo* is also supported by experiments using microdialysis in the hypoglossal (XII) nucleus of unanesthetized mice, which showed that increasing inhaled CO₂ to 7% causes a 2.4–2.6 fold increase in extracellular 5-HT levels (Kanamaru and Homma, 2007). Thus, in unanesthetized mammals *in vivo* there is a substantial increase in firing rate and neurotransmitter release in at least a subset of 5-HT neurons in response to hypercapnia, including some that project to respiratory nuclei.

2.4. Focal acidosis in the raphé increases respiratory output *in vivo* and *in vitro*

Focal acidosis has been induced within the medullary raphé *in vivo* using either pressure microinjection of the carbonic anhydrase inhibitor acetazolamide or microdialysis of artificial cerebrospinal fluid (aCSF) equilibrated with high levels of CO₂. Both approaches lead to an increase in ventilation in rats (Bernard et al., 1996; Nattie and Li, 2001; Feldman et al., 2003). Similarly, the latter approach increases ventilation in goats, and the degree of ventilatory stimulation is greater with a greater degree of acidosis in the raphé (Hodges et al., 2004a, 2004b, 2005b).

An analogous approach has also been applied to brain slices of the rostral medulla containing the pre-Bötzinger complex (pre-BötC). These slices, which spontaneously generate respiratory output (Smith et al., 1991), contain a large number of 5-HT neurons in the midline that project directly to the pre-BötC and the XII nucleus (Ptak et al., 2006). Focal acidosis within the medullary raphé in these slices increases the frequency of respiratory output in the hypoglossal nerve (Peever et al., 2001; Ptak et al., 2006).

2.5. Disruption of 5-HT neurons blunts the hypercapnic ventilatory response

It is difficult to lesion all 5-HT neurons, because they are so widely distributed throughout the brainstem. Early attempts to do this used the 5-HT neuron selective neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) in neonatal rats and found that as adults there is an increase in apneic threshold, and a large decrease in both baseline ventilation and the slope of the relationship between ventilation and arterial P_{CO₂} (Mueller et al., 1984). More recently, midline 5-HT neurons were specifically lesioned in adult rats using the toxin saporin conjugated to an antibody against the 5-HT transporter. This also leads to a decrease in the HCVR (Nattie et al., 2004; Dias et al., 2007). Focal injection of non-selective agents such as muscimol or ibotenic acid into the medullary raphé also results in blunting of the ventilatory response to CO₂ (Messier et al., 2002; Hodges et al., 2004c; Dias et al., 2007). In addition, injection of 8-hydroxy-2-(dipropylamino)-tetralin (8-OH-DPAT) to silence 5-HT neurons leads to a decrease in the HCVR (Messier et al., 2004; Taylor et al., 2005).

More recently, efforts have been made to determine the relative contributions of individual raphé nuclei to the HCVR. Selective and non-selective lesions of ~35% of 5-HT neurons in the raphé magnus reduce the HCVR by 24% (Dias et al., 2007). Such experiments have not been performed on the more caudal raphé obscurus, however new studies suggest that this nucleus also plays a modulatory role on central responses to hypercapnia (see below).

2.6. Insights from transgenic and knock-out mice

Since 5-HT neurons are so widely distributed throughout the brainstem, none of the methods described above for lesioning or silencing them are able to affect them all. To affect more 5-HT neurons, novel genetic methods have recently been used. Mice lacking the Pet-1 ETS transcription factor (Pet-1^{-/-}), which is expressed only in 5-HT neurons, exhibit a 70% decrease in central 5-HT neurons (Hendricks et al., 2003). Pet-1^{-/-} mice display heightened anxiety-like behaviors and aggression, poor maternal behavior, and a neonatal respiratory phenotype in which the respiratory rhythm is slow and unstable when compared to wild type mice (Hendricks et al., 2003; Erickson et al., 2007; Lerch-Haner 2008). Adult males have normal resting ventilation, but have a blunted HCVR (Hodges et al., 2005a). This abnormality does not occur in female Pet-1^{-/-} mice, consistent with other evidence that there can be sex differences in the ventilatory response to hypercapnia (Penatti et al., 2006).

Conditional knockout of the transcription factor *Lmx1b* selectively in Pet-1 expressing cells (*Lmx1b*^{ff/p}) causes complete (>99%) and specific loss of 5-HT neurons in the central nervous system (CNS) without affecting gross brain morphology or other amine systems (Ding et al., 2003; Zhao et al., 2006). This leads to a decrease in the HCVR to 50% of wild type mice (Hodges et al., 2008a), demonstrating that 5-HT neurons are required for normal central chemoreception. In contrast, when exposed to an extreme level of 10% ambient CO₂, ventilation increases similarly in both genotypes (Hodges et al., 2008c). This suggests that non-5-HT chemoreceptor neurons may play their most significant role in response to unusually high or pathological levels of CO₂.

Interestingly, the deficit in the HCVR in *Lmx1b*^{ff/p} mice was reversed by intracerebroventricular infusion of exogenous 5-HT, indicating that 5-HT can enhance the response of non-5-HT neurons to hypercapnia (Hodges et al., 2008a). The site of action of this effect is unknown, but potentially could include the RTN (see below), the NTS where afferent inputs from peripheral chemoreceptors are processed, other putative central chemoreceptors, or other respiratory nuclei. This result does not contradict the conclusion that there is a contribution of intrinsic chemosensitivity of 5-HT neurons to the HCVR, since it is not clear whether the concentration of 5-HT that reaches the relevant receptors is physiological or supra-physiological. However, it does identify a novel contribution of 5-HT neurons to the HCVR, enhancing chemosensitivity of non-5-HT neurons. It remains unclear how important that contribution normally is compared to intrinsic chemosensitivity.

The HCVR has also been studied in mice in which the 5-HT transporter (5-HTT) has been genetically deleted. These mice differ from Pet-1^{-/-} and *Lmx1b*^{ff/p} mice which lack 5-HT neurons in that 5-HTT knockouts presumably undergo development with excess 5-HT in the brain extracellular fluid. This results in decreased 5-HT neuron activity and decreased 5-HT_{1A} receptor binding as adults (Li et al., 2000; Gobbi et al., 2001). 5-HTT knockout mice have a reduced HCVR when compared to wild type mice (Li and Nattie, 2008). This blunting is greater in males whose response is ~30% that of wild type, compared to females whose HCVR is ~80% of wild type, providing support for sex differences in the role of 5-HT in respiratory chemosensitivity.

The hypercapnic response is also affected in Necdin knock out (*Ndn*-KO) mice (Zanella et al., 2008). Irregularities in the NECDIN gene has been linked to Prader-Willi syndrome and affects network maturation and function (Pagliardini et al., 2005). *Ndn*-KO mice also display abnormalities of the serotonin system, and as adults are 40% less response to inhalation of 4% CO₂ than wild-type mice (Zanella et al., 2008).

While genetic mutants provide a powerful tool in our ability to assess the role of the 5-HT system under physiological conditions, it is important to recognize that 1) other neurons may

also be central chemoreceptors, and 2) there may be significant compensation by other putative chemoreceptors during development of these animals. Thus, the data described above probably underestimate the importance of 5-HT neurons for the HCVR, because compensatory changes, such as an increased CO₂ response of peripheral and/or other central chemoreceptors, likely act to normalize the respiratory control system. Developmental compensation may also lead to changes in the hierarchy of mechanisms and sites involved in CO₂ chemoreception.

2.7. The hypercapnic ventilatory response of rodents develops with age in parallel with chemosensitivity of 5-HT neurons

In rodents, the ability to respond to hypercapnia is present at birth, however sensitivity to CO₂ is significantly less than that of adults. Two studies have reported a decrease in the HCVR during the first week post-natal, reaching a nadir around P8 (Serra et al., 2001; Stunden et al., 2001). A more recent study by Davis et al. (2006) examined the development of CO₂ sensitivity during the neonatal period in three strains of rat. No decline in the HCVR was observed during the first week in any strain. Despite this difference during early development, the HCVR increased in all of these studies after ~P12 until the adult level was reached sometime after P20 (Fig. 4A) (Serra et al., 2001; Stunden et al., 2001; Putnam et al., 2005; Davis et al., 2006). This developmental pattern could be due to maturation of central chemoreceptors over the first 2–3 weeks of life. Consistent with this, chemosensitivity of 5-HT neurons in rats and mice follows a similar time course (Fig. 4B). It is rare to find 5-HT neurons that are chemosensitive until around P12 in slices and in culture (Wang and Richerson, 1999; Wu et al., 2008). After that age, chemosensitivity increases, reaching full maturity sometime after P20. In contrast, robust chemosensitivity is established early in development for neurons of both the locus coeruleus and NTS *in vitro* (Conrad et al., 2009; Nichols et al., 2009; Stunden et al., 2001). The correlation between the *in vitro* response of 5-HT neurons and *in vivo* data suggests that the HCVR depends primarily on non-serotonergic central chemoreceptors in early life, then increases from P12 to P20 as 5-HT neurons mature and develop chemosensitivity (Nichols et al., 2009). Messier et al. (2004) also found evidence for developmental changes in 5-HT neuron chemosensitivity. Inhibition of 5-HT neurons by microdialysis of 8-OH-DPAT in the medullary raphe decreases the HCVR in 10–16 day old conscious piglets, while the opposite effect is observed in younger animals.

Additionally, experiments performed on younger animals do not result in similar blunting of the HCVR when 5-HT neuron function is disrupted. After lesioning 5-HT neurons in the medullary raphe, Penatti et al. (2006) did not observe a change in the HCVR in newborn conscious piglets (4–12 days old), which contrasts with earlier work done in older animals (Dreshaj et al., 1998). 5,7-DHT causes an increase in the HCVR in neonatal rats (Cummings et al., 2008). Also, neonatal (P4.5) Pet-1^{-/-} mice have the same HCVR as wild type mice (Erickson et al., 2007); however, as adults there is a significant difference between the two genotypes in males (Hodges et al., 2005a). It is not yet known at what age this difference appears, but it is in agreement with the hypothesis that 5-HT neurons play a more important role in the central response to hypercapnia in adults than in neonatal mice.

3. Arguments against a role of 5-HT neurons as chemoreceptors

Although there is strong evidence for a role of 5-HT neurons in central respiratory chemoreception, this hypothesis has recently been challenged. Here we evaluate the two main lines of evidence that have been cited as support for the conclusion that 5-HT neurons are not chemoreceptors, and discuss their validity.

3.1. Attenuated chemosensory response of 5-HT neurons in TASK channel knockout mice

The first argument that 5-HT neurons are not involved in central chemoreception comes from data obtained from TASK (TWIK-related acid-sensitive potassium) channel knockout (TASK KO) mice (Mulkey et al., 2007b). *In vitro* evidence was presented that was interpreted as showing that TASK channels mediate pH sensitivity of 5-HT neurons. The HCVR was normal in TASK KO mice *in vivo*. Therefore, it was concluded that neither TASK channels nor 5-HT neurons are critical for respiratory chemosensitivity.

The assertion that chemosensitivity of 5-HT neurons is mediated by TASK channels is based on recordings from dorsal raphe neurons in brain slices (Mulkey et al., 2007b). It was found that 5-HT neurons from wild type mice increase their firing rate approximately 2-fold in response to a change in pH from 7.3 to 6.9. This response was absent in 5-HT neurons from mice with genetic deletion of TASK-1 and/or TASK-3 channels, leading to the conclusion that chemosensitivity of medullary raphe neurons is mediated by TASK channels. However, this conclusion may not be valid for the following reason. In order to obtain a response in 5-HT neurons from wild type mice it was necessary to use a pH of 6.9. There was no response when pH was changed from 7.5 to 7.3 (Fig. 5A). In contrast, there are multiple previous reports that 5-HT neurons have a robust response to a decrease in pH from 7.4 to 7.2 (see above).

This is an important point as it is unrealistic to believe that a mammal would ever exhibit a pH of 6.9 *in vivo* except under severe pathological conditions such as cardiopulmonary arrest. To achieve an acute respiratory acidosis to pH < 7.0, PaCO₂ would have to exceed 100mmHg (Keele et al., 1982; Hare et al., 2003). High levels of PaCO₂ are often observed in patients with chronic obstructive pulmonary disease (COPD) and chronic sleep apnea, and can also occur in breath-hold divers (Koo et al., 1975; Sari et al., 1992; Guardiola et al., 2004; Binks et al., 2007), but in such cases, PaCO₂ typically only increases up to 50 – 70mmHg, leading to a concomitant decrease in pH to only 7.2–7.3 (Koo et al., 1975; Keele et al., 1982). It has been estimated that a person would need to hold their breath for 20 minutes to cause PaCO₂ to rise to higher than 100 mm Hg (Frumin et al., 1959). Thus, it seems advisable to limit *in vitro* studies of putative central chemoreceptor neurons to this “physiological” or moderately severe pathological range. In addition, levels above ~80 mmHg causes leveling off of the ventilatory response to PaCO₂ (Lambertsen, 1980), suggesting that any cellular response to changes in P_{CO2}/pH beyond this range may not be relevant to respiratory control.

Comparing the responses reported by Mulkey et al. (2007b) with one of the datasets previously reported for 5-HT neurons (Wang et al., 2002), it is apparent that there is a large difference in the degree of chemosensitivity (Fig. 5B). The most likely explanation for the discrepancy is that Mulkey et al. (2007b) made recordings from P7-12 mice, whereas rat and mouse 5-HT neurons do not begin to develop a response to changes in pH between 7.4 and 7.2 until they are P12 (see above). Thus, there may be a small non-specific response of some immature 5-HT neurons to a pathological level of acidosis that may be mediated by TASK channels. However, there is no evidence that TASK channels mediate the large changes in firing rate that occur in mature raphe neurons in response to small changes in pH near 7.4.

Although TASK channels are sensitive to pH (Rajan et al., 2000; Morton et al., 2003), there are reasons to believe they should not be important for central respiratory chemoreception. TASK channels are widely expressed in the brain (Karschin et al., 2001; Talley et al., 2001) and yet most neurons and brain functions are not stimulated by acidosis (Richerson, 1998; Richerson et al., 2001). For example, TASK channels are expressed at high levels in non-respiratory motor neurons (Karschin et al., 2001; Talley et al., 2001), and yet hypercapnia does not cause a generalized increase in muscle tone. TASK channels are also expressed at high levels in hippocampal principal neurons (Taverna et al., 2005), and yet those neurons (and seizures) are inhibited, not stimulated, by physiologically relevant acidosis (Somjen and

Tombaugh, 1998; Wang and Richerson, 2000). The reason for this disconnect is probably that pH-sensitive TASK currents seen during voltage clamp recordings are not activated under normal conditions and/or are of too small a magnitude to influence neuronal firing rate. In fact, TASK currents are often very small under basal conditions, so they are typically studied after being activated by inhalational anesthetics like halothane to increase their amplitude (Washburn et al., 2002).

The large response of mature 5-HT neurons in the medullary raphé to physiologically relevant changes in pH are unlikely to be due to TASK channels for several reasons. First, chemosensitivity of 5-HT neurons is mediated by changes in intracellular pH (Wang et al., 2002), whereas TASK channels are sensitive to changes in extracellular pH (Rajan et al., 2000; Morton et al., 2003). In addition, TASK channel mediated currents in 5-HT neurons of the dorsal raphé are relatively insensitive to changes in pH between 7.6 and 7.2 (Washburn et al., 2002), a range that has a particularly large effect on the firing rate of mature 5-HT neurons (Wang & Richerson, 2002) and that is relevant to respiratory chemoreception. Finally, our preliminary data indicates that the current that mediates pH sensitivity in mature 5-HT neurons of the medullary raphé is not a leak K⁺ channel, but instead is a calcium-activated non-selective cation (CAN) current (Richerson, 2004). Thus, it may not be valid to make conclusions about the role of 5-HT neurons in central chemoreception on the basis of a preserved HCVR in TASK KO mice.

3.2. Small response of 5-HT neurons to CO₂ in halothane anesthetized rats

The second argument that 5-HT neurons are not central chemoreceptors is based on the assertion that 5-HT neurons do not have a large response to CO₂ *in vivo* (Guyenet, 2008b). This conclusion is based on recordings from rats under halothane anesthesia in which 5-HT neurons in the rostral VLM have a small response to hypercapnia (Mulkey et al., 2004). However, as discussed above, other studies from unanesthetized animals have shown that there is a substantial response of 5-HT neurons to hypercapnia (see above).

It is also hard to explain why 5-HT neurons would be highly chemosensitive in brain slices and in culture, but have little or no response *in vivo*. The most likely explanation for the small CO₂ response of 5-HT neurons in the rostral VLM seen *in vivo* by Mulkey et al. (2004) is the use of halothane. Anesthesia is well known to have major effects on respiratory control. The experiments performed by Mulkey et al. (2004) were performed with halothane anesthesia, whereas other studies of chemosensitivity of 5-HT neurons *in vivo* were performed in unanesthetized animals (Larnicol et al., 1994; Veasey et al., 1995, 1997; Haxhiu et al., 2001; Pete et al., 2002; Johnson et al., 2005; Kanamaru and Homma, 2007). This suggests that chemosensitivity of 5-HT neurons is blunted by halothane anesthesia. A potential mechanism for this effect is that 5-HT neurons abundantly express TASK channels (Washburn et al., 2002). These channels are not very active at rest, but are strongly activated by halothane (Sirois et al., 1998, 2000). This would lead to a large current shunt, reducing the effect on membrane potential of changes in other currents. Since TASK channels are less sensitive to changes in pH near 7.4 than the pH-sensitive CAN current identified in raphé neurons (see above), activation of TASK channels would lead to a reduction in pH sensitivity of 5-HT neurons. In contrast, any neuron whose pH response is primarily due to a leak potassium current, such as has been reported for RTN neurons (Mulkey et al., 2007b), would have an increase in response if that current is activated by halothane. Consistent with this, it has previously been reported that the relative importance of the VLM increases under halothane anesthesia (Forster et al., 1995, 1997; Ohtake et al., 1995).

Another possibility is that 5-HT neurons in the VLM may be less pH sensitive than those in the midline raphé. So far, *in vitro* chemosensitivity has only been studied in 5-HT neurons of the midline raphé, so it is not known whether those in the VLM are as highly chemosensitive.

As discussed above, 5-HT neurons with less chemosensitivity might modulate non-respiratory brain functions that are relatively less sensitive to hypercapnia. 5-HT neurons with a low degree of chemosensitivity may not have been activated by the stimulus used by Mulkey et al. (2004), which was to increase end-tidal CO₂ to 10%. Some 5-HT neurons are activated in unanesthetized cats with a relatively high threshold (Veasey et al., 1997), and an even larger stimulus may be required under anesthesia. There was a subset of 5-HT neurons reported by Mulkey et al. (2004) in which 10% CO₂ did induce a small response (10–30% increase). Although this is a small increase in firing rate, it may still be significant even for respiratory chemoreceptors, since there are many ways for a small response of a neuron to be amplified downstream and cause a large increase in breathing (Richerson et al., 2005).

4. Evidence for other central chemoreceptors

There is compelling evidence that there are additional central chemoreceptors located in other brainstem nuclei, including the RTN, locus coeruleus, nucleus tractus solitarius (NTS), lateral hypothalamus and cerebellum. The idea of a chemoreceptor system with more than one site is supported by data that has been discussed in several recent reviews (Nattie, 1998, 1999; Feldman et al., 2003; Nattie and Li, 2008a).

The RTN has long been recognized as being important in respiratory control (Feldman et al., 2003) and central chemoreception (Li and Nattie, 1997, 2002; Li et al., 1999; Guyenet, 2005). There are data supporting the hypothesis that RTN neurons are central respiratory chemoreceptors. For example, RTN neurons have a large response to CO₂ inhalation *in vivo* (Nattie et al., 1993; Mulkey et al., 2004). However, as discussed in a previous review (Richerson et al., 2005), it is not yet clear how important these neurons are as chemoreceptors, because it is not known how much of their response is intrinsic. In fact, there is evidence that RTN neurons are synaptically driven by other chemoreceptors. RTN neurons receive input from peripheral chemoreceptors, as demonstrated by an increase in firing rate in response to hypoxia (Guyenet, 2008b). They also receive input from other regions containing central chemoreceptor candidates, including the medullary raphé, NTS and lateral hypothalamus (Rosin et al., 2006). These converging inputs could mediate some or all of the response of RTN neurons to CO₂. One hypothesis that is consistent with the existing data is that the RTN is not a major chemoreceptor site itself, but instead acts primarily to integrate input from other chemoreceptors (Guyenet, 2008b). In support of this alternative, lesioning RTN neurons in rats *in vivo* leads to a shift in the apneic threshold, but causes an increase in slope of the ventilation vs. CO₂ curve (Guyenet et al., 2008a). This would not be expected if RTN neurons play a critical role as chemoreceptors themselves.

It is now clear that RTN neurons play a more complicated role in respiratory control than just being chemoreceptors. For example, the RTN is involved in respiratory rhythm generation (Onimaru, 2008), especially during the neonatal period. Recently, pre-inspiratory neurons within the parafacial respiratory group (pFRG) have been discovered to possess rhythmic properties, and contribute to respiratory rhythm generation (Onimaru and Homma, 2003). Onimaru (2008) confirmed that these pFRG neurons are Phox2b-immunoreactive (a marker of RTN neurons) and that they depolarize in response to increased CO₂. Thus, there may be extensive anatomical and functional overlap between the RTN and pFRG, and RTN neurons may be intimately involved in rhythm generation, making it difficult to parse out an independent role in chemoreception.

5. Relationship between the RTN and medullary raphé

As discussed above, evidence for widespread distribution of chemosensitivity lends support to the hypothesis that central chemoreception is the result of interaction between many sites, with some more dominant under certain conditions (eg., arousal state, gender, pathology).

There is good evidence in favour of both the RTN and 5-HT neurons being chemoreceptors. Comparison of this evidence (Table 1) reveals many similarities, and some differences.

While it is likely that both nuclei, along with others, contribute to the overall chemosensitivity of the CNS, recent work has revealed a significant interaction between the RTN and the medullary raphé. Li et al. (2006) first explored this possibility by focally inhibiting these nuclei, first separately and then simultaneously. Microinjection of muscimol into the RTN alone decreases the ventilatory response to 7% CO₂ by 24%. Inhibition of the more caudal raphé obscurus with 8-OH-DPAT does not significantly affect the CO₂ response by itself (Li et al., 2006), whereas inhibition of this region with ibotenic acid slightly reduces the HCVR by ~10% (da Silva et al., 2008). Simultaneous inhibition of the raphé obscurus and RTN results in a substantial decrease in the response to CO₂ by 51%. Furthermore, simultaneous stimulation of these sites with focally applied CO₂ causes an increase in ventilation by 22%; compared to an increase of only 15% when the RTN alone is stimulated, and no change in ventilation when the raphé obscurus alone is stimulated (Dias et al., 2008). These results indicate that there is a substantial interaction between this specific portion of the raphé obscurus and the RTN. The functional consequence of this interaction is unclear, but this portion of the raphé appears to potentiate the response of the RTN to hypercapnia.

There is also an interaction between 5-HT neurons and RTN neurons *in vivo* and *in vitro*. Mulkey et al. (2007a) found that 5-HT projections exist from many raphé nuclei to RTN neurons. Each of the neurotransmitters that are released by 5-HT neurons, SP, TRH and 5-HT, activate RTN Phox2b chemosensitive neurons *in vivo* and *in vitro* (Cream et al., 1999; Mulkey et al., 2007a). The role of these inputs is not clear, but recent experiments were performed in which chemosensitivity of RTN neurons in slices was measured in the presence and absence of exogenous 5-HT (Mulkey et al., 2007a). It was found that 5-HT excites RTN neurons, but does not change the slope of the firing rate vs. pH curve. It was concluded that chemosensitivity of RTN neurons is not dependent on input from 5-HT neurons. However, that possibility can only be ruled out by demonstrating that the degree of chemosensitivity of RTN neurons is not decreased after blocking 5-HT and NK1 receptors.

6. Conclusions

In evaluating the existing data, we find considerable support of the hypothesis that 5-HT neurons are central respiratory chemoreceptors. Novel genetic methods have made a large contribution to this conclusion and lend further support to the concept that there are gender differences in the role of 5-HT and CO₂ sensitivity. Additionally, increasing evidence points to a functional interaction between the RTN and raphé 5-HT neurons, supporting the growing theory that RTN neurons act to integrate input from multiple other chemoreceptor sites.

Given the support for the hypothesis that central chemoreceptors are present in many brainstem regions, and the lack of conclusive evidence that any one of them is dominant, it will now be important to determine under what conditions each of them plays their most important role. It is possible that they are all equivalent and redundant, but it is equally possible that they are structured in a hierarchy, or that some are more important during development, during sleep, or under pathological conditions. Continuing to address these questions will not be easy, and will require a careful and critical analysis of data obtained from a variety of experimental approaches.

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Figure 1.

Serotonergic neurons have properties expected of central respiratory chemoreceptors. A) Confocal image of the ventral surface of the medulla. 5-HT neurons (green and yellow) are concentrated near the basilar artery and its main branches (red). Reproduced with permission from Bradley et al. (2002). B) Patch-clamp recording of a medullary raphe 5-HT neuron at a baseline pH of 7.4 (control) and exposed to pH 7.2. Acidosis causes an increase in firing rate. Reproduced with permission from Wang et al., (2002).

Figure 2.

Raphé neurons are intrinsically sensitive, responding to hypercapnic acidosis after acute dissociation. Shown is a 13 day old 5-HT neuron acutely dissociated from a genetically modified mouse that expresses yellow fluorescent protein (YFP) in 5-HT neurons (Scott et al., 2005). Images on the left were taken using differential interference contrast (DIC) and fluorescence microscopy (YFP). The recording was made 3 days after acute dissociation, which is long enough to allow synaptic terminals and glial processes to die off, but not long enough for new synaptic connections to be made or for regrowth of glial processes. This approach ensures that the response to pH is intrinsic to the recorded neuron. Shown on the right is the firing rate response to hypercapnic acidosis. The magnitude of the response was typical for raphé neurons of the same age recorded in slices and in culture.

Figure 3.

Raphé neurons are chemosensitive in unanesthetized animals. A) *In vivo* recordings of individual 5-HT raphé neurons in cats in response to increasing inhaled CO_2 . Some neurons display a non-linear response to CO_2 , requiring a threshold level of CO_2 to be reached before increasing their firing rate. B) There is a close correlation between the firing rate of some raphé neurons recorded *in vivo* and the increase in minute ventilation in response to inhaled CO_2 . Adapted with permission from Veasey et al. (1995).

Figure 4.

A) Development of the hypercapnic ventilatory response in Sprague-Dawley rats *in vivo*. Note that there is very little response to 7% CO₂ (expressed as a percentage of eucapnia) during the first two weeks post-natal, and increases significantly after around P12–P14. Adapted with permission from Davis et al. (2006). B) Sprague-Dawley rat raphé neurons recorded in slices and in tissue culture do not develop significant chemosensitivity until they are older than P12. Modified with permission from Wang et al. (1999).

Figure 5.

A) Midbrain 5-HT neurons from wild type mice have no response to a decrease in pH from 7.5 to 7.3, and a two-fold response to a decrease in pH from 7.3 to 6.9. In contrast, midbrain 5-HT neurons from mice with genetic deletion of TASK-1 and/or TASK-3 do not respond to changes in pH between 6.9 and 7.5. Adapted with permission from Mulkey et al. (2007b). B) Comparison of responses of mouse midbrain raphé neurons reported by Mulkey et al. (2007b) and values previously reported in rat medullary raphé neurons (Wang et al., 2002). The small response for wild-type mice is re-plotted from part A, and is contrasted with the large response in rats.

Table 1

Comparison of evidence that 5-HT and RTN neurons are central respiratory chemoreceptors.

	5-HT Neurons	RTN Neurons
Increased firing rate <i>in vivo</i>	Yes	Yes
Intrinsic chemosensitivity	Yes	Not known
Increase in firing rate <i>in vitro</i> in response to a change of 0.2 pH unit (% control)	413% (at 30°C)	248% (at 35°C)
Increased response with increased temperature	Yes	Yes
Project to respiratory neurons	Yes	Yes
Neurotransmitters stimulate breathing	Yes 5-HT, TRH, SP	Yes Glutamate
Present in classical VLM chemosensitive zones	Rostral & caudal	Rostral only
Decrease in chemoreception after specific lesions	Yes	No
Focal acidosis stimulates ventilation	Yes	Yes
Anatomical specialization	Large arteries	Marginal glia